THE PROKARYOTES

Third Edition

A Handbook on the Biology of Bacteria:
Symbiotic Associations, Biotechnology,
Applied Microbiology

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Volume 1

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A Handbook on the Biology of Bacteria

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Volume 1: Symbiotic Associations, Biotechnology, Applied Microbiology

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Preface

Each of the first two editions of *The Prokaryotes* took a bold step. The first edition, published in 1981, set out to be an encyclopedic, synoptic account of the world of the prokaryotes—a collection of monographic descriptions of the genera of bacteria. The Archaea had not yet been formalized as a group. For the second edition in 1992, the editors made the decision to organize the chapters on the basis of the molecular phylogeny championed by Carl Woese, which increasingly provided a rational, evolutionary basis for the taxonomy of the prokaryotes. In addition, the archaea had by then been recognized as a phylogenetically separate and distinguishable group of the prokaryotes. The two volumes of the first edition had by then expanded to four. The third edition was arguably the boldest step of all. We decided that the material would only be presented electronically. The advantages were obvious and persuasive. There would be essentially unlimited space. There would be no restrictions on the use of color illustrations. Film and animated descriptions could be made available. The text would be hyperlinked to external sources. Publication of chapters would be seriati—the edition would no longer have to delay publication until the last tardy author had submitted his or her chapter. Updates and modifications could be made continuously. And, most attractively, a library could place its subscribed copy on its server and make it available easily and cheaply to all in its community. One hundred and seventy chapters have thus far been presented in 16 releases over a six-year period. The virtues and advantages of the online edition have been borne out. But we failed to predict the affection that many have for holding a bound, print version of a book in their hands. Thus, this print version of the third edition shall accompany the online version.

We are now four years into the 21st century. Indulge us then while we comment on the challenges, problems and opportunities for microbiology that confront us.

Moselio Schaechter has referred to the present era of microbiology as its third golden age—the era of "integrative microbiology." Essentially all microbiologists now speak a common language. So that the boundaries that previously separated subdisciplines from each other have faded: physiology has become indistinguishable from pathogenesis; ecologists and molecular geneticists speak to each other; biochemistry is spoken by all; and—mirabile dictu!—molecular biologists are collaborating with taxonomists.

But before these molecular dissections of complex processes can be effective there must be a clear view of the organism being studied. And it is our goal that these chapters in *The Prokaryotes* provide that opportunity.

There is also yet a larger issue. Microbiology is now confronted with the need to understand increasingly complex processes. And the modus operandi that has served us so successfully for 150 years—that of the pure culture studied under standard laboratory conditions—is inadequate. We are now challenged to solve problems of multimembered populations interacting with each other and with their environment under constantly variable conditions. Carl Woese has pointed out a useful and important distinction between empirical, methodological reductionism and fundamentalist reductionism. The former has served us well; the latter stands in the way of our further understanding of complex, interacting systems. But no matter what kind of synoptic systems analysis emerges as our way of understanding host–parasite relations, ecology, or multicellular behavior, the understanding of the organism as such is sine qua non. And in that context, we are pleased to present to you the third edition of *The Prokaryotes*.

Martin Dworkin
Editor-in-Chief

Contents

Volume 2

1. Ecophysiological and Biochemical Aspects

Volume 3

A: Archaea

Volume 4

1. Firmicutes (Gram-Positive Bacteria)

1.2 Firmicutes with Low GC Content of DNA

Volume 6

Volume 7

3. Proteobacteria

3.4. Delta Subclass

3.5. Epsilon Subclass

4. Spirochetes

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PART 1

Essays in Prokaryotic Biology

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CHAPTER 1.1

How We Do, Don't and Should Look at Bacteria and Bacteriology

CARL R. WOESE

Background

To try to plan for the future without a sense of the past is like trying to plant cut flowers.
(Daniel Boorstein)

Microbiology today has a new-found wealth far greater than any it possessed before. The source of that wealth is the universal phylogenetic tree—the framework essential for understanding organismal relationships. The power that flows from phylogenetic ordering permeates the field. Microbiologists now accomplish with ease things that were previously impossible and approach bacteria in ways that 20 years ago were unthinkable. Microbial ecology is no longer the *faux* ecology it had been—when defining a niche in organismal terms was not an option. Today the field rests on a par with plant and animal ecology and exceeds them in importance, for it is in the microbial realm that the base and fount of the global ecosystem lie. Studying microbial diversity used to be the equivalent of hunting through antique shops for curios—which resulted in a collection of species no more connected to one another than the items in a bower bird's nest. Now all organisms sit on the well-ordered tips of branches on the universal phylogenetic tree (Woese, 1987; Olsen et al., 1994), and the study of one, far from being an isolated adventure, can contribute to the study of all. An interest in bacterial evolution used to be perceived as metaphysical and worthless. Today evolutionary relationships are the foundation and motive force behind a new and resurgent microbiology, and thence biology as a whole.

Microbial genomes can be sequenced today in their entirety, and when measured against the information gained, the cost of so doing is actually small—and that cost is continually decreasing. An exploration of a given bacterium can now start with knowledge of all of its genes. Not only are we (potentially) privy to the full range of the organism's biochemistry, but we possess a partial but very useful record of its evolutionary past. This historical record, it turns out, extends so far back into biological antiquity that the study of bacterial evolution fuses with the study of the origins of modern cells (Woese, 2002).

Unfortunately, the universal phylogenetic tree, bacteriology's essential framework, arrived rather late in the course of events. Its belated arrival adversely affected not only the development of microbiology but that of all biology (see below). Consequently, the introductory chapter to the third edition of *The Prokaryotes* is largely devoted to understanding the historical development of bacteriology, for in its twists and turns lie insights into much of what microbiology and biology are today, as well as a guide to their future development.

The editors of the second edition of *The Prokaryotes* wisely decided to give the volume phylogenetic underpinnings (as best they could at the time). In this third edition, the trend continues, and the book's phylogenetic bent is more pronounced. Bacterial taxonomy, which only two decades ago was a dry subject whose main if not only purpose was determinative classification, identification of species, has now blossomed into an intriguing, meaty study in its own right. As you use this third edition of *The Prokaryotes* realize that the book is not merely a manual for identification, cultivation, and determinative classification. Use it for what it really is, a long overdue treatise on comparative bacteriology. If you let your imaginations follow the evolutionary trails the book lays out, they will lead you to the edge of microbiology's future. Most of all, let this work by its structure open your eyes to the emerging world of bacterial evolution and the challenge that poses to all of biology. This is not the evolution that Darwin and the classical evolutionists had in mind. It is centered on physiologies rather than forms, on molecules rather than gross morphologies. And it extends the scientific reach much further into the past than was ever before possible. But to begin charting the future, we need a map of the past. As in so many walks of life, a knowledge of history is the best guide we have.

Microbiology's Halting Development

Prior to its phylogenetic liberation, microbiology had long been mired down conceptually, sinking ever deeper as twentieth century biology unfolded. This descent into innocence started long before that, however, when microbiologists failed to develop a phylogenetic, or "natural," classification system for bacteria based upon the classical characteristics available to them. As a consequence, bacteriology's development became increasingly one-sided, so much so that from an organismal perspective, the discipline developed not at all. The condition was chronic, progressing over several generations of microbiologists, and so, was scarcely felt, and to the extent that it was felt, the condition was accepted as normal.

One of the rare (if not only) times the problem was brought to the fore was in 1962 when Stanier and van Niel lamented: "... the abiding intellectual scandal of bacteriology has been the absence of a clear concept of a bacterium." Unfortunately, the authors' suggested remedy for the malaise was untenable, as we shall see, and as a consequence served to encourage the unfortunate changeover from the name "bacteriology" to "microbiology," a change that dogs microbiology to this day. A label that had denoted the study of what seemed a naturally defined grouping of organisms, "bacteriology," was replaced by one that encompassed an organismal *potpourri*. One can in principle have a biologically valid, unified concept of "bacteria," but a comparable unifying concept of the artificial grouping "microorganisms" is impossible. The semantic thimblerig involved in this name change was one of the factors that helped to push the problem of a "concept of a bacterium" off the table. From a basic scientific perspective, bacteriology in 1992 was worse off than it had been in 1962. But by 1992, bacteriology had a powerful remedy for the situation.

What Is Bacteriology?

Whatever else it is, bacteriology is first and foremost an *organismal science*, just as are zoology and botany. As such, its focus is understanding particular naturally defined groupings of organisms. A critical difference between bacteriology and the other two organismal sciences, however, is that the other two could develop as organismal sciences in the absence of molecular characterizations, whereas bacteriology could not. Bacterial morphologies are too simple and variable to have much phylogenetic significance, but phylogeny is embedded in the complex morphologies and variations on morphological themes characteristic of animal and plant life. In the bacterial world it is physiology (not morphology) that predominates and distinguishes. But, physiological characteristics have so far proven phylogenetically intractable. Whereas amongst eukaryotes, organization manifests itself predominately in structure, organization in the bacterial world occurs mainly in the shifting and subtle biochemical states of the system—something the eye cannot directly apprehend. In the absence of a knowledge of phylogenetic relationships, a concept of bacteria was unattainable.

What exactly is an organismal science? Simply put, one that seeks to understand naturally defined groups of organisms in *biological terms*. Such an understanding has four principal components: 1) structure/function—how the organisms in the group are built (organized) and how they work; 2) diversity—how many and what kinds of organisms the group comprises, and the ways in which the various kinds are similar and different; 3) ecology—how the organisms interact with their environments (including other organisms); and 4) evolution—the origins of the group and how the organisms therein are ancestrally related. This last, evolution, is what underlies and enables the development of a biological concept of a group. As Dobzhansky famously put it "... nothing in biology makes sense except in the light of evolution" (Dobzhansky, 1963). And it was the lack of this essential evolutionary framework that prevented bacteriology from developing as an organismal science.

With no understanding of phylogenetic relationships, there can be no effective study of ecology or diversity. Ecological niches cannot be defined in organismal terms, and studies in bacterial diversity amount to no more than a catalog of disconnected vignettes. It is inconceivable that a zoologist or botanist could go into the field and not be able to distinguish the animals from the plants. Yet would-be microbial ecologists were in this exact position or worse all the time: they couldn't distinguish their "animals" from their "plants" or (with a few exceptions) from representatives of any others of the many kingdom-level bacterial taxa.

Blocked from developing into a full-fledged organismal science throughout most of the twentieth century, bacteriology wandered aimlessly, not knowing what it was, where it came from, or what it should become. The field was driven by the winds of scientific fad and other outside influences. Any development our science did undergo was confined to its structure/function aspect and its applied side. With exceptions too rare to impact, twentieth century microbiologists (and biologists in general) remained unaware of microbiology's "identity crisis."

Dumbing Down

What more than anything served to obscure bacteriology's structural problem was the molecular perspective dominating twentieth century biology. Molecular biology embodies a reductionist fundamentalism that rules out any holistic perspective. The paradigm assumed that the age-old problem of biological organization would be automatically solved when comprehensive molecular parts-lists for cells were generated. Yet even with the parts-lists now in hand, the problem remains with us, awaiting a fresh, constructive, and holistic outlook. The molecular perspective took evolution for granted, found it intellectually wanting, and dismissed its study as trivial. In such a fundamentalist milieu, the organism *per se* has only a secondary existence, shadowy and temporal. For molecular biology, the organism lies essentially in its collective parts (which molecular biologists felt no compulsion to reassemble into a whole). Little wonder that under the molecular aegis, bacteriology felt no need to develop into an organismal science, even if it *could* have done so. Instead, microbiology followed molecular biology's lead and slipped into a mechanistic reductionism.

Much of the important biochemistry of the last century was done in bacterial systems. Bacteria—with their enormous population numbers, rapid growth rates, and general ease of handling—also proved well suited to many of the contemporary studies in genetics and molecular biology. In one sense, great progress occurred in microbiology over the last five or so decades. None of it, however, contributed to the development of a concept of bacteria.

Bacteriology's Wandering Course

It is difficult to know where you are going if you don't know where you came from. And if you don't know where you are going, the saying goes, any path will take you there. The future course of bacteriology (microbiology) is not something that can be left to the vagaries of chance and necessity. How bacteriology now develops is a matter of utmost concern. *The future of all biology turns upon it.*

Although Leeuwenhoek discovered the microbial world during the time of Newton, microbiology did not emerge as an effective science until two centuries later. In the last half of the nineteenth century the works of scientists like Cohn, Koch, and Pasteur laid the groundwork. And with the next generation of microbiologists, the Beijerincks, Winogradskys, and Orla-Jensens, the study of microorganisms began in earnest. The grouping of microbiologists that grew around Beijerinck and then Kluyver, known as the Delft School, became the dominant influence in microbiology for most of the twentieth century. Among the School's members, one finds articulate spokesmen with an overview of their discipline. So, it is to the Delft School that we mainly turn to learn the early history of microbiology.

Martinus Beijerinck is the actual founder of the Delft School, although Leeuwenhoek, who had lived in Delft, is portrayed as its spiritual father (van Niel, 1949). Beijerinck is credited, along with Winogradsky, with the development of enrichment culturing, through which methodology microbiologists could begin a major exploration of the nature and scope of the microbial world. Beijerinck's contributions were many and varied (van Iterson et al., 1940): he added greatly to the understanding of bacterial physiology and diversity. Through his studies on iron bacteria and the like, he was one of the first to appreciate the intimate role bacteria play in geologic processes. He was keenly aware of developments in genetics (in the higher forms) and was perhaps first to suggest that some of the variation seen in the microbial world was of mutational origin. His discovery of a filterable factor associated with tobacco mosaic disease made him a pioneer in virology. And he was keenly aware of Darwin. How did this enlightened Dutch scientist view the study of microorganisms? On the occasion of his being awarded the Leeuwenhoek Medal (microbiology's highest and his most prized honor), Beijerinck addressed the question:

> [M]icrobial ecology . . . is the most necessary and fruitful direction to guide us in organizing our knowledge of that part of nature which deals with the lowest limits of the organic world, and which constantly keeps before our minds the profound problem of the origin of life itself (Beijerinck, 1905; van Iterson et al., 1940; translated by van Niel, 1949).

This is a sophisticated view of microbiology. The importance of the microbe-environment relationship is foremost, with (microbial) evolution constantly in the background, shaping and deepening the outlook. To me, Beijerinck's concept of the microbial world seems deeper and more holistic than any that followed until the present day, when the realities of genomics are bringing us once more to a similarly broad and inspiring perspective on bacteria and their world.

In this quote (and in his studies), Beijerinck did not (to my knowledge) speak to the importance of determining the natural relationships among microorganisms. It is possible he did not believe (as others of his day did not) that bacteria evolved in a Darwinian fashion, as animals and plants do. I would prefer to believe, however—especially given his familiarity with Darwin's writings (van Iterson et al., 1940)—that

Beijerinck saw no way to determine these relationships given the primitive state of bacteriology in his day. Only with the later accumulation of large numbers of bacterial species (mainly through enrichment culturing) would the problem of their classification become a choking one, and only then, when traditional taxonomic approaches failed to yield a satisfactory (natural) classification system, would the problem become genuinely acute (Stanier and van Niel, 1962).

Beijerinck's successor at Delft was Albert Jan Kluyver. A brilliant and sophisticated scientist, Kluyver was by training a biochemist, not a microbiologist. Only upon assuming Beijerinck's chair did he transform himself into the latter (Kamp et al., 1959). It is in Kluyver that I see the beginning of bacteriology's drift away from striving to become an organismal discipline. Kluyver is noted, and rightly so, for his fundamental, unifying contributions to biochemistry (Kluyver and Donker, 1926; Kluyver, 1931). He pioneered what he called "comparative biochemistry," a field he envisioned as "... benefit[ing] biochemistry in a manner similar to that in which the concept of 'comparative anatomy' had helped to bring order into [anatomy]" (Kluyver, 1931; van Niel, 1949). Yet in one way, the analogy deceives. Comparative anatomy is basically an organismal, evolutionary pursuit. Comparative biochemistry is not. The latter is simply a way to bring some chemical order to the plethora of biochemistries that abound in nature. Kluyver's simile here veils the important distinction between the organism and its parts. My assertion that Kluyver represented (or came to represent) the biochemical dissection of bacteria rather than an organismal comprehension thereof finds indirect support in van Niel's historical account of the Delft School, which details Kluyver's contributions to biochemistry while failing once to mention Kluyver's (and his own) concerns with developing a natural bacterial classification (van Niel, 1949).

The Gordian Knot of Bacterial Classification

The Linnean system had proven extremely useful in structuring our knowledge of zoology and botany, making both into respectable organismal sciences even before Darwin's time. Darwin's theory did not change the Linnean classification of animals and plants all that much; it merely provided it theoretical justification (Darwin, 1859).

Early microbiologists were aware of the benefits of "natural" classification. Yet I have never satisfied myself as to the degree of their commitment to or the depth of their appreciation of evolution. Was a natural system merely the most useful classification, or were bacteria given the evolutionary significance Beijerinck (quoted above) seemed to accord them? Clearly, most microbiologists of the time simply wanted some kind, any kind, of useful pigeonholing system. *Bergey's Manual*, a (much criticized though popular) determinative system, provided precisely that (Stanier and van Niel, 1941).

Kluyver and van Niel's classic paper of 1936 on bacterial classification represents the Delft School outlook. The paper was a reasoned discussion of the problems faced in contemporary bacterial classification, in the context of which the authors then proposed a system of their own, which they hoped would overcome some of the problems and, so, move bacteriology closer to a genuine natural bacterial system. At the time, classifications were largely morphological and their purpose for the most part utilitarian. A salient exception to these approaches had been the system of Orla-Jensen (see Kluyver and van Niel, 1936), which was based on an evolutionary conjecture, namely that the first organisms to evolve were necessarily autotrophs (Oparin and his ocean had yet to enter the picture). Like Beijerinck, Orla-Jensen appeared to believe that microorganisms held the key to understanding life's origin, and he constructed a classification that hopefully would help to bring out the origin of metabolism. At this time, Kluyver and van Niel seemed to share Beijerinck's belief in the importance of bacteria as evolutionary beacons; at least they said: "A true reconstruction of the course of evolution is the ideal of every taxonomist" (Kluyver and van Niel, 1936).

In the Kluyver-van Niel article, one readily senses bacteriology's ongoing struggle regarding the bases upon which to develop bacterial classification. Because zoological and botanical classifications are morphologically based, a strong precedent existed for putting bacterial classification on a similar footing—despite the fact that microbiologists intuitively knew that bacteria are as fundamentally physiological as animals and plants are morphological. On what levels, to what extent, in what ways, then, are the morphological and various physiological properties of bacteria taxonomically significant? In the following, we see Kluyver and van Niel worry the problem (Kluyver and van Niel, 1936, pp. 370, 371):

> The question then arises in which [bacterial] characters phylogeny expresses itself. There is no doubt that in this respect morphology remains the first and most reliable guide ... [although] the indispensability of physiological characters for the purpose of classification has also been generally accepted. ...
>
> A lack of insight in the fundamentals of metabolism has thus far been the great stumbling-block for a rational application of physiological characteristics in taxonomy and it also explains the horror with which many systematicians have

witnessed their ever increasing use. . . . The fundamental nature of the energy providing processes justifies the view that they should be rated first amongst the physiological characters suitable for classification. . . .

Because the classification sought was a natural one, the kinds of choices Kluyver and van Niel were entertaining required the answers to certain evolutionary questions. But there were no *a priori* answers—even hints of them. Natural classification then had to rest on a foundation of conjecture—bolstered by the hope that if the scheme were anywhere near phylogenetically valid, it could bootstrap itself into a true natural classification. (Only today is knowledge of evolutionary relationships independent of [and so can precede the construction of] a taxonomy rather than follow from it.)

There is little point in detailing Kluyver and van Niel's attempt at a natural classification. But since the tenor of their thinking is so instructive as to the microbiological gestalt of their day, it is worth sampling a bit more. After debating the relative significance and utility of morphological and physiological characteristics in bacterial classification, the authors (tentatively) opt for ". . . the use of morphological criteria as [the] main guiding principle . . . above the rank of genera" (Kluyver and van Niel, 1936).

It is basic to biological thought that the complex tends to evolve from the simple, and given the presumed primitive nature of bacteria, Kluyver and van Niel carried this notion to the extreme:

> It seems acceptable that the diversity of bacterial forms is the outcome of various independent morphological evolutions which have had their starting-point in the simplest form both existent and conceivable: the sphere (Kluyver and van Niel, 1936, p. 387).

From this starting assumption they picture the aboriginal spherical bacterium as (somehow) giving rise to four basic morphological types: 1) coccoid (the Micrococcaceae), 2) cells with polar flagella (Pseudomonadaceae), 3) cells with peritrichous flagella (Bacteriaceae), and 4) a nonmotile line whose beginnings are streptococci. Each of the four primary lineages in turn evolves in a quasi-ontogenetic fashion through stages of increasing complexity to some "highest stage of development," e.g., the coccoid forms giving rise to sarcinae (packets of cocci), and thence sarcinae that form spores, or the "streptococcal" lineage developing through short Gram-positive rods to mycobacteria and ultimately the complex actinomycetes (Kluyver and van Niel, 1936).

Within each main line of morphological descent, morphology increasingly gives way to physiological characters in defining the lower taxonomic levels (Kluyver and van Niel, 1936). In retrospect, unfortunately, bacterial classification in the early twentieth century comes to be little more than a tapestry of *Just So Stories*.

The Beginning of the End

We need to consider one final attempt to devise a natural bacterial system, which was proposed by Stanier and van Niel five years after the Kluyver and van Niel system (Stanier and van Niel, 1941). Again our reason for doing so does not lie in the system itself but in the discussion that accompanied it. Theirs was the last blush of enthusiasm among microbiologists for developing a comprehensive natural bacterial classification, and the last time that having an evolutionary overview of the bacteria of any kind was vigorously defended (Stanier and van Niel, 1941).

Bacterial taxonomists had long been split into two camps: the *idealists*, represented especially by members of the Delft School, and the *realists*, represented by the majority of other bacteriologists, e.g., the board of *Bergey's Manual* (Breed, 1939; Stanier and van Niel, 1941). The overarching perspective of the idealists recognized the importance to the discipline's future of a phylogenetically based taxonomy. The realists' practical perspective was content with the intellectual pauper's gruel provided by a taxonomy merely enabling species identification and a convenient pigeon-holing system. Stanier and van Niel deplored this mercantile mentality (Stanier and van Niel, 1941):

> In most biological fields it is considered a truism to state that the only satisfactory basis for the construction of a rational system of classification is the phylogenetic one. Nevertheless, "realistic" bacteriologists show a curious aversion to the attempted use of phylogeny in bacterial systematics. . . . To what may we ascribe this distrust of phylogeny? In part it is undoubtedly due to the unsatisfactory nature of certain systems, purportedly based on phylogeny, which have been proposed in the past. However, the mere fact that a particular phylogenetic scheme has been shown to be unsound by later work is not a valid reason for total rejection of the phylogenetic approach.

The authors then turn to the drawbacks of the alternative:

> . . . There is good reason to prefer an admittedly imperfect natural system to a purely empirical one. A phylogenetic system has at least a rational basis, and can be altered and improved as new facts come to light; its very weaknesses will suggest the type of experimental work necessary for improvement. On the other hand, an empirical system is largely unmodifiable because the differential characters employed are arbitrarily chosen and usually cannot be altered to any great extent without disrupting the whole system. . . . [When the] wide separation of closely related groups caused by the use of arbitrary differential characters . . . makes it impossible to tell with certainty in what order a given organism belongs, an empirical system loses its value. . . .

This is incisive commentary! But unfortunately, it is just about the last one hears the idealist perspective, the last time the attitude "if it can't be solved today, then we will try anew tomorrow," is expressed.

That final time seems to be 1946, when van Niel gave a major address concerning bacterial classification at that year's memorable post-war Cold Spring Harbor Symposium. In the address, he analyzed in detail the failures of current and past attempts at natural bacterial classification. He pointed out the difference between the successful use of morphological characters in plant and animal classification, and their lack of utility in the bacterial case (as mentioned above). He detailed the frustrations bacteriologists had experienced in trying to use physiological characteristics for classificatory purposes, concluding that there is no way one can determine natural relationships in the bacterial world as things then stood (van Niel, 1946). But at the end of this depressing critique, van Niel held firm to the idealist position: "[Thus, since] the morphology of a bacterium is of no more use in [classification] than is its physiology . . . the search for a basis upon which a 'natural system' can be constructed must continue" (van Niel, 1946, p. 290).

Where To Play? The Sand Castles Have All Washed Away

The year was 1955 when van Niel returned for the last time (to my knowledge) to the subject of natural bacterial classification (van Niel, 1955). By that time biochemists, following Kluyver's pioneering synthesis of microbial biochemistry, had turned their attentions in earnest to bacterial systems; sexual recombination had been discovered in bacteria by Lederberg and Tatum (1946), and geneticists were poised to take advantage, and (as mentioned) molecular biologists too were finding bacterial systems highly suitable for their studies. The emphasis in bacteriology was definitely shifting strongly in the reductionist structure/function direction. And van Niel returned to his topic, with a changed, jaded outlook.

Van Niel's still hopeful (and scientifically proper) perspective of 1946—"it hasn't been done yet, so we must continue"—had now become the pessimistic (and scientifically unacceptable)—"it hasn't been done yet, so it can't be done," which he expressed this way:

> What made Winogradsky . . . grant that the systematics of plants and animals on the basis of the Linnean system is defensible, while contending that a similar classification of bacteria is out of the question? The answer must be obvious to those who recognize in the former an increasingly successful attempt at reconstructing a phylogenetic history of the higher plants and animals . . . and who feel that comparable efforts in the realm of the bacteria (and bluegreen algae) are doomed to failure because it does not appear likely that criteria of truly phylogenetic significance can be devised for these organisms (van Niel, 1955, pp. 101–102).

The idealists were now in full retreat, and in the process took an untenable fallback position, namely that the concept of a bacterium could be developed *without* resort to phylogenetic relationships. It could be developed simply by knowing the structure/function differences between eukaryotes and "prokaryotes." A little thought shows the folly in this. As emphasized above, an organismal science *must* be founded on evolutionary relationships. Yet, that is far from what the next generation of microbiologists were taught. In *The Microbial World* first edition, 1957:

> An eminent contemporary bacteriologist, van Niel, who is noted for his taxonomic studies on several groups of bacteria, has expressed the opinion that it is a waste of time to attempt a natural system of classification for bacteria, and that bacteriologists should concentrate instead on the more humble practical task of devising *determinative keys* (Stanier et al., 1957, p. 296).

In *Archives of Microbiology*, 1962:

> It is now clear that among organisms there are two different organizational patterns of cells . . . the eucaryotic and procaryotic type. *The distinctive property of bacteria and bluegreen algae is the procaryotic nature of their cells*. . . . The remaining pages of this essay will be devoted to a discussion of the essential differences between these two cell types, upon which rests our only hope of more clearly formulating a "concept of a bacterium" (Stanier and van Niel, 1962, pp. 20–21).

In *The Microbial World* second edition, 1963:

> All [bacteria] share the distinctive structural properties associated with the procaryotic cell, and we can therefore safely infer a common origin for the whole group in the remote evolutionary past; we can also discern four principal sub-groups, blue-green algae, myxobacteria, spirochetes, and eubacteria, which seem to be distinct from one another. . . . Beyond this point, however, any systematic attempt to construct a detailed scheme of natural relationships becomes the purest speculation, completely unsupported by any sort of evidence. The only possible conclusion is, accordingly, that *the ultimate scientific goal of biological classification cannot be achieved in the case of bacteria* (Stanier et al., 1963, p. 409).

A similarly dark and contrived picture was painted in the third, 1970, edition of *The Microbial World* and carried through essentially unchanged into the fourth (Stanier et al., 1970b; Stanier et al., 1976).

Here Comes the (Molecular) Cavalry

The sad irony in all this is that while the idealist position was in retreat, with defeat masquerading as victory, and while new recruits were taught

not to look back, as the importance of a "concept of a bacterium" (and with it any interest in bacterial evolution) slipped from view, the ground for a genuine phylogeny of bacteria, which could transform the discipline, was simultaneously being prepared. In the early 1950s, Sanger had sequenced the first proteins (Sanger and Tuppy, 1951; Sanger and Thompson, 1953), and the notion that molecular sequence comparisons were a rich source of evolutionary information was beginning to take hold:

> Biologists should realize that before long we shall have a subject which might be called "protein taxonomy"—the study of amino acid sequences of proteins of an organism and the comparison of them between species. It can be argued that these sequences are the most delicate expression possible of the phenotype of an organism and that vast amounts of evolutionary information may be hidden away within them (Crick, 1958, p. 142).

However, among microbiologists Crick's prescience fell on deafened ears. The molecular and bacteriology paradigms were somehow on different conceptual planes. However, some "macrobiologists" did see the new molecular royal road to phylogeny (Zuckerkandl and Pauling, 1965), and a cottage industry arose around confirming and (slightly) extending the classical phylogenetic tree through sequence comparisons of cytochrome *c*s and of a few other molecules (Fitch and Margoliash, 1967). Yet the record shows that even among these first generation molecular evolutionists there was no recognition of the importance of the larger and far more important problem of using molecular sequence comparisons to infer *bacterial* phylogenies, which would have opened the door to the universal phylogenetic tree.

Microbiologists did get on the molecular evolution bandwagon, but belatedly and halfheartedly. In the main, their efforts relied upon simple and relatively uninformative methods, such as nucleic acid hybridization (Gillespie and Spiegelman, 1965), and their interests were confined largely to weeding out misclassified species from genera and in properly grouping genera into families. A few protein sequences were attempted and superficially analyzed, but those responsible saw nothing of evolutionary value in the comparisons (Ambler et al., 1979a; Ambler et al., 1979b; Meyer et al., 1986)! The compelling vision of the idealists, the overriding concern with building a concept of bacteria on a comprehensive natural bacterial taxonomy, was gone. (Two prominent exceptions to the above characterization of microbiologists' attitudes toward bacterial taxonomy appear to be expressed by the Europeans J. De Ley and O. Kandler, in whose works and writings the candle of hope still flickered for a broad reaching, if not comprehensive, natural bacterial system [Heberlein et al., 1967; Schleifer and Kandler, 1972].)

Cleaving the Gordian Knot It was only a matter of time before the sleeping giant of bacterial phylogeny would be roused by a dose of molecular medicine. By the mid-1960s Sanger's "oligonucleotide cataloging" method had come along (Sanger et al., 1965). By 1970, my laboratory was using it on ribosomal RNAs (Sogin et al., 1971; Woese et al., 1974). The universal distribution of rRNA, its large size, its high degree of sequence conservation, and a presumed refractoriness of rDNA to horizontal gene displacement argued that the molecule could be used to derive a universal phylogeny (Fox et al., 1977b; Woese, 1987). By 1975, we had characterized about 30 rRNAs (mainly bacterial) by the method (Woese et al., 1975), a number that approached a hundred by the end of the decade. With the 1980s, newer and faster methods permitted effectively full sequencing of an rRNA molecule (Lane et al., 1985), which significantly sharpened branching orders in the universal tree. Today the sequencing of rRNA (or rDNA) has become trivial, and the collection of rRNA sequences now numbers in the tens of thousands (Maidak et al., 2001).

The most important findings to come out of this new and revolutionary approach to bacterial taxonomy (and taxonomy in general) were that, yes, phylogenetic relationships among bacteria (distant or not) could be determined, and so, developing a clear concept of a bacterium was now feasible, second in importance only to the determining of the universal phylogenetic tree itself (Fox et al., 1980)—one of the great problems that the nineteenth century in biology bequeathed to the twentieth. As Darwin had said (Burkhardt and Smith, 1990): "The time will come I believe, though I shall not live to see it, when we shall have very fairly true genealogical trees of each great kingdom of nature" (Darwin Corresp. 6:456). The discovery of the Archaea (Fox et al., 1977a; Woese and Fox, 1977b) once and for all exploded the prokaryote/eukaryote phylogenetic myth. The universal tree shows prokaryotes to comprise not one, but two, unrelated major organismal groups (primary lines of descent), the Archaea and the Eubacteria. The new perception of microbial diversity that came out of the universal tree, in addition to the unexpected discovery of a third highest level grouping of organisms, found biology organizationally unprepared. Academic department structures, their organization and courses, did not and still do not reflect the new reality. Most textbooks do not do so adequately even today. And the funding priorities for governmental supported biol-

ogy remain structured in a way that recognizes a bipartite, not tripartite, division of life.

Reaching into the Unknown While rRNA phylogenies put bacteriology on the road to becoming a full fledged organismal discipline, they alone did not completely resolve bacterial ecology's core problem: a phylogenetic framework is a necessary but not sufficient condition for developing bacterial ecology. To identify a species, the bacterial ecologist has first to detect and isolate it. Proper identification of a bacterial species was firmly believed to require its cultivation (in pure culture) in the laboratory. Yet the vast majority of bacterial species in the typical niche go undetected, and among those that can be detected, most defy laboratory (pure) culture. (Just how severe a problem this was did not become apparent until relatively recently, well after the fact.)

But, contrary to established belief, isolation of bacteria in laboratory culture is not an absolute requirement for bacterial identification and classification. Since bacteria can now be identified and related merely through the sequence of one molecule, rRNA, only that molecule needs to be isolated from the environment to detect, identify, and classify organisms. This realization seems first to have occurred to Norman Pace, in the early 1980s. Over the next several years, he and his coworkers published a series of articles pointing out in principle and demonstrating in practice the new molecular sequence-based approach to microbial ecology (Stahl et al., 1985; Olsen et al., 1986). The crippling limitations that organismal detection and laboratory isolation had previously imposed on microbial ecology had vanished!

Pace and coworkers went further, showing that for certain purposes it was not even necessary to *isolate* a molecule (or its gene) from the environment: "phylogenetic stains" (sets of fluorescent nucleic acid probes for rRNA) could be developed to identify individual cells microscopically *in situ by species, genus, family, etc.* (DeLong et al., 1989). Using this powerful new methodology, a microbiologist could identify any and virtually all microbial species in a given niche in terms of phylogenetic relationships and morphology (and that, obviously, is only the beginning for such a methodology). At last a genuine bacterial ecology is emerging! It is impossible to overestimate the importance of phylogeny and direct detection of genes to bacterial ecology's development.

Perspective

Throughout its history molecular biology has been guided in one way or another by "the problem of the gene," which basically defined twentieth century biology. What would molecular biology have been like had the gene not been its focus? I ask this simply to provide perspective in posing a second question: what would bacteriology have been like if developing "a concept of a bacterium" *had* been its central focus, and it had succeeded in doing so—allowing bacteriology to become a full fledged organismal science?

Early on, microbiologists were not averse to asking evolutionary questions: are all bacteria at base specifically related to one another? Did bacterial evolution follow a course primarily laid out by morphological or physiological development? Were the first bacteria heterotrophs or autotrophs? Were they mesophiles or thermophiles? Are all photosynthetic bacteria related to one another to the exclusion of nonphotosynthetic species? How did the many diverse bacterial metabolisms come into being? How many times did this or that phenotypic feature evolve? Does this or that common feature signify relationship or convergent evolution? Unfortunately, all such questions (necessarily) were answered only by (untestable) conjectures—not a very reliable foundation upon which to base a concept of bacteria. A fabric of conjecture soon comes to be viewed as metascience (Stanier, 1970a), and this has the effect (most unfortunate in microbiology's case) of squelching inquiry. In this way, bacteriology came to abandon its evolutionary curiosity—much to its and biology's detriment.

Just as unsubstantiated conjecture leads to stifling curiosity, phylogenetic knowledge does the opposite. Fairly reliable responses to the early microbiologists' evolutionary musings would have led to the asking of new, more detailed questions. Had a reasonable natural bacterial classification come on the scene early enough, the spirit of evolutionary inquiry would not have withered but intensified, and a genuine concept of bacteria would naturally have followed. The development of all twentieth century biology would have been different, retaining to some extent a focus on the organism as a whole and its evolution. Ah well. . . .

Archaea and Eubacteria

As discussed above, the character of a prokaryotic type of cell is expressed far more in physiology (dynamic pattern) than in morphology (static pattern), which underlies why bacterial morphologies and the like are so uninformative (even deceiving) phylogenetically. It also accounts for why the two prokaryotic cell types appear so similar upon superficial analysis. At

the molecular level, however, this impression of kinship between the two quickly evaporates. In their histories, the two prokaryotic types have followed very different paths that from time to time have crossed, leading to the occasional transfer of genes (especially early on), which accounts for much of the similarity often taken to signify specific relationship at the organismal level. Yet in looking at the core functionality of the cell, the enormous gulf between archaea and eubacteria leaps out.

Metabolism, Membranes and Walls

Let us begin a comparison of the two types with their metabolisms, wherein the differences are relatively few, but still significant. The eubacteria are far and away the most metabolically diverse and versatile group of organisms on the planet. The metabolic uniqueness of the archaea is most prominently displayed in methanogenesis, a metabolism confined to the Euryarchaeota. The most interesting thing about archaeal metabolism may be the unique set of cofactors it employs (mainly in methanogenesis). To mention some: the C-1 carriers, coenzyme M, methanofuran, and methanopterin; F_{420}, an electron carrier analogous to NAD; F_{430}, a nickel-containing porphyrin akin to heme, vitamin B_{12}, and chlorophyll; and methanophenazine, a membrane-bound carrier that functions like a quinone (Wolfe, 1992; Beifuss et al., 2000). Viewed metabolically these cofactors are interesting but not all that unusual in the reaction types they catalyze. What is unusual and intriguing, however, are their biosyntheses (the study of which is still in its beginning phases). While the pathways in archaeal cofactor synthesis themselves tend to be fairly standard in a chemical sense, the enzymes catalyzing the reactions more often than not have no homologs outside of the archaea. As suggested by Graham and White (2002), the unity of biochemistry we have known since the time of Kluyver may be as much a matter of evolutionary convergence on common biochemical themes as it is retention of ancestral metabolic ways.

Archaeal membrane structure is unique as well. It is based upon ether-linked branched-chain lipids, whereas in the eubacterial and eukaryotic cases, straight-chain ester-linked lipids predominate (Kates, 1964; Kates et al., 1968; Torrnabene and Langworthy, 1979). Cell wall structures differ too. The eubacterial wall characteristically comprises murein (peptidoglycan), while archaeal walls are most often proteinaceous (Kandler and Hippe, 1977; Kandler and König, 1985). In Methanobacteriales species, however, walls comprise pseudomurein, a compound that, as the name suggests, resembles eubacterial murein (König and Kandler, 1979). Yet the sugars from which murein and pseudomurein are built are largely different, and when the biosynthetic mechanisms involved in wall formation are considered, it is clear that murein and pseudomurein are another example of biochemical convergence (Hartmann and König, 1990).

Translation

It is in information processing that the differences between eubacterial and archaeal cell designs appear in their full glory. Of the three main information processing systems (translation, transcription, and genome replication), translation exhibits the most universality in its componentry, the most homology between archaea and eubacteria (and eukaryotes). Ribosomal RNAs, elongation factors, ribosomal proteins, aminoacyl-tRNA synthetases are all basically universal. However, among these universal protein components, a clear distinction exists between the eubacterial and archaeal versions. The distinction is characteristically so blatant that it tends to stand out even upon gross inspection of a sequence alignment (Woese et al., 2000). (For example, the eubacterial versions of a given sequence often contain moderate to large blocks of amino acids [located terminally or in the interior of the molecule] not seen in the archaeal versions and *vice versa*. In addition, for those columns in an alignment where composition is highly conserved, one often sees that the characteristic eubacterial composition differs from that characteristic of the archaea.) This nearly qualitative distinction between archaeal and eubacterial versions of a sequence, this difference in genre between the two, has been called the "canonical phylogenetic pattern" (Woese et al., 2000). Differences this extreme are never encountered among the various eubacterial taxa or among different archaeal taxa (although in each case there have been three billion years or so, most of this planet's history, in which such differences could have arisen). When eubacterial and archaeal sequences show these differences in genre, their eukaryotic counterparts are almost always of the archaeal genre (Woese et al., 2000).

A minority of ribosomal proteins, however, are not universally distributed. A relatively small cadre are characteristic of and found only in eubacteria, while a somewhat larger set is common and confined to the archaea and eukaryotes, with yet a few others being found only in eukaryotic ribosomes. Overall, though, translation gives the impression of a system that existed in basically modern form at the root of the phylogenetic tree.

Transcription

Transcription presents a similar evolutionary picture, but with more, and more pronounced, exceptions to universality (Langer and Zillig, 1995). The two largest (the catalytic) subunits of the DNA-dependent RNA polymerase, β and β′ in eubacterial nomenclature, are clearly universal and show the canonical pattern (with again eukaryotic sequences being of the archaeal genre). But that's about it for homology. For the remaining main subunit of the eubacterial RNA polymerase (α), homology between the eubacterial and archaeal versions is only partial. Eubacterial α occurs in two copies in the holoenzyme, whereas its archaeal counterpart (found also in eukaryotes) comprises two separate proteins of very different size, each present in single copy in the holoenzyme, with parts of each showing homology to (somewhat) different parts of eubacterial α (Langer and Zillig, 1995). The archaeal transcription polymerase has a number of additional (smaller) subunits, all of which occur in the eukaryotic enzyme(s), but not in the eubacterial. And as in the case of translation, the eukaryotic version(s) of the transcription polymerase contain a few additional subunits specific to themselves (Tjian, 1996). When it comes to transcription initiation, one sees no homology between the eubacteria and the archaea: the two mechanisms are different and use different componentry (Bell and Jackson, 2001). Once again the eukaryotes possess an embellished archaeal version.

Genome Replication

Genome replication is the extreme example of archaeal/eubacterial difference. The closely related archaeal and eukaryotic systems are to a first approximation totally distinct from their eubacterial counterpart (Olsen and Woese, 1996). And, as now might be expected, the mechanism of initiation of chromosome replication in the eubacteria is fundamentally different from that basically common to the archaea and eukaryotes (Kelman, 2000). In the past it was common to draw phylogenetic significance (i.e., specific relationship) from the fact that archaea and eubacteria both have circular chromosomes, in distinction to eukaryotic chromosomes, which are linear. In light of the similarity between the archaea and eukaryotes in both chromosome replication and the initiation thereof, that conclusion needs revisiting.

It is also of interest that both the eukaryotic and archaeal (euryarchaeal) chromosomes show nucleosome organization—the single archaeal histone responsible being a homolog of the four (related) histones that structure the eukaryotic nucleosome (Reeve et al., 1997). This is an example of a theme that repeatedly occurs in the chromosome replication mechanism and elsewhere, namely: a job is done by a particular multimeric protein complex. The many subunits therein, however, represent only one or a few gene families. In the eukaryotic case, a number of different members of a family tend to be represented in the complex, each present in *single* copy. In the archaeal case, there tends to be only one member of the corresponding family, and that present in *multiple* copies.

Comparisons between the information processing systems of Archaea and Eukarya are what give rise to the strong impression of fundamental relationship between the two. However, that specific relationship is reflected in relatively few molecules other than those involved in information processing, one example being HSP-60. Overall, the relationships among the three basic cell types present a complex, fascinating conundrum—a delightful challenge.

The close resemblance between archaeal and eukaryotic versions of the three information processing systems has been the source of much speculation as to the origin of the eukaryotic cell, speculation that usually takes the form of invoking a fusion of a particular bacterial cell (such as an α proteobacterium) with a particular archaeal cell (say, a methanogen; Martin and Mueller, 1998) or sees some hypothetical ancient "protoeukaryotic" cell as horizontally acquiring certain metabolic capabilities from the eubacteria and information processing capabilities from the archaea (Hartman, 1984). I do not like either type of explanation. The first type gives the eukaryotic cell no character of its own before it emerges from the fusion of the two prokaryotic types. In the second, the protoeukaryotic cell suddenly acquires information processing systems that it has never before encountered, and its basic cellular design is suddenly altered. All such models miss the essential point in cellular evolution: the core machinery of the three primary cell types evolved and became established in each case well before any cells achieved their "modern" type of organization (Woese, 2002). Evolution does not proceed by suddenly and drastically altering a given cell design (at least a fairly advanced one). These models ignore the possibility (likelihood) that a cell design is stable, homeostatic, in an evolutionary sense.

Horizontal Gene Transfer: How Cells Evolved

It might seem that horizontal gene transfer (HGT) would be a complicating factor in understanding how the various major cell types

evolved. Yet, far from being a complicating factor, HGT turns out to be the essence of the process (Woese, 2000). As is now well known, HGT appears to have had (at certain times in evolution) the potential not only to introduce entirely new functions into cells, but to displace any constituent (gene) in the cell with an alien equivalent. What effect has HGT had on the genetic record of organismal descent? What is its relationship to canonical pattern? Understanding here pivots on what the characteristics of HGT are and what factors determine them.

The interested reader will find a more extensive and useful discussion of the major problem of evolving cellular organization and its relevance to the universal phylogenetic tree structure in Woese (Woese, 2000; Woese, 2002).

The most obvious thing about HGT is its extreme variability, in both frequency and phylogenetic range, from one gene to another, from one major taxon to another, and *likely* from one evolutionary stage to another (Woese et al., 2000). It turns out that the dominant factor shaping HGT is the organization (design) of the potential recipient cell (Woese, 2002). The various components of a cell are in one way or another, to one extent or another, interconnected, variously integrated into the cell's "design fabric." Some of them are weakly and simply integrated into the fabric, others tightly and complexly so (Woese and Fox, 1977a; Woese et al., 2000). Components of the first type are in effect modular: functionally and structurally they are pretty much self-contained and self-defining, i.e., their interactions with the rest of the cell componentry are minimal. It stands to reason that such a modular, loosely coupled, component is far more likely to undergo horizontal gene displacement by some alien equivalent than is highly and idiosyncratically integrated componentry.

An ideal system for assessing the character of HGT is the translation apparatus, for while most of its componentry, e.g., the ribosomal proteins and elongation factors, are tightly coupled into the rest of the mechanism (and hence the cell fabric), the tRNA charging enzymes (aminoacyl-tRNA synthetases) are not; the latter are modular, are loosely coupled into the cellular fabric. And as might then be expected, the ribosomal proteins and elongation factors have been largely unaffected by HGT, whereas the aminoacyl-tRNA synthetases have been quite highly buffeted by it, both in terms of the frequency with which HGT has affected them and the (broad) phylogenetic range of many of the alien displacements that have occurred (Woese, 2000). It is common, for example, to see the archaeal genre of a given aminoacyl-tRNA synthetase in one or a few eubacterial lineages (Woese et al., 2000).

A detailed phylogenetic analysis of the 20-odd aminoacyl-tRNA synthetases (Woese et al., 2000) shows: 1) that about two thirds of the tRNA charging enzymes exhibit the canonical pattern typical of the other components of the cellular information processing systems; and 2) that in all these cases the canonical pattern has to one extent or another been eroded by HGT; but 3) that the degree to which and the ways in which that pattern has been eroded are idiosyncratic, qualitatively different for each of the enzymes. From these facts it was concluded that the canonical phylogenetic pattern itself was not produced by HGT, but reflects some earlier evolutionary dynamic (Woese et al., 2000). Thus, the canonical pattern represents a (partial) genetic record of organismal histories that extends back to the root of the universal tree. In other words, while HGT has been a powerful, pervasive, and shaping force in organismal evolution (especially early on), it has not completely obliterated the ancestral organismal trace that the universal tree represents.

As the reader can see, I cannot discuss similarities and differences among the major organismal types without the discussion shifting into an evolutionary framework. This, however, is no peculiarity of mine, but rather the fact that such matters cannot be productively discussed in any other context. So, like it or not, microbiology is going to be in the center of evolutionary study in the future—and *vice versa*.

Microbiology on the Move

A great deal has happened in bacteriology in the last decade or so. The four pillars upon which a true bacteriology must be built are now in place, and the long sought "concept of bacteria" is beginning to take shape. Bacteria are now routinely seen in terms of their natural (phylogenetic) relationships; discussions often turn on various aspects of their evolution. The sleeping giant of microbial ecology has finally awakened and we are beginning to see the microbial base of the biosphere. Geomicrobiology, which increasingly ties bacteria to geological processes, is a burgeoning field. Marine microbial ecology is bringing to the fore the important role that bacteria play in determining the character of the oceans. Genomic microbiology is beginning to tackle some of the knotty problems associated with bacterial evolution, adaptation to environments, and the evolution of the global ecosystem itself. These and similar developments are the potential building blocks of a new microbiology. But the concrescence of twenty-first century microbiology will not occur unless we, its practitioners, understand *what bacteriology is and, thus, what its future goals are*.

To me, microbiology today resembles a peasant who has suddenly become wealthy. The chains of poverty are gone, but the cobwebs that are the habits of poverty remain and hold him fast. Obviously there are no longer scientific barriers to bacteriology's becoming the organismal discipline it should long ago have become. But, who cares anymore? The idealists among us, whose crucial role it had been to define and develop the character of bacteriology, to keep the discipline on track, are long gone. The character of bacteriology (like that of the peasant) is essentially defined by overlords, external circumstances, be they scientific or practical (societal). Contrast bacteriology to disciplines such as genetics and molecular biology, disciplines that once were strong in being defined from within—disciplines whose paradigms embodied major scientific goals, disciplines that had a grand vision. Microbiology was never that. Today's microbiologists are, unfortunately, the realists of yesteryear. The field is without overview, without a concept of itself, and its goals remain shaped by practical considerations and influences from the stronger disciplines. Only an agonizing awareness of the situation will change that.

Where Is Our Essence?

It is time to take stock. Microbiology today is not the discipline microbiologists knew 30 years ago. Everything is new—the technology, the problems that the discipline faces, our training, our conceptual framework, and the way microbiology is perceived by the other sciences. Only the organisms remain the same. But is this last even true? The organisms *per se* may be the same, but our perception of them surely isn't. What is a bacterium to us except a synthesis of the various ways in which we experience it? That surely has changed, dramatically so. (Is one's concept of bacteroides and cytophagas, for example, the same as it previously was once one knows that these two phenotypically dissimilar types are actually specifically related to one another? What are *Deinococcus* and *Thermus* doing in the same stable? Are the myxobacteria that we know today as members of the δ-proteobacteria the same as the classical myxobacteria, which if they had any affiliation at all, it was to the cyanobacteria?) The only things that remain at all constant are the prejudices we have inherited from our intellectual forbears. Time to clear the table!

There are two fundamental challenges that microbiology faces today, challenges deep and basic enough that they speak to the whole of biology. First is the age-old question "where do we come from," which can now be addressed in the form of how cellular organization evolved. The other is the problem of the biosphere: what is this incredibly complex yet smooth functioning web of interactions among this incredibly diverse collection of organisms—this coherent, self-sustaining, overarching state of the whole? Actually these two problems are at base the same. And the understanding of both lies in the microbial world, in its diversity, in the (functional) structure of microbial communities, in the organization of microbial cells, in evolutionary and other adaptive responses of microorganisms to environmental perturbations, and in the incredible interconnectedness of microbial life in general. In a way, twenty-first century microbiology is a return to microbiology's roots, a rediscovered and development of the grand view of microorganisms that Beijerinck and other early microbiologists shared.

The View from the Top (see The Phylogeny of the Prokaryotes: A Living Tree in Volume 2)

We have discussed above the major features that distinguish the Archaea and Eubacteria. What remains to be done is provide an organismal overview of the individual domains. We will go about this in a top down fashion, starting with the major eubacterial and archaeal taxa, leaving the details and definitions of the lower, subordinate taxa to you and the next generation of microbiologists. I do not claim that these high level taxa as currently defined are entirely correct (are genuine naturally defined groupings) in all cases, but most of them surely are, and, therefore, our discussing them at this point is useful. Let us start with:

The Organismal Structure of the Archaea

As we have seen above (and elsewhere), the Archaea represent a unique cell design (Graham et al., 2000). In their cellular organization they stand apart from the Eubacteria and, of course, the Eukarya. The Archaea are also unique in their overall phylogenetic structure: the domain comprises two characterized kingdoms, the Euryarchaeota and the Crenarchaeota—with the possibility of a third kingdom, the "Korarchaeota," lurking among the unwashed masses of species that have been identified only through direct isolation of rRNA genes from the environment (Barns et al., 1996). Even a fourth archaeal kingdom is suggested by the recent discovery of a bizarre nano-sized exosymbiont associated with particular ones of the Crenarchaeota (Huber et al., 2002). What makes archaeal phylogeny stand apart from that of the Eubacteria and Eukarya is the pronounced phenotypic and

genotypic distinction between the kingdoms Euryarchaeota and Crenarchaeota—the depth of their phylogenetic split, the genomic differences between them. Interkingdom differences of this magnitude are not seen in the other two domains.

The euryarchaeota appear phenotypically constituted (designed) around a methanogenic metabolism. They are also characteristically thermophilic and show relatively high intracellular concentrations of monovalent ions. To our present knowledge, the kingdom has spawned four main methanogenic branches (phyla), the *Methanococcales,* the *Methanobacteriales,* the *Methanomicrobiales,* and the *Methanopyrales* (Olsen et al., 1994; see Methanococcales, Methanobacteriales and Methanopyrales and Methanomicrobiales in Volume 3).

The euryarchaeal kingdom also harbors other, non-methanogenic, phenotypes. Interestingly, all but one of these are offshoots of the lineage that gives rise to the Methanomicrobiales. The non-methanogenic phenotypes in question are the extreme halophiles, the sulfate reducing archaea (Archaeoglobales) and the Thermoplasmatales. The lone non-methanogen group not stemming from this lineage is the Thermococcales (which comprises the genera *Thermococcus* and *Pyrococcus*); see The Order Halobacteriales, Archaeoglobus, Thermoplasmatales and The Order Thermococcales in Volume 3.

The extreme halophiles are facultative aerobes that grow in highly saline environments and have been known to microbiologists for ages: they are known for putrefying salted fish among other things. The extreme halophiles are also notable for the reddish pigment(s) they produce, which are in part responsible for the striking red color in salt evaporation ponds. Among these pigments is bacterial (now archaeal) rhodopsin, which provides them a photochemistry. Archaeal rhodopsin is central to a light driven transmembrane pump, by means of which the extreme halophiles can derive energy; see The Order Halobacteriales in Volume 3.

Beyond their extremely high (5M) intracellular potassium ion levels and their photochemical ability (unique among cultured bacteria), the extreme halophiles offer little of interest, unless it be the fact that as an organismal group they are phylogenetically very tightly clustered—and so, of relatively recent origin.

One naturally assumes, given the specific (though somewhat distant) phylogenetic relationship to the Methanomicrobiales, that the metabolism of the halophiles is probably a derived, degenerate form of the metabolism characteristic of the Methanomicrobiales. But, the halophile genome shows something different. It is rife with bacterial genes and others that have no relationships functionally or phylogenetically to methanogens and methanogenesis.

The second grouping in the Methanomicrobiales cluster comprises the anaerobic sulfate reducing organisms of the family Archaeoglobales—see Archaeoglobus in Volume 3. Iron reduction is seen in the genus *Ferroglobus* (Hafenbradl et al., 1996). The members of this order, though anaerobic, produce no methane, except for a trickle thereof. The biochemical reason is obvious: the gene for the terminal enzyme in the methanogenic pathway, methyl CoM reductase, is missing from their genomes. Yet the rest of the methanogenic pathway appears intact in these organisms. However, *Archaeoglobus sp*. run the pathway backward (oxidatively), producing carbon dioxide as the end product. The Archaeoglobales are highly thermophilic, with no known exceptions, which distinguishes them from their sister lineages, the Methanomicrobiales and extreme halophiles, lineages that do not appear to harbor hard-core thermophiles.

The final aberrant phenotype within the Methanomicrobiales segment of the euryarchaeal tree is the Thermoplasmatales—see Thermoplasmatales in Volume 3. They are ostensibly wall-less thermophiles (unique among the archaea), whose thermoacidophilic phenotype superficially resembles that of the crenarchaeota. And again iron metabolism surfaces in the genus Ferroplasma (Golyshina et al., 2000). Direct environmental sampling for rRNA genes reveals that the Thermoplasmales also encompass a low temperature marine grouping (DeLong, 1992)—whose phenotype is otherwise unknown. In their genomic makeup these organisms are the most phylogenetically cosmopolitan of all the archaea.

I find it intriguing that among all euryarchaeota, it is only the Methanomicrobiales (and their ancestral lineage) that have proven so evolutionarily inventive. In addition to spawning the above-described aberrant phenotypes, the Methanomicrobiales proper have been inventive in terms of methanogenesis itself. These are the only methanogens that utilize certain carbon sources other than carbon dioxide (e.g., acetate) in methanogenesis. And they seem to be able to utilize the methanogenesis pathway in both directions—which they do in metabolizing acetate (e.g., see The Order Methanomicrobiales in Volume 3). Interestingly, their genomes are quite large by archaeal standards (Deppenmeier et al., 2002; Galagan et al., 2002)—and they contain a number of non-archaeal genes!

Among the euryarchaeotes one finds both autotrophic and heterotrophic organisms (which is typical of large organismal groupings, of course). In the present case, the phylogenetic arrangement and genomic analysis of species

strongly suggest that the heterotrophs are derived from the autotrophs, not the reverse.

The Crenarchaeota, the other of the (classically recognized) archaeal kingdoms, cannot be usefully characterized at this point in time. The reason is two-fold. For one, the group has been relatively poorly studied in the laboratory. For another, the crenarchaeal species isolated in laboratory culture form only a phylogenetically restricted and nonrepresentative subset of the kingdom as a whole: direct environmental gene probing has shown that the crenarchaeal tree contains many branchings that are far more deeply placed than those of the cultured crenarchaeal species (DeLong, 1992; Barns et al., 1994). I will leave you with this impression: crenarchaeota are basically thermophilic organisms whose metabolisms somehow feature sulfur prominently. They are basically anaerobic, though they can be facultatively aerobic in some cases. And, as an evolutionist might expect, mesophilic (even psychrophilic) phenotypes also exist. Indeed, one of the major phenotypes in the oceans—the largest biotope on the planet—represents one particular crenarchaeal group, the Order Cenarchaeales, which abounds throughout the breadth and depth of the oceans, being remarkably in evidence in Antarctic waters (DeLong, 1997).

I have one closing thought concerning the archaea. The depth of the phylogenetic split between the two archaeal kingdoms seems the tip of an evolutionary iceberg. We have much to learn evolutionarily from this group of organisms. It saddens me to see how little interest goes into studying the archaea as an organismal group. And the fact that there exists an entire archaeal kingdom, the Korarchaeota, not a single representative of which has been isolated, is doubly distressing.

The Organismal Structure of the Eubacteria

The eubacteria are the consummate metabolists: sheer metabolic power and versatility. They are the masters of metabolic theme and variation—just as eukaryotes are masters of structural theme and variation. Some day we will understand just how eubacteria could have acquired such a varied metabolic repertoire. Their versatility is the more interesting when one realizes (from genomic analysis) that much of the more limited metabolic repertoire of the Archaea beyond that characteristic of the methanogens and methanogenesis is (as alluded to above) the product of metabolic capabilities (enzymes and pathways) imported from the eubacterial world.

The phylogenetic structure of the Eubacteria is rather different from the archaeal one just discussed. Viewed from afar, the main eubacterial lineages appear to radiate out from a "crown group," with a few atavistic forerunners branching from the eubacterial stem slightly earlier (examples being the Aquificales and the Thermotogales). (It is the crown radiation that spawns the phenotypic richness we characteristically associate with the Eubacteria.)

Among the major (kingdom level) eubacterial taxa—those dominant by virtue of numbers, phylogenetic diversity, and ecological impact—there exist several kingdoms and phyla about which we know next to nothing. Only a few, if any, cultured species represent them, their evolutionary breadth and ecological prominence being demonstrated by the large numbers of environmentally derived rRNA sequences from the various groupings (Pace, 1997). The lesson that has yet to sink in is that microbiologists *know relatively little about the bacterial world*. We clearly have no useful idea of what some of the major environmentally important bacterial groups are and are doing. You would think that a great deal of work would now be going into remedying this situation. This is not the case, however. Ask yourselves: do microbiologists have better things to do?

Let us begin with the major (most significant) eubacterial kingdoms and phyla, and then, when appropriate, extend to their subordinate classes. I more or less lump kingdoms and phyla here, because—until the proper genomic criteria have been developed to define taxonomic rank on the basis of the properties of naturally defined organismal groups—it is not in some cases possible to distinguish the two.

THE PROTEOBACTERIA One of the most, if not the most, ecologically dominant eubacterial kingdoms is the Proteobacteria (see Introduction to the Proteobacteria in Volume 5). The kingdom comprises five phyla, distinguished by the Greek letters αε. The α, β, and γ phyla obviously cluster, which easily emerges by any number of different phylogenetic analyses, while δ and ε, which may be specifically related, stand apart—but still within the proteobacterial kingdom. The β-phylum is actually a major branching within γ. I still consider β to be of phylum rank because of the apparent phenotypic diversity within it and its branching from a section of the γ-tree which, except for the β-branch, contains no photosynthetic representatives. (A taxonomic precedent for such a ranking is given by the class of mammals arising from within the class of reptiles.)

The α-phylum (see The Phototrophic Alpha-Proteobacteria in Volume 5) to a first approximation comprises (purple) photosynthetic lineages, nonphotosynthetic derivatives thereof, and a variety of pathogenic groups, mainly intra-

cellular pathogens. The most notable of the latter is the rickettsial group (see The Order Rickettsiales in Volume 5) (which comprises the genera *Rickettsia*, *Wolbachia*, *Ehrlichia*, *Anaplasma*, and *Cowdria* [see Introduction to the Rickettsiales and Other Intracellular Prokaryotes; The Genus *Wolbachia*; The Order Rickettsides; and The Genus *Bartonella* all in Volume 5]). What seem to be free-living relatives of these pathogens have been detected by rRNA analyses from marine habitats, although none have been cultured.

While the closely related agrobacteria and rhizobacteria, which play such important roles in the plant kingdom, are not members of the rickettsial group proper, the mitochondria are (Gray et al., 2001). At this point I'm going to leave further exploration of this interesting eubacterial phylum to the tastes of the reader—but not without first mentioning an evolutionary peculiarity of the α-proteobacteria. The α-phylum appears (anecdotally) to be (to have been) more prone to the horizontal import of genes than are most or all other proteobacterial phyla, as judged by the number of archaeal genre aminoacyl-tRNA synthetase genes they contain (Woese et al., 2000). A systematic study of HGT in the α-phylum is definitely called for, especially an accounting of when in the phylum's evolutionary history the bulk of these transfers occurred.

Little more needs be said than already has been about the β-phylum (see The Phototrophic Betaproteobacteria in Volume 5). The photosynthetic phenotype therein (represented in several lineages) is unlike the photosynthesis found in the γ-phylum (the latter are the purple sulfur bacteria), but akin to the purple non-sulfur types seen in the α-phylum. The β-phylum is again a haven for pathogens (e.g., chapters on *Burkholderia* [see The Genus *Burkholderia* in Volume 5], *Neisseria* [see The Genus *Neisseria* in Volume 5], and *Bordetella* [see The Genus *Bordetella* in Volume 5]) but also for a varied collection of metabolisms (e.g., chapters on the lithotrophic ammonia and nitrite oxidizers [see The Lithoautotrophic Ammonia-Oxidizing Bacteria and Nitrate-Oxidizing Bacteria in Volume 5], *Thiobacillus* [see The Genus *Thiobacillus* in Volume 5], and the dinitrogen-fixing *Derxia* [see The Genus *Derxia* also in Volume 5]). I am inclined to believe, on the basis of the relative uniqueness of the characteristic β-rRNA sequence, that the grouping may have begun as a rapidly evolving off-shoot of the γ-lineage.

The γ-proteobacteria do live up to the implication of the designation "proteo-"; a great deal of morphological and other phenotypic variation is housed therein. Thus, one can make few generalizations about the phylum as a whole. The purple photosynthesis characteristic of the γ-phylum is, as just mentioned, biochemically unique, and the lineages harboring it, the genera *Chromatium* (see The Chromatiaceae in Volume 6), *Ectothiorhodospira* (see The Family Ectothiorhodospiraceae in Volume 6), and closely related kin branch early from the γ-stem. These photosynthetic γ-proteobacteria deserve far more genomic attention than they have been given to date. I stress this because if photosynthesis is ancestral to the proteobacteria, at least to the α-β-γ cluster therein, then a genomic understanding of the photosynthetic representatives of the kingdom should prove key in the longer term to an understanding of the group's evolution.

This leaves the δ- and -phyla of the proteobacteria. Again a lack of significant genomic coverage of this assemblage—except, of course, for a notable preoccupation with the pathogenic helicobacteria—leaves little to be said. Perhaps the most significant phenotypic characteristic of the group is a lack of photosynthetic representatives. The reduction of sulfur compounds is a predominant theme among the δ-lineages. One would like to see a representative genomic sweep of the δ-phylum and some genomic emphasis on the nonpathogenic members of the -phylum, such as *Wolinella* (see The Genus *Wolinella* in Volume 7) and *Thiovulum*.

Mention must be made, of course, of the myxobacteria (see The Myxobacteria in Volume 7), which are one of the four major classically recognized bacterial groups (see quote from *The Microbial World* second edition, above). Under molecular scrutiny, however, the myxobacteria turn out to be subordinate to, a lineage within, the δ-proteobacteria. Yet, their complex and beautiful morphologies and complicated life cycles give cause to wonder. How could such morphologically rich and metabolically versatile entities phylogenetically reside among the otherwise morphologically drab δ-proteobacteria? The genomes of the myxobacteria are inordinately large, much larger than other δ-proteobacteria. Within their excess DNA must lie the answer.

The Gram-Positive Eubacteria This ecologically and phylogenetically major eubacterial kingdom (see The Genus *Eubacteria* in Volume 4) is one of the few classical eubacterial groupings to survive relatively unscathed the molecular phylogenetic assault on bacterial taxonomy. A fair bit of taxonomic reorganization has occurred within the kingdom, and some new members that do not possess typical Gram-positive walls have been added. But the two classical traits, endospore formation and Gram-positive wall, turn out to be phylogenetically

valid characteristics. The only real taxonomic surprise has been the addition of the photosynthetic heliobacteria to the group (Woese et al., 1985a; see The Family Heliobacteriaceae in Volume 4).

The main phylogenetic split among the Gram-positive eubacteria separates the "high G+C" group (the actinomycetes [see Introduction to the Classification of the Actinomyces in Volume 3], mycobacteria [see The Genus Mycobacterium–Medical and Non-Pathogenic Mycobacteria in Volume 3], and a number of others) from the "low G+C" group. The phylogenetic split between the two is great enough that their specific relationship might be questioned. While the coherence of the entire kingdom was apparent in phylogenetic analyses of the small subunit rRNAs (Olsen et al., 1994), it cannot be reliably demonstrated in my experience using the large subunit rRNAs. What is called for here is an appropriate test of the coherence of this kingdom based upon genomic analysis of the proper Gram-positive genomes—key ones of which have yet to be sequenced, however.

While most eubacterial genomes are sequenced for medical reasons—and this is surely true in the Gram-positive kingdom—one would still not single out the kingdom as an evolutionary training ground for pathogens. Metabolic versatility is its hallmark. The clostridia (not a genus or family, but a phylogenetically widely dispersed agglomerate of many different low G+C Gram-positive lineages) are classically notorious for the variety of degradative biochemistries of which they are collectively capable. Several clostridial genomes have now been sequenced, but many more are needed, and I would hope chosen in a systematic, phylogenetically comprehensive manner.

The Cyanobacterial Kingdom This grouping (see Cyanobacteria–Isolation, Purification, and Identification in Volume 4 and Cyanobacteria: Ecology, Physiology, and Molecular Genetics also in Volume 4) is one of the very few high-level eubacterial taxa that are anywhere near phenotypically uniform. Morphological variety abounds (as expected for a high-level taxon), but chlorophyll-based photosynthesis appears a constant metabolic theme in all. Classically isolated cyanobacteria contained only chlorophyll *a*, whereas the chloroplasts of plants, which are domesticated cyanobacteria, contain both chlorophylls *a* and *b*. Cyanobacteria that contain both chlorophylls have more recently been found (Lewin, 1984). Although the chloroplast lineage(s) traces to the general cyanobacterial cluster, no lineage representing a free-living cyanobacterium shows a specific relationship to the chloroplast lineage. Indeed the cyanobacterial phylogenetic tree has a remarkably bushy appearance. Why this is so is again the type of question that an evolutionarily oriented bacteriology finds interesting. Did the invention of oxygenic photosynthesis in the ancestor of the whole group kick off a major evolutionary radiation, open a vast new evolutionary world for exploration?

It should be noted that chlorophyll *g*, characteristic of the Gram-positive photosynthetic heliobacteria, is the closest in structure to the cyanobacterial chlorophylls of all the eubacterial chlorophylls. Tantalizing molecular clues have also suggested a specific relationship between the Gram-positive eubacteria and the cyanobacteria—none as yet convincing, however.

The Chlorobium-Cytophaga Kingdom This grouping and the drawing together of the cytophaga-bacteroides phylum (by whatever name) (see The Family Chlorobiaceae; The Order Cytophagales; and the Medically Important Bacteroides ssp. in Health and Disease all in Volume 7) is one of the more significant triumphs of rRNA-based molecular phylogeny of the bacteria. As one can infer from the glut of generic names that constitute the phylum (*Cytophaga*, *Flexibacter*, *Flavobacterium*, *Microscilla*, *Bacteroides*, and a host of lesser known genera), the grouping lay in separate taxonomic pieces before the advent of this molecular approach. The bringing together of the aerobic cytophagas and their ilk with the anaerobic bacteroides was an impressive join. Interestingly, the phylum does not comprise two major classes, split along aerobic/anaerobic lines. The bacteroides cluster forms a rather superficial branching from the main stem of the phylum, and has close specific relatives having a cytophaga phenotype. (None of the latter have piqued the interest of genome sequencers yet.) I would note that the rRNA analysis reveals the coherence of this phylum very clearly: the members of the group exhibit a clean rRNA signature (Woese et al., 1985b).

The specific relationship between cytophagas and the green photosynthetic *Chlorobium* species (the latter an unexpectedly tight phylogenetic cluster for a phylum) was only weakly suggested by the small-subunit rRNA comparisons. To demonstrate the relationship convincingly, we had to resort to sequencing the large subunit rRNA (Burggraf et al., 1991). And although expected, it is most reassuring to see the coherence of the kingdom spelled out in full genomic detail by the comparison of the genome of a *Chlorobium* species (Eisen et al., 2002) with that of *Porphyromonas*, a member of the bacteroides contingent (Paster et al., 1994). In its entirety this kingdom, with its spectacular admixture of phenotypes, demands some sort of

evolutionary rationalization. So, as you might by now expect, I would encourage a concerted, systematic genomic assault on the problem.

THE SPIROCHETES Since bacteriology's beginnings, this kingdom has been recognized as a major taxon (again see above quote from the second edition of *The Microbial World*). The unusual morphology of these organisms together with their unique mode of locomotion makes them stand out among all bacteria. The kingdom is phenotypically (morphologically) pure, monolithic—with one minor exception, an organism originally described as mycoplasma-like, but otherwise uncharacterized (K. Stetter, unpublished observation) except for an rRNA sequence that places it clearly among the spirochetes (Maidak et al., 2001). See Free Living Saccharolytic Spirochetes: The Genus *Spirochaeta* in Volume 7.

The spirochete grouping is deeply split phylogenetically into two major lineages, phyla. The one encompasses the more typical spirochetes such as *Spirochaeta halophila* (and houses the spirochete pathogens to which so much genomic attention has been paid, i.e., *Borrelia* and *Treponema* [see The Genus *Borrelia* and The Genus *Treponema* both in Volume 7]). The other is basically a lineage of aerobic spirochetes, the leptospiras (see The Genus *Leptospira* in Volume 7) and their relatives (some pathogenic).

At some early point in the evolutionary history of the spirochete kingdom (exactly how early cannot be pinned down yet, because genome sequences are not available for the leptospira lineages), the spirochetes experienced a considerable amount of HGT. This assertion, however, is based on a very small sampling of genes, namely the aminoacyl-tRNA synthetases. The spirochetes have replaced more of their ancestral aminoacyl-tRNA synthetases by synthetases of the archaeal genre than has any other eubacterial group (Woese et al., 2000).

THE PLANCTOMYCETES, CHLAMYDIAE, AND VERRUCOMICROBIA These three phenotypically disparate and phylogenetic distant groupings are combined here because fragmentary (unpublished) evidence suggests that they may well turn out to be specifically related to one another (at the kingdom level) when the appropriate genomes are available. Only the chlamydiae have been given significant attention to date (again for reasons of their pathogenicity). The reader is invited to peruse this third edition of *The Prokaryotes* for evidence of this relationship (see The Order Planctomycetales; The Genus *Chlamydia*–Medical; and The Phylum Verrucomicrobia all in Volume 7).

As their name suggests, the *Planctomyces* were originally thought to be eukaryotic. Their walls, as are the walls of chlamydiae and verrucomicrobia, are not typically eubacterial. It is unfortunate that so few examples of the verrucomicrobia exist in culture, for in nature they are clearly a major and ecologically important group of organisms. The planctomyces and relatives also appear to be playing a significant environmental role(s), although perhaps not at the level of the verrucomicrobia.

It has recently been discovered that the *Prosthecobacterium* subgroup of the verrucomicrobia carries two tubulin genes, the only known bacterial sources of these molecules, heretofore considered unique and essential components of the eukaryote cytoskeleton (Jenkins et al., 2002).

THE DEINOCOCCUS-THERMUS AND CHLOROFLEXUS GROUP Here again are two phylum level taxa that, I feel, will some day be united into a eubacterial kingdom. The phylogenetic grouping of Deinococcus and Thermus (see The Genus Thermus and Relatives in Volume 7), two disparate phenotypes in their own right, is another triumph of molecular (rRNA-based) phylogenetics (Weisberg et al., 1989). And the genomic sequence data now available more than confirm the relationship. The *Chloroflexus* grouping (see The Family Chloroflexaceae in Volume 7) likewise was pulled together through rRNA analysis (Olsen et al., 1994). A specific relationship between the two phyla, however, is not convincingly demonstrated on this level, merely vaguely suggested. So far, a lack of sufficient genomic sequence data prevents the hypothesis being put to critical test—but early results from the genomic front are encouraging, however (R. Overbeek, personal communication).

The ecological significance of these two phyla is still being explored (by default as it were, because no one knows *a priori* what will be uncovered when new niches are probed by Pace's methodology). Environmental sampling of rRNA sequences shows there to exist additional major lineages in both the Deinococcus-Thermus and the Chloroflexus phyla, none as yet with isolated representatives (N. Pace, personal communication).

THE REMAINING EUBACTERIAL KINGDOMS AND PHYLA With the above, I have come nowhere near to covering the full range of eubacterial kingdoms and phyla. However, those that remain fall into one or both of two categories: 1) demonstrated to be ecologically and phylogenetically major groupings by direct rRNA analyses of niches, but not represented by any (or very few) cultivated examples, and 2) represented by

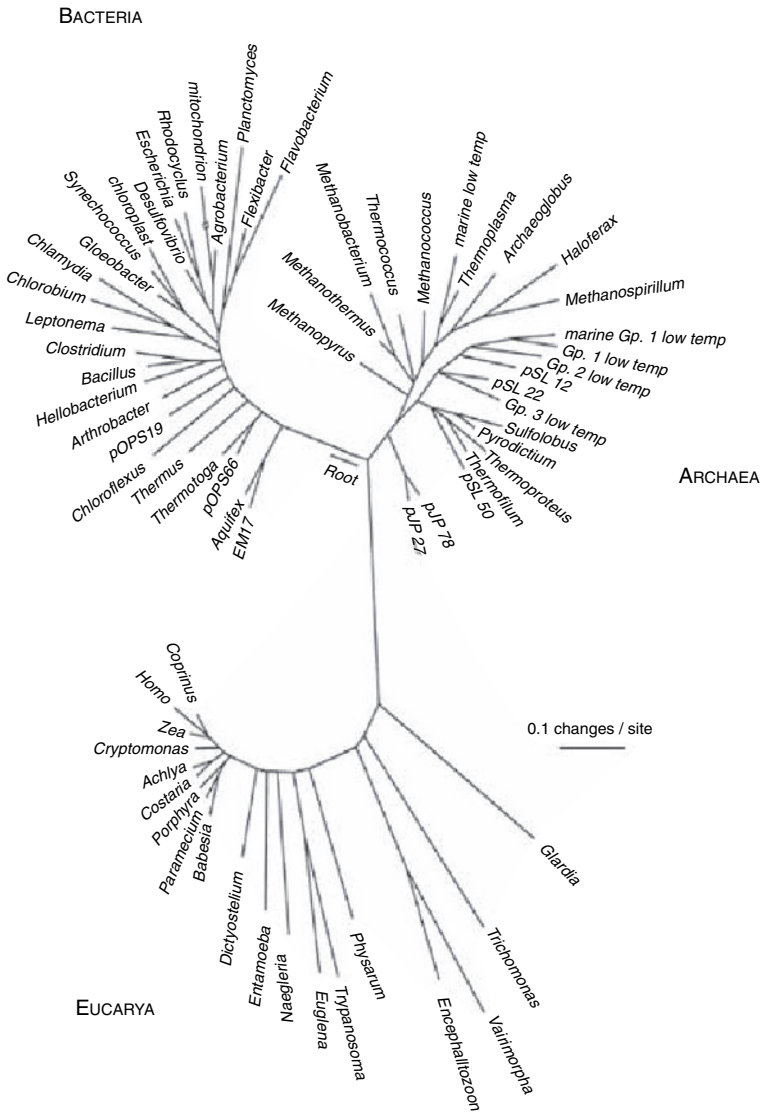

Fig. 1. Universal phylogenetic tree based on small subunit (SSU) rRNA sequence. Sixty-four rRNA sequences representative of all known phylogenetic domains were aligned and a tree was produced using FASTD NAML. That tree was modified, resulting in the composite one shown, by trimming lineages and adjusting branch points to incorporate results of other analyses. The scale bar corresponds to 0.1 changes per nucleotide.

few directly isolated rRNA clones and no or few cultivated species. These bear little comment at this point in time. A phylogenetic tree (Fig. 1) generated by N. Pace and colleagues gives some idea of the overall scope of the major eubacterial lineages (Pace et al., 1997).

I would end this brief, incomplete survey of the major organismal groupings by mentioning two eubacterial kingdoms that have rather limited ecological distribution, but are represented by genome sequences. Both are among the deepest branching eubacterial lineages (see Fig. 1). The first is represented by *Aquifex* (Huber et al., 1992; Deckert et al., 1998; see Aquificales in Volume 7). The second comprises what are now called the *Thermotogales* (Huber et al., 1986; Nelson et al., 1999). The number of generic names in the group, *Thermotoga*, *Geotoga*, *Petrotoga* and so on, does suggest a somewhat varied ecological distribution for the various members of the kingdom (see Thermotogales in Volume 7).

Now, on to the feast that awaits you in the rest of this third edition of *The Prokaryotes*.

Literature Cited

Ambler, R. P., M. Daniel, J. Hermoso, T. E. Meyer, R. G. Bartsch, and M. D. Kamen. 1979a. Cytochrome c_2 sequence variation among the recognised species of purple nonsulphur photosynthetic bacteria. Nature 278: 659–660.

Ambler, R. P., T. E. Meyer, and M. D. Kamen. 1979b. Anomalies in amino acid sequence of small cytochromes c and cytochromes c' from two species of purple photosynthetic bacteria. Nature 278:661–662.

Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. USA 91:1609–1613.

Barns, S. M., C. F. Delwiche, J. D. Palmer, and N. R. Pace. 1996. Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc. Natl. Acad. Sci. USA 93:9188–9193.

Beifuss, U., M. Tietze, S. Baumer, and U. Deppenmeier. 2000. Methanophenazine: Structure, total synthesis, and function of a new cofactor from methanogenic Archaea. Angew. Chem. Int. Ed. Engl. 39:2470–2472.

Beijerinck, M. W. 1905. Versl. Kon. Akad. Wetensch. Amsterdam 14:168–169.

Bell, S. D., and S. P. Jackson. 2001. Mechanism and regulation of transcription in archaea. Curr. Opin. Microbiol. 4: 208–213.

Breed, R. S. 1939. *In:* Bergey's Manual of Systematic Bacteriology, 2nd ed.

Burggraf, S., A. Ching, K. O. Stetter, and C. R. Woese. 1991. The sequence of Methanospirillum hungatei 23S rRNA confirms the specific relationship between the extreme halophiles and the Methanomicrobiales. Curr. Opin. Microbiol. 14:358–363.

Burkhardt, F., and S. Smith. 1990. The Correspondence of Charles Darwin. Volume 6: 1856. Cambridge University Press. Cambridge, UK.

Crick, F. H. C. 1958. The biological replication of macromolecules. Symp. Soc. Exp. Biol. 12:138–163.

Darwin, C. 1859. The Origin of Species by Means of Natural Selection, Or the Preservation of Favoured Races in the Struggle for Life. John Murray. London, UK. 69.

Deckert, G., P. V. Warren, T. Gaasterland, W. G. Young, A. L. Lenox, D. E. Graham, R. Overbeek, M. A. Snead, M. Keller, M. Aujay, R. Huber, R. A. Feldman, J. M. Short, G. J. Olsen, and R. V. Swanson. 1998. The complete genome of the hyperthermophilic bacterium Aquifex aeolicus. Nature 392:353–358.

DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363.

DeLong, E. F. 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA 89:5685–5689.

DeLong, E. F. 1997. Marine microbial diversity: The tip of the iceberg. Trends Biotechnol. 15:203–207.

Deppenmeier, U., A. Johann, T. Hartsch, R. Merkl, R. A. Schmitz, R. Martinez-Arias, A. Henne, A. Wiezer, S. Baumer, C. Jacobi, H. Bruggemann, T. Lienard, A. Christmann, M. Bomeke, S. Steckel, A. Bhattacharyya, A. Lykidis, R. Overbeek, H. P. Klenk, R. P. Gunsalus, H. J. Fritz, and G. Gottschalk. 2002. The genome of Methanosarcina mazei: Evidence for lateral gene transfer between bacteria and archaea. J. Molec. Microb. Biotechnol. 4:453–461.

Dobzhansky, T. 1963. Evolution, Genetics and Man. John Wiley. New York, NY.

Eisen, J. A., K. E. Nelson, I. T. Paulsen, J. F. Heidelberg, M. Wu, R. J. Dodson, R. Deboy, M. L. Gwinn, W. C. Nelson, D. H. Haft, E. K. Hickey, J. D. Peterson, A. S. Durkin, J. L. Kolonay, F. Yang, I. Holt, L. A. Umayam, T. Mason, M. Brenner, T. P. Shea, D. Parksey, W. C. Nierman, T. V. Feldblyum, C. L. Hansen, M. B. Craven, D. Radune, J. Vamathevan, H. Khouri, O. White, T. M. Gruber, K. A. Ketchum, J. C. Venter, H. Tettelin, D. A. Bryant, and C. M. Fraser. 2002. The complete genome sequence of Chlorobium tepidum TLS, a photosynthetic, anaerobic, green-sulfur bacterium. Proc. Natl. Acad. Sci. USA 99: 9509–9514.

Fitch, W. M., and E. Margoliash. 1967. Construction of phylogenetic trees. Science 155:279–284.

Fox, G. E., L. J. Magrum, W. E. Balch, R. S. Wolfe, and C. R. Woese. 1977a. Classification of methanogenic bacteria by 16S ribosomal RNA characterization. Proc. Natl. Acad. Sci. USA 74:4537–4541.

Fox, G. E., K. R. Pechman, and C. R. Woese. 1977b. Comparative cataloging of 16S ribosomal ribonucleic acid: Molecular approach to procaryotic systematics. Int. J. Syst. Bacteriol. 27:44–57.

Fox, G., E. Stackebrandt, R. B. Hespell, J. Gibson, J. Maniloff, T. A. Dyer, R. S. Wolfe, W. E. Balch, R. S. Tanner, L. J. Magrum, L. B. Zablen, R. Blakemore, R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl, K. R. Luehrsen, K. N. Chen, and C. R. Woese. 1980. The phylogeny of prokaryotes. Science 209:457–463.

Galagan, J. E., C. Nusbaum, A. Roy, M. G. Endrizzi, P. Macdonald, W. FitzHugh, S. Calvo, R. Engels, S. Smirnov, D. Atnoor, A. Brown, N. Allen, J. Naylor, N. Stange-Thomann, K. DeArellano, R. Johnson, L. Linton, P. McEwan, K. McKernan, J. Talamas, A. Tirrell, W. Ye, A. Zimmer, R. D. Barber, I. Cann, D. E. Graham, D. A. Grahame, A. M. Guss, R. Hedderich, C. Ingram-Smith, H. C. Kuettner, J. A. Krzycki, J. A. Leigh, W. Li, J. Liu, B. Mukhopadhyay, J. N. Reeve, K. Smith, T. A. Springer, L. A. Umayam, O. White, R. H. White, E. Conway de Macario, J. G. Ferry, K. F. Jarrell, H. Jing, A. J. Macario, I. Paulsen, M. Pritchett, K. R. Sowers, R. V. Swanson, S. H. Zinder, E. Lander, W. W. Metcalf, and B. Birren. 2002. The genome of M. acetivorans reveals extensive metabolic and physiological diversity. Genome Res. 12:532–542.

Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Molec. Biol. 12:829–842.

Golyshina, O. V., T. A. Pivovarova, G. I. Karavaiko, T. F. Kondrateva, E. R., Moore, W. R. Abraham, H. Lunsdorf, K. N. Timmis, M. M. Yakimov, and P. N. Golyshin. 2000. Ferroplasma acidiphilum gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the Archaea. Int. J. Syst. Evol. Microbiol. 50:997–1006.

Graham, D. E., R. Overbeek, G. J. Olsen, and C. R. Woese. 2000. An archaeal genomic signature. Proc. Natl. Acad. Sci. USA 97:3304–3308.

Graham, D. E., and R. H. White. 2002. Elucidation of methanogenic coenzyme biosyntheses: From spectroscopy to genomics. Nat. Prod. Reports 19:133–147.

Gray, M. W., G. Burger, and B. F. Lang. 2001. The origin and early evolution of mitochondria. Genome Biol. 2:REVIEWS1018.

Hafenbradl, D., M. Keller, R. Dirmeier, R. Rachel, P. Rossnagel, S. Burggraf, H. Huber, and K. O. Stetter. 1996. Ferroglobus placidus gen. nov., sp. nov., A novel hyperthermophilic archaeum that oxidizes Fe^{2+} at neutral pH under anoxic conditions. Arch. Microbiol. 166:308–314.

Hartman, H. 1984. The origin of the eukaryotic cell. Spec. Sci. Technol. 7:77–81.

Hartman, H., and A. Fedorov. 2002. The origin of the eukaryotic cell: A genomic investigation. Proc. Natl. Acad. Sci. USA 99:1420–1425.

Hartmann, E., and H. König. 1990. Comparison of the biosynthesis of the methanobacterial pseudomurein and the eubacterial murein. Naturwissenschaften 77:472–475.

Heberlein, G. T., J. De Ley, and R. Tijtgat. 1967. Deoxyribonucleicacid homology and taxonomy of Agrobacterium, Rhizobium, and Chromobacterium. J. Bacteriol. 94:116–124.

Huber, R., T. A. Langworthy, H. Konig, M. Thomm, C. R. Woese, U. B. Sleytr, and K. O. Stetter. 1986. Thermotoga maritima sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. 144:324–333.

Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. Konig, R. Rachel, I. Rockinger, H. Fricke, and K. O. Stetter. 1992. Aquifex pyrophilus gen. nov., sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst. Appl. Microbiol. 15:340–351.

Huber, H., M. J. Hohn, R. Rachel, T. Fuchs, V. C. Wimmer, and K. O. Stetter. 2002. A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. Nature 417:63–67.

Jenkins, C., R. Samudrala, I. Anderson, B. P. Hedlund, G. Petroni, N. Michailova, N. Pinel, R. Overbeek, G. Rosati, and J. T. Staley. 2002. Genes for the cytoskeletal protein tubulin in the bacterial genus Prosthecobacter. Proc. Natl. Acad. Sci. USA 99:17049–17054.

Kamp, A. F., J. W. M. La Riviere, and W. Verhoeven (Eds.). 1959. Albert Jan Kluyver: His Life and Works. North Holland. Amsterdam, The Netherlands.

Kandler, O., and H. Hippe. 1977. Lack of peptidoglycan in the cell walls of Methanosarcina barkeri. Arch. Microbiol. 113:57–60.

Kandler, O., and H. König. 1985. Cell envelopes of archaebacteria. *In:* C. R. Woese and R. S. Wolfe (Eds.) The Bacteria. Volume 8: Archaebacteria. Academic Press. New York, NY. 413–458.

Kates, M. 1964. Bacterial lipids. Adv. Lipid Res. 2:17–90.

Kates, M., M. K. Wassef, and D. J. Kushner. 1968. Radioisotopic studies on the biosynthesis of the glyceryl diether lipids of Halobacterium cutirubrum. Can. J. Biochem. 46:971–977.

Kelman, Z. 2000. DNA replication in the third domain (of life). Curr. Protein Pept. Sci. 1:139–154.

Kluyver, A. J., and H. J. L. Donker. 1926. Die Einheit in der Biochemie. Chem. Zell. Gewiss. 13:134–190.

Kluyver, A. J. 1931. The Chemical Activities of Microorganisms. University Press. London, UK.

Kluyver, A. J., and C. B. van Niel. 1936. Prospects for a natural system of classification of bacteria. Zentralbl. Bakteriol. Parasitenkd. Infektionskrankh. 94:369–403.

König, H., and O. Kandler. 1979. The amino acid sequence of the peptide moiety of the pseudomurein from Methanobacterium thermoautotrophicum. Arch. Microbiol. 121:271–275.

Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA 82:6955–6959.

Langer, D., J. Hain, P. Thuriaux, and W. Zillig. 1995. Transcription in archaea: Similarity to that in eucarya. Proc. Natl. Acad. Sci. USA 92:5768–5772.

Lederberg, J., and E. L. Tatum. 1946. Gene recombination in E. coli. Nature 158:558.

Lewin, R. A. 1984. Prochloron—a status report. Phycologia 23:203–208.

Maidak, B. L., J. R. Cole, T. G. Lilburn, C. Y. Parker Jr., P. R. Saxman, R. J. Farris, G. M. Garrity, G. J. Olsen, T. M. Schmidt, and J. M. Tiedje. 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. 29:173–174.

Martin, W., and M. Mueller. 1998. The hydrogen hypothesis for the first eukaryote. Nature 392:37–41.

Meyer, T. E., M. A. Cusanovich, and M. D. Kamen. 1986. Evidence against use of bacterial amino acid sequence data for construction of all-inclusive phylogenetic trees. Proc. Natl. Acad. Sci. USA 83:217–220.

Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, C. M. Fraser, et al. 1999. Evidence for lateral gene transfer between Archaea and bacteria. from genome sequence of Thermotoga maritima. Nature 399:323–329.

Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl. 1986. Microbial ecology and evolution: A ribosomal RNA approach. Ann. Rev. Microbiol. 40:337–365.

Olsen, G. J., C. R. Woese, and R. Overbeek. 1994. The winds of (evolutionary) change: Breathing new life into microbiology. J. Bacteriol. 176:1–6.

Olsen, G. J., and C. R. Woese. 1996. Lessons from an archaeal genome: What are we learning from Methanococcus jannaschii. Trends Genet. 12:377–379.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. Science 276:734–740.

Paster, B. J., F. E. Dewhirst, G. J. Olsen, and I. Fraser. 1994. Phylogeny of Bacteroides, Prevotella, and Porphyromonas spp. and related bacteria. J. Bacteriol. 176:725–732.

Reeve, J. N., K. Sandman, and C. J. Daniels. 1997. Archaeal histones, nucleosomes, and transcription initiation. Cell 89:999–1002.

Sanger, F., and H. Tuppy. 1951. The amino-acid sequence in the phenylalanyl chain of insulin. Biochem. J. 49:481–490.

Sanger, F., and E. O. P. Thompson. 1953. The amino-acid sequence in the glycyl chain of insulin. Society for General Microbiology Symposium 53:353–374.

Sanger, F., G. G. Brownlee, and B. G. Barrell. 1965. A two-dimensional fractionation procedure for radioactive nucleotides. J. Molec. Biol. 13:373–398.

Schleifer, K.-H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36:407–477.

Sogin, S. J., M. L. Sogin, and C. R. Woese. 1971. Phylogenetic measurement in procaryotes by primary structural characterization. J. Molec. Evol. 1:173–184.

Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace. 1985. Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl. Environ. Microbiol. 49:1379–1384.

Stanier, R. Y., and C. B. van Niel. 1941. The main outlines of bacterial classification. J. Bacteriol. 42:437–466.

Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. 1957. The Microbial World, 1st ed. Prentice-Hall. Engelwood Cliffs, NJ.

Stanier, R. Y., and C. B. van Niel. 1962. The concept of a bacterium. Arch. Mikrobiol. 42:17–35.

Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. 1963. The Microbial World, 2nd ed. Prentice-Hall. Engelwood Cliffs, NJ.

Stanier, R. Y. 1970a. Organization and Control in Prokaryotic and Eukaryotic Cells. *In:* H. P. Charles and B. C. J. G. Knight (Eds.) Society for General Microbiology Symposium 20. Cambridge University Press. Cambridge, UK. 1–38.

Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. 1970b. The Microbial World, 3rd ed. Prentice-Hall. Engelwood Cliffs, NJ.

Stanier, R. Y., E. A. Adelberg, and J. L. Ingraham. 1976. The Microbial World, 4th ed. Prentice-Hall. Engelwood Cliffs, NJ.

Tjian, R. 1996. The biochemistry of transcription in eukaryotes: A paradigm for multisubunit regulatory complexes. Philos. Trans. R. Soc. Lond. B51:491–499.

Tornabene, T. G., and T. A. Langworthy. 1979. Diphytanyl and dibiphytanyl glycerol ether lipids of methanogenic archaebacteria. Science 203:51–53.

van Iterson Jr., G., L. E. den Dooren de Jong, and A. J. Kluyver. 1940. Martinus Willem Beijerinck: His Life and His Work. Delftsch Hoogeschoolfonds. Delft, The Netherlands.

van Niel, C. B. 1946. The classification and natural relationships of bacteria. Cold Spring Harbor Symp. Quant. Biol. 11:285–301.

van Niel, C. B. 1949. The "Delft School" and the rise of general microbiology. Bacteriol. Rev. 13:161–174.

van Niel, C. B. 1955. Classification and taxonomy of the bacteria and bluegreen algae. A Century of Progress in the Natural Sciences 1853–1953. California Academy of Sciences. San Francisco, CA. 89–114.

Weisberg, W. G., S. J. Giovannoni, and C. R. Woese. 1989. The Deinococcus-Thermus phylum and the effect of mRNA composition on phylogenetic tree construction. Syst. Appl. Microbiol. 11:128–134.

Woese, C. R., M. L. Sogin, and L. A. Sutton. 1974. Procaryote phylogeny. I: Concerning the relatedness of Aerobacter aerogenes to Escherichia coli. J. Molec. Evol. 3:293–299.

Woese, C. R., G. E. Fox, L. Zablen, T. Uchida, L. Bonen, K. Pechman, B. J. Lewis, and D. Stahl. 1975. Conservation of primary structure in 16S ribosomal RNA. Nature 254:83–96.

Woese, C. R., and G. E. Fox. 1977a. The concept of cellular evolution. J. Molec. Evol. 10:1–6.

Woese, C. R., and G. E. Fox. 1977b. The phylogenetic structure of the prokaryotic domain: The primary kingdoms. Proc. Natl. Acad. Sci. USA 74:5088–5090.

Woese, C. R., B. A. Debrunner-Vossbrinck, H. Oyaizu, E. Stackebrandt, and W. Ludwig. 1985a. Gram-positive bacteria: Possible photosynthetic ancestry. Science 229: 762–765.

Woese, C. R., E. Stackebrandt, T. J. Macke, and G. E. Fox. 1985b. A phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. 6:143–151.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Woese, C. R. 2000. Interpreting the universal phylogenetic tree. Proc. Natl. Acad. Sci. USA 97:8392–8396.

Woese, C. R., G. J. Olsen, M. Ibba, and D. Söll. 2000. Aminoacyl-tRNA synthetases, the genetic code, and the evolutionary process. Microbiol. Molec. Biol. Rev. 64:202–236.

Woese, C. R. 2002. On the evolution of cells. Proc. Natl. Acad. Sci. USA 99:8742–8747.

Wolfe, R. S. 1992. Biochemistry of methanogenesis. Biochem. Soc. Symp. 58:41–49.

Zuckerkandl, E., and L. Pauling. 1965. Molecules as documents of evolutionary history. J. Theoret. Biol. 8:357–366.

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CHAPTER 1.2

Databases

WOLFGANG LUDWIG, KARL-HEINZ SCHLEIFER AND ERKO STACKEBRANDT

Introduction

Computerization of microbiological data was first introduced by Sneath (1957) to handle the enormous amount of phenetic data collected to analyze the numerical phenetic taxonomy (NT) of the genus *Chromobacterium* (105 characters of 45 operational taxonomic units [OTUs]). This work paralleled the work of Sokal and Michener (1958), who used electric tabulating machinery and an electric mechanical desk calculator to generate a classification of bees of the *Hoplitis* complex (122 characters of 97 species). Sokal and Sneath joined forces to develop the *Principles of Numerical Taxonomy* (Sokal and Sneath, 1963) and they, together with Florek et al. (1951), Cain and Harrison (1958), and Rogers and Tanimoto (1960), were the first to develop and apply clustering methods (such as single and average-linkage clustering), probabilistic distance coefficients in NT, Jaccard's coefficient, scaling of multistate characters, parallelism and convergence, and equal weighting. Many of these algorithms and their modifications are still used today to analyze DNA and RNA electrophoretic patterns (Riboprint, denaturing gradient gel electrophoresis [DGGE], thermal gradient gel electrophoresis [TGGE], amplified fragment length polymorphism [AFLP], restriction fragment length polymorphism [RFLP], and the like), protein patterns, fatty acid methyl ester patterns, and evaluation of ecological parameters, to name a few. As mentioned by Sokal (1985), most larger universities acquired their first computer in 1956, but it took another 15–20 years before PCs were provided to biologists.

The present easy accessibility of public databases of biological resources and their sequences of nucleic acids and proteins let us forget how cumbersome comparative sequence analysis was 30 years ago. Similarities were determined from sequences, scattered in the literature, which had to be searched for, copied, and aligned by hand. By the late 1970s, dozens of short oligonucleotides that constituted a 16S rRNA catalogue (Uchida et al., 1974; Fox et al., 1977) were compared using a simple average linkage cluster analysis. For those of us who had no access to a personal computer, the calculation of similarities was done on paper. Nevertheless, as long as the fragments to be compared were short and the number of organisms analyzed were small (<20), it was the sequence analysis, not the determination of similarity coefficients (S_{AB}), that constituted the bottleneck of the approach. The discoveries of the phylogenetic uniqueness of the Archaea that led to the recognition of the three primary kingdoms (Woese and Fox, 1977) and thus revolutionized many fields in biology were achieved without the use of PCs and sophisticated treeing algorithms. Though hypotheses of phylogeny were derived mainly from similarity (phenetic) comparisons of rRNA catalogues and the extent of the homoplasy (parallelisms and reversals) was underestimated, most of the general findings from the 1970s and 1980s were proven right when analysis of more complete genes and genome fragments was evaluated by more sophisticated evolutionary models.

Public Databases

From the late 1970s on, availability of the genome sequences of the bacteriophages PhiX 174 and G4 required computers to store and analyze the data (McCallum and Smith, 1977; Staden, 1977; Staden, 1978; Godson et al., 1978). The strategy of shotgun cloning used computers to design the sequencing strategy (Staden, 1982). Tools used routinely these days (such as functions for alignment, edit, search, contig assembly, database comparison, folding, homologies, and translation functions) were developed. The increasing number of sequences in the literature required a coordinated storing and retrieving system. In 1980 the European Molecular Biology Laboratory (EMBL) Data Library was founded—the first central depository of nucleotide sequence data in the world (precursor to

EMBL's European Bioinformatics Institute [EBI] Outstation). But at that time it was not essential to deposit sequences, and the older literature still contains sequences that have not yet been transferred to an electronic platform. In 1982, *Nucleic Acids Research* devoted a whole issue to the use of computers in the analysis of nucleic acid sequences (Söll and Roberts, 1982). The role of this journal continues to inform scientists about the development of established databases and the availability of the rapidly proliferating new databases on individual markers (e.g., bacterial virulence factors, protein families, and pathways), individual species (e.g., *Pseudomonas aeruginosa* and *Candida albicans*), and specific groups of organisms (e.g., Yeast and *Caenorhabditis*). The journal also gives an overview of molecular biology database collections in the first issue of each volume (http://nar.jour nals.org/cgi/content/full/33/suppl_1/D5), listing more than 700 entries, ordered according to determination and application. Three of the major databases constitute an international nucleic acid sequence database cooperative: DDBJ (http://www.ddbj.nig.ac.jp/), Japan; EMBL (http://www.ebi.ac.uk), Europe; and GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), United States. They are regularly exchanging new additions.

The Swiss-Prot (now UniProt; http://www.expasy.uniprot.org/) database plays a crucial role in that it provides the user with integrated databases (nucleic acid sequences, protein sequences, protein tertiary structure, and specialized databases). Besides a high level of integration, UniProt allows a high level of annotation on functions of proteins (secondary to quaternary structure, modifications, and domain sites) and a low level of redundancy, indicating conflicting entries which occur in multiple databases. The UniProt/TrEMBL database (http://www.uniprot.org/entry/P99999, http://www. expasy.uniprot.org/index.shtml) complements the UniProt database by providing translations of all open reading frames of nucleic acid sequences provided by the three major databases indicated above.

Another entry to information retrieval is via the Entrez browser, giving access to major databases. These may be broad databases such as GenBank and protein sequence database, complete genomes, three dimensional structures, single nucleotide polymorphism, and population study data sets to chemical structures of small molecules and bioactive chemical substances. Entrez is known best as the gate to PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). The pendant at the EBI data library is the sequence retrieval system (SRS; http://srs.ebi. ac.uk).

Ribosomal RNA Databases

Ribosomal rRNA sequence data still provide a solid basis for microbial taxonomy and identification. Three major projects are maintaining databases of processed rRNA primary structure data.

The Ribosomal Database Project (RDP) II initially founded by Carl Woese and coworkers in 1992 is now maintained at the Center for Microbial Ecology at Michigan State University (http://rdp.cme.msu.edu). The RDP maintains databases of aligned large and small subunit rRNA sequences. The alignments in RDP databases are optimized by specialists taking into account higher structure features and can be used as guides for the alignment of user provided data. The data can be accessed by the browsing of databases (http://rdp.cme.msu.edu/hierarchy/hb_intro.jsp) according to RDP's or National Center for Biotechnology Information (NCBI)'s taxonomic hierarchy. The RDP hierarchy is based upon that proposed in the recent edition of *Bergey's Manual of Systematic Bacteriology.* The databases or user defined subsets can be downloaded as compressed files in GenBank (*.gb.bz2) or FASTA (*.fasta.bx2) format and either as aligned or non-aligned datasets on (http://rdp.cme.msu.edu/misc/resources.jsp). The RDP also provides facilities for on-line sequence checking of user-provided sequences and database entries such as searching most similar primary structures, preliminary alignment and phylogenetic analysis, chimera checking, and oligonucleotide (probe) matching. Additional databases are indicated in Tables 1 and 2.

The European Ribosomal RNA Database located at the University of Ghent (Belgium; http://www.psb.ugent.be/rRNA/) maintains small and large subunit rRNA sequences in aligned format. The alignment takes into account secondary structure prediction based upon comparative analysis of the database sequences. Data selection and download can be done by using a query interface or a taxonomy browser. An rRNA BLAST service and some stand alone software for primary and secondary structure editing are available at or from this site.

The two major tasks of the ARB Project (*arbor*, Latin: tree; http://www.arb-home.de) initiated in 1992 at the Technical University of Munich are 1) the maintenance of a structured integrative database combining processed primary structures and any type of additional data assigned to the individual sequence entries and 2) a comprehensive selection of software tools (controlled via a common graphical interface) directly interacting with one another and the central database (Ludwig et al., 2004). Initially designed for rRNA data, the ARB software

Table 1. Selected databases providing rRNA gene sequences of taxonomic significance.

Tool	Content	References
5S rRNA	5S rRNA sequences	http://biobases.ibch.poznan.pl/5SData/
European rRNA database	All complete or nearly complete rRNA sequences	http://www.psb.ugent.be/rRNA/
RRNDB	rRNA operon numbers in various prokaryotes	http://rrndb.cme.msu.edu
Ridom	rRNA based differentiation of medical microorganisms	http://www.ridom-rdna.de
RDP-II	Ribosomal database project	http://rdp.cme.msu.edu
ARB	rRNA and conserved protein gene sequences	http://www.arb-home.de
ProbeBase	In vitro evaluated probes	http://www.microbial-ecology.de/probebase/index.html
rRNA Probes for Protists and Cyanobacteria	In vitro evaluated probes	http://www.sb-roscoff.fr/Phyto/Databases/RNA_probes_introduction.php
ARB Probe Library	All probes, in silico evaluated	http://www.arb-home.de

Abbreviations: RRNDB, Ribosomal RNA Operon Copy Number Database; Ridom, Ribosomal Differentiation of Microorganisms Web Server; and RDP-II, Ribosomal Database Project-II.

Table 2. Other selected databases of interest to taxonomy.

Tool	Content	References
CB	Gyr database for identification and classification of bacteria	http://seqsquit.mbio.co.jp/icb/index.php
PANDIT	Protein and associated nucleotide domains with inferred trees	http://www.ebi.ac.uk/goldman-srv/pandit
HGT-DB	Putative horizontally transferred genes in prokaryotic genomes	http://www.fut.es/-debb/HGT/
TIGR microbial database	Lists of completed and ongoing genome projects with link to complete genome sequences	http://www.tigr.org/tdb/mdb/mdbcomplete.html
TIGR comprehensive microbial resource	Uniform annotation, properties of DNA and predicted proteins of various complete microbial genomes	http://www.tigr.org/CMR
NCBI taxonomy	Names of all organisms represented in GenBank	http://www.ncbi.nlm.nih.gov/Taxonomy
Tree of life	Information on phylogeny and biodiversity	http://phylogeny.arizona.edu/tree/phylogeny.html
PGTdb	Prokaryotic growth temperature database	http://pgtdb.csie.ncu.edu.tw

Abbreviations: CB, Computational Biology database; PANDIT, protein and associated nucleotide domains with inferred trees; HGT-DB, Horizontal Gene Transfer Database; TIGR, The Institute of Genome Research; NCBI, National Center for Biotechnology Information; and PGTdb, Prokaryotic Growth Temperature database.

package can also be applied to DNA, protein and full genome sequences. ARB software and databases are accessible to the public (http://www.arb-home.de) and have to be installed locally. The ARB project provides databases for large and small subunit rRNA, a selection of evolutionarily conserved protein genes such as the genes for elongation and initiation factors, ATPase subunits, recA, RNA polymerases, DNA gyrases, heat shock proteins, and amino acyl tRNA synthases as well as full genome sequences. The primary structures representing organisms, genes or gene products are stored in individual database fields. The primary data are processed with respect to alignment, higher order structure prediction, conservation profiles, and other criteria. According to the ARB concept of an integrative database, any type of additional data can be stored within user defined database fields assigned to the respective sequence. It is either stored in the local database or linked to it via local networks or the internet. The database is hierarchically structured and highly compressed. The data can be imported and exported in a variety of commonly used formats. Easy data access is possible via powerful search and retrieval tools or by mouse click at the nodes of graphically visualized (phylogenetic) trees. The ARB software package comprises a comprehensive set of cooperating tools for database maintenance and analysis. Among these tools are powerful primary and secondary editors as well a 3-D structure tool allowing visualization of any type of

sequence-associated information such as search strings, probe and primer sites, any type of profiles, in situ accessibility maps, position filters, and functional sites by user defined background colors (Kumar et al., 2005). Further important coordinated software tools concern automated and user supported sequence alignment, a selection of alternative treeing methods, and phylogenetic probe design and evaluation. ARB-genome is designed for functional genomics. The integrated database allows the user to assign experimental parameters as well as results from proteomics and transcriptomics to the respective organism and gene data. The interacting software tools allow comparative genome as well as experimental data visualization and analysis.

rRNA Targeted Probe Databases

Taxon specific probe technologies nowadays are central tools in microbial identification, taxon specific enrichment, and ecological studies. Against the background of many examples of redundant, ineffective, and incorrect probe design, databases for evaluated taxon specific probes are urgently needed.

ProbeBase (http://www.microbial-ecology.de/probebase/index.html) was an initiative of the microbial ecolog group at the Lehrstuhl für Mikrobiologie of the Technical University of Munich (http://www.mikro.biologie.tu-muenchen.de) and is now located at the University of Vienna. It contains published rRNA-targeted oligonucleotide probe sequences, DNA microarray layouts, and associated information. The probes were not only evaluated in silico using the ARB program package and databases (http://www.arb-home.de) but also experimentally tested.

The Roscoff database maintains experimentally evaluated rRNA targeted probes specific for eukaryotes, protists (mostly photosynthetic), and cyanobacteria at the Station Biologique de Roscoff. The data are accessible at (http://www.sb-roscoff.fr/Phyto/Databases/RNA_probes_introduction.php).

The ARB probe library gives access to all possible phylogenetic probes against the full background of the current ARB databases. A platform-independent client which can be downloaded from (http://www.mikro.biologie.tu-muenchen.de) visualizes a phylogenetic tree. This tree provides mouse click access to any potential node or subtree specific probe target and additional descriptive information. Users have to be aware that the potential probe targets are designed and evaluated in silico but not tested in vitro.

Concluding Remarks

Despite the progress in bioinformatics and genomics as well as the collaboration of some of the databases, the concept of integrative databases—the assignment of any information (be it primary data of expression studies, physiological pathways, or conventional descriptive data such as a picture of the habitats or recordings of the voices of higher organisms) to the sequence data—is not yet optimally implemented. As with data-bases, a similar situation is seen with respect to interoperability of the vast number of software tools. The ARB concept is an attempt to combine direct interaction of various tools and databases with a maximum of accepted interventions by the individual user.

Literature Cited

Cain, A. J., and G. A. Harrison. 1958. An analysis of the taxonomist's judgement of affinity. Proc. Zool. Soc. Lond. 131:85–98.

Florek, K., J. Lukaszewicz, J. Perkal, H. Steinhaus, and S. Zubrzycki. 1951. Taksonomia Wrocllawska. Przegl Antropoliczny 19:193–211.

Fox, G. E., L. J. Magrum, W. Balch, R. Wolf, and C. R. Woese. 1977. Classification of methanogenic bacteria by 16S ribosomal RNA characterization. Proc. Natl. Acad. Sci. (Washington) 74:4537–454.

Godson, G. N., B. G. Barrell, R. Staden, and J. C. Fiddes. 1978. Nucleotide sequence of bacteriophage G4 DNA. Nature 276:236–247.

Kumar, Y., R. Westra, S. Behrens, B. Fuchs, F. O. Glöckner, R. Amann, H. Meier, and W. Ludwig. 2005. Graphical representation of ribosomal RNA probe accessibility data using ARB software package. Online: (http://dx.doi.org/10.1186/1471-2105-6-61). BMC Bioinformatics.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W., Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer. 2004. ARB: A software environment for sequence data. Nucleic Acids Res. 32:1363–1371.

McCallum, D., and M. Smith. 1977. Computer processing of DNA sequence data. J. Molec. Biol. 15:29–30.

Rogers, D. J., and T. T. Tanimoto. 1960. A computer program for classifying plants. Science 132:1115–1118.

Sneath, P. H. A. 1957. The applications of computers to taxonomy. J. Gen. Microbiol. 17:201–226.

Sokal, R. R., and C. D. Michener. 1958. A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull. 38:1409–1438.

Sokal, R., and P. H. A. Sneath. 1963. Principles of Numerical Taxonomy. San Francisco, CA.

Sokal, R. R. 1985. The priciples of numerical taxonomy: Twenty years later. *In:* M. Goodfellow, D. Jones, and

F. G. Priest (Eds.) Computer-assisted Bacterial Systematics. Academic Press. London, UK. 1–20.

Söll, D., and R. J. Roberts. 1984. The Applications of Computers to Research on Nucleic Acids, Part 2. IRL Press. Oxford, UK.

Staden, R. 1977. Sequence data handling by computer. Nucleic Acids Res. 4:4037–4051.

Staden, R. 1978. Further procedures for by computer. Nucleic Acids Res. 5:1013–1016.

Staden, R. 1982. Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nucleic Acids Res. 10: 4731–4751.

Uchida, T., L. Bonen, H. W. Schaup, B. J. Lewis, L. Zablen, and C. Woese. 1974. The use of ribonuclease U2 in RNA sequence determination: Some corrections in the catalog of oligomers produced by ribonuclease T1 digestion of Escherichia coli 16S ribosomal RNA. J. Molec. Evol. 28:63–77.

Woese, G., and E. Fox. 1977. Phylogenetic structure of the prokaryotic domain: The primary kingdoms. Proc. Natl. Acad. Sci USA 74:5088–5090.

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CHAPTER 1.3

Defining Taxonomic Ranks

ERKO STACKEBRANDT

Systematics and Classification

"Well, in *our* country," said Alice, still panting a little, "you'd generally get to somewhere else—if you ran very fast for a long time, as we've been doing."

"A slow sort of country!" said the Queen, "Now, *here*, you see, it takes all the running *you* can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!"

—Lewis Carroll, *Through the Looking Glass*, (1872)

Those who have chosen systematics, classification, and taxonomy as research topics have learned to consider the complexities as exciting and important. For others, these topics are mainly boring and through changes in names of microbial taxa, may cause confusion. Indeed, the problem of changing names of taxa is inherent. Classification is motivated by the desire of taxonomists to provide the user with a system that in their opinion optimally reflects the natural relatedness between the taxa. Now finally, the determination of phylogenetic relationships is achievable (Stackebrandt, 1992). Looking back in the history of microbiology, the lack of interest in classification can be traced to the enormous difficulty of past generations of systematists to put in order the phenotypic and genotypic properties of the steadily growing numbers of bacterial strains. The user of taxonomy was confronted with constantly changing classification concepts and systems, taxonomic rearrangements, and synonymy of names. Problems also arose from the terminology: while some regard systematics and taxonomy as synonymous, others define taxonomy as the theory and practice of classifying organisms and systematics as broader, including the evolutionary and phylogenetic components. For many researchers, nomenclature is their only contact with taxonomy, and the contact occurs only when they are confronted with name changes. However, systematics includes more than naming of organisms (Stackebrandt et al., 1999).

Classification is done by generating as much data on the properties of novel isolates as possible and by the process of identification, e.g., comparing the data with the database of previously classified organisms and by affiliating the isolate with a previously described or a new species. Classification includes the theory and process of ordering the characterized organisms into one or more systems. Nomenclature is the naming of the appropriate taxon within a classification system, and it includes subjective changes that occur whenever novel insights alter the taxonomic weight of characters, and thereby the rank of taxa.

As outlined (Stackebrandt et al., 1992), several classification systems exist in parallel and no classification system can claim predominance. No two systems of clustering need to match. As long as a system succeeds in doing what it sets out to do, it cannot be described as wrong or in error.

There are systems that group microorganisms on the basis of their increasing degrees of risk to humans, animals and plants. Here, organisms are artificially, pragmatically classified into risk groups according to degree of pathogenicity or risk potential, and this system serves no other purpose (Table 1).

Another system focuses on the rapid and reliable identification of bacteria for which knowledge about phylogenetic relatedness is not mandatory (Table 2). In such a diagnostic system, used in the past, affiliation of an isolate to a genus and species was based on Gram-stain reaction, oxygen requirement and morphology, chemotaxonomy, numerical phenetic analyses, usage of rapid diagnostic kits (e.g., API, Merieux, and BIOLOG), and combinations of selected physiological tests. (API System, La Balme les Grottes, 38390 Montalieu Vercieu, France. BIOLOG, Biolog, Inc., 3989 Trust Way, Hayward, CA, 94545 USA.)

Yet another system considers similarities in homologous molecules. Organisms are grouped according to their phylogenetic relatedness, which is then circumscribed by a wide range of genomic and epigenetic characteristics. This

Table 1. Examples of prokaryotic species classified by risk.

Genus	Risk group 1	Risk group 2	Risk group 3
Chlamydia	not known	*C. trachomatis*	*C. psittaci*
Bacillus	*B. circulans*	*B. cereus*	*B. anthracis*
Burkholderia	*Bu. cocovenenans*	*Bu. Cepacia*	*Bu. mallei*
	Bu. antropogonis	*Bu. Vietnamensis*	*Bu. pseudomallei*
Francisella	not known	*F. tularensis* subsp. *mediasiatica*	*F. tularensis* subsp. *tularensis*
Mycobacterium	*M. asiaticum*	*M. avium*	*M. leprae*
	M. fallax	*M. chelonae*	*M. tuberculosis*

Table 2. Examples of Bacteroidaceae classified by phenotypic properties.

Family	Genera	Main diagnostic differences
Bacteroidaceae[a]	*Bacteroides*	Peritrichous straight rod; produces a mixture of fermentation products from carbohydrate and peptone; butyrate not a major product
	Fusobacterium	Nonmotile straight rod; butyrate is a major product
	Leptotrichia	Nonmotile straight rod; lactate is the sole major fermentation product
	Butyrivibrio	Motile, not peritrichous, curved rod; butyric acid is the major fermentation product
	Succinimonas	Short, motile rod or coccobacilli; single polar flagellum; succinate and acetate are major fermentation products
	Succinivibrio	Motile helical or spiral-shaped cell; single polar flagellum; succinate and acetate are major fermentation products
	Anaerobiospirillum	Motile helical or spiral-shaped cell; bipolar tufts of flagella; succinate and acetate are major fermentation products
	Wolinella	Motile, helical to curved, or straight rod, single polar flagellum; either hydrogen or formate as electron donor for reduction of fumarate to succinate; carbohydrates not fermented
	Selenomonas	Motile, crescent-shaped cell, tufts of flagella on concave side; fermentation products are propionate and acetate
	Anaerovibrio	Motile curved cells, single polar flagellum, lipolytic; fermentation products are propionate and acetate
	Pectinatus	Motile curved cells, lateral flagella aligned on concave side; fermentation products are propionate and acetate

[a]Members of the family Bacteroidaceae are described as Gram-negative, fermentative anaerobic organisms (Holt et al., 1994).

genealogically based classification system is the most comprehensive one in overall understanding of the biology of the organisms, including the evolution of core processes of genetics, biochemistry, and physiology. This approach, which was outlined two decades ago, is applied by the vast majority of microbiologists.

Thus, classification in bacteriology is based on the principle of degree of relatedness. The grouping of organisms, whether or not they are formally designated taxa (e.g., genera, phyla, domains, groups, clusters, etc.), generally brings together those organisms on the basis of shared properties. Of the many systems that have been described in the past, the one that is based on genealogical relatedness offers the greatest potential, as it explains the widest range of genetic and biochemical properties. Genealogy derived from gene sequence similarity has the added advantage of working with a reliable, objective and stable basis for identification and classification. The dramatic changes that occurred in the classification of species of *Bacteroides*, lumped together in the past on the basis of a few superficial properties, are an excellent example of a shift to the concept that regards phylogenetic relatedness as the most reliable basis for classification (Table 3). The reclassification of a species requires redefinition of its properties. Analysis of the natural relatedness of a species will provide information on its phylogenetic position, i.e., its nearest neighbor(s). However, in many cases the position will not provide information on other properties needed to decide whether this species can be considered a species of a known genus or the nucleus of a novel genus. These conclusions depend upon the results of a wide array of phenotypic and genomic properties.

This chapter introduces the importance of gene and gene product sequence homology as a basis for an objective framework in which to order lineages of prokaryotic organisms. It then describes the (subjective) decision-making by which bacteriologists, on the basis of a phylogenetic framework, define the ranks of species and genera, and it deals with the problem of delineating ranks above the genus level. Special emphasis is placed here on the pragmatic definition of the species. An example of the process of

Table 3. Changes in higher classification as exemplified by the fate of some species of Bacteroides (see Table 2) after phylogenetic-polyphasic taxonomic analysis (Shah and Collins, 1989).

Traditional classification	Phylogenetic classification	Affiliation to higher taxon
Bacteroides furcosus	*Anaerorhabdus furcosus*	Bacteroidaceae
Bacteroides bivius	*Prevotella bivia*	Bacteroidaceae
Bacteroides endodentalis	*Porphyromonas endodentalis*	Bacteroidaceae
Bacteroides microfuscus	*Rikenella microfuscus*	Bacteroidaceae
Bacteroides amylophilus	*Ruminobacter amylophilus*	Gamma subclass of Proteobacteria
Bacteroides gracilis	*Campylobacter gracilis*	Epsilon subclass of Proteobacteria
Bacteroides hypermegas	*Megamonas hypermegas*	N.D.
Bacteroides nodosus	*Dichelobacter nodosus*	Beta subclass of Proteobacteria
Bacteroides termitidis	*Sebaldella termitidis*	N.D.
Bacteroides succinogenes	*Fibrobacter succinogenes*	Phylum Fibrobacter

Abbreviation: N.D. not determined.

modern classification is given for actinomycete taxa. This chapter should be studied in conjunction with the one by Wolfgang Ludwig (Ludwig, 1999), as many examples of lineages defined by 16S rDNA analysis, of the phylogenetic coherence or incoherence of taxa, and the delineation of higher taxa, can be judged best by having the phylogenetic tree available.

Past Classification Attempts

Ranks or taxa have been introduced in the classification of biological specimens to facilitate communication among men and to arrange living matter by morphological, physiological, ecological and genomic features. The basis of any system is the species and the genus, and according to the binomial system (Linnaeus, 1753), the description of a type species is not possible without describing a genus, and a genus cannot be described without a species. This simplicity explains why the binomial system is still used for the naming of organisms within the three domains Archaea, Bacteria and Eucarya (Woese, 1987). Moreno (1997) states: "The wisdom of Linnaeus was not only to create a comprehensive classification system, but more importantly, a useful one." The definition of a species has been debated extensively since the publication of the key work—*On the Origin of Species* (Darwin, 1859). At that time, the debate centered on animals and plants but excluded the prokaryotes mainly because they had no evolutionary record.

Bacterial classification as a science began with the contribution of Cohn (1872, 1875), who was the first to ask if bacteria, like plants and animals, can be arranged in species and genera. He presented a classification scheme composed of six genera that were distinguished on the basis of morphological criteria. However, he clearly pointed out that morphological properties are insufficient, inasmuch as similarly shaped bacteria may have different physiological characters. Cohn regarded the genera as natural entities but the species he described as largely provisional. With hindsight, it is possible to conclude that early microbial systematists were in no position to judge the importance of such simple properties in evolutionary terms. It was not known until the late 1970s that taxa defined by superficial properties such as morphology did not necessarily match taxa defined by traits that arise in the course of long evolutionary processes.

In the early twentieth century, the number of determinable properties expanded dramatically and, consequently, the number of species increased. New strains of medical importance were described as new taxa and the classification systems placed emphasis on these organisms and their identification. New systems were proposed in which the accent shifted from morphology to physiology, metabolism, pigments, and pathogenicity (Migula, 1900; Orla-Jensen, 1909; Pringsheim, 1923; Prévot, 1938; Kluyver and van Niel, 1936; Stanier and van Niel, 1941). To incorporate the wealth of information and to integrate the different prokaryote systems with those of higher animals and plants, a single unified formal system of bacterial classification was established by Buchanan (Buchanan, 1916, 1918). This system provided the basis for *Bergey's Manual of Determinative Bacteriology*, which, in the many editions that followed, presented better than any other source the most useful references for identification but retained a nomenclature that connoted phylogenetic relationships, in the tradition of Buchanan's system. Attempts to construct another single formal classification system or to work with several systems in parallel were criticized. Kluyver and van Niel (1936) suggested that rather than searching for a natural system, taxonomists should develop determinative keys to provide the easiest possible identification of species and genera. However, because the choice of characters used to establish the system was subjective, it was recognized that empirical sys-

tems would be largely unmodifiable. Consequently, the whole system was disrupted when novel characters were taken as the basis for the establishment of a new classification system. The main advantage of the empirical system was its immediate practical utility, but even this advantage disappeared when differential characters were not actually mutually exclusive. The period during which the importance of developing a natural classification system was recognized (Stanier and van Niel, 1941) but considered unachievable lasted until 1975. The question then remains why past generations of microbiologists could not develop a phylogenetic framework for prokaryotes. In hindsight, the answer is quite easy: Early attempts were prone to failure because scientists lacked fundamental genetic information, understanding of mechanisms of heredity, and the technical ability to find out the structure of genes and chromosomes.

Phylogeny Is Based on Homology

Phylogenetic systematics seeks congruence between the lines of descent evolved over time and the supraspecific taxa described by taxonomists. Prerequisite for the description of a taxon of any rank in a phylogenetic system is the recognition that all members originate from one ancestral form and that homologous traits of the ancestral form are found also in their descendants. The question then remained which of the several thousand semantides in a prokaryotic cell are useful for phylogenetic studies. The establishment of a system which is set up to subsume all species must include phylogenetic markers that are ubiquitously distributed, functionally equivalent, and homologous housekeeping molecules. These markers should be homologous apomorphic characters that evolved only once (synapomorphy) but not by convergence. Homology is the sharing by two taxa of a property derived from the same or equivalent property of the nearest common ancestor. Deciding whether a property is homologous or the product of convergence has been the greatest problem, and one that could have been solved only by comparing the course of evolution of each property as laid down in the fossil record. It is obvious that lack of a substantial fossil record and thus the inability to use that record to draw conclusions on genomic and phenotypic properties have prevented the inductive derivation of genealogical lines. Furthermore, morphological complexity and comparative anatomy, extremely useful properties of eukaryotes for determining homologies, are absent in the morphologically and developmentally simple prokaryotes. As a consequence, a phylogenetic classification system only became available after the theoretical and methodological basis had been laid about 30 years ago.

One of the main intellectual breakthroughs that helped microbiologists solve the problems of phylogeny was provided by Zuckerkandl and Pauling (1965), who recognized that organisms are the products of historical events and that all cellular structures reflect their evolutionary history. These scientists also commented that in the case of microorganisms, early evolutionary events can be documented only at the primary structural level of homologous and phylogenetically informative molecules. When comparing organisms, the number and composition of sequence differences between corresponding proteins and genes coding for rRNA reflect phylogenies and consequently allow the recognition of pairs or groups of organisms that originated from a common ancestor. Determining relatedness is based on sequence analysis of genes or their transcripts, also known as the semantides.

There are three categories: primary (DNA), secondary (RNA), and tertiary (proteins) semantides. Sequences of these molecules are molecular chronometers, records of evolution, as they indirectly measure the time lapsed since their origin, and the comparative analysis of primary structures is a powerful tool to measure evolutionary relationships. Episemantic molecules to be used in comparative studies are synthesized under the control of tertiary semantides, and above all it is the chemical composition of cell constituents that have received considerable attention (Schleifer and Kandler, 1972). Asemantic molecules (e.g., exogenous vitamins, phosphate ions, oxygen, viruses) are not produced by the organisms themselves and do not express any of the historic information that organisms contain.

Zuckerkandl and Pauling state that "at any level of integration, the amount of history preserved will be the greater, the greater the complexity of the elements at that level and the smaller the parts of elements that have to be affected to bring about a significant change. Under favorable conditions of this kind, a recognition of many differences between two elements does not preclude the recognition of their similarity." The correctness of this hypothesis was demonstrated by the impressive phylogenetic trees of gene and protein sequences. Episemantic molecules were not considered useful for deriving evolutionary conclusions because enzymes with different primary structures can lead to the synthesis of identical episemantic or similar molecules in different organisms as long as the active enzymatic sites are similar.

The Main Phylogenetic Parameters for Classification

The two main tools [sequence analysis of the semantides DNA, RNA and proteins (Zuckerkandl and Pauling, 1965) and hybridization of genomes] for determining phylogenetic relationships in prokaryotes were developed in the mid-1960s. Historically, the molecular approaches used were sequence analyses of proteins, such as cytochrome C, fibrinopeptides, and ferrodoxins, as well as immunological approaches, such as immunodiffusion and microcomplement fixation. However, the latter methods as well as protein sequencing became less significant with the availability of rapid sequencing techniques for DNA. The branching patterns based upon 16S rRNA and 16S rDNA sequences were surprising, mainly because these patterns showed that characters used traditionally to cluster organisms have in reality restricted phylogenetic meaning. Prominent examples of phenotypic characteristics shown not to circumscribe higher taxa in the past are now examples of characteristics whose evolutionary development has recently been or is soon to be unraveled. These are cell walls (Kandler and König, 1985; Stackebrandt et al., 1985; Schleifer et al., 1990), aerobic metabolism (Fox et al., 1980; Seewaldt et al., 1982), spore formation (Ash et al., 1991; Collins et al., 1994; Stackebrandt and Rainey, 1997), biosynthetic pathways (Balch et al., 1979; Fowler et al., 1986; Stackebrandt et al., 1989), and photosynthesis (Gibson et al., 1985; Woese et al., 1985a; Stackebrandt et al., 1988; Imhoff et al., 1998a; Imhoff et al., 1998b). Today, sequencing of 16S rDNA in bacteriology is so widely accepted that it is considered a classical approach. The sequencing and analysis strategies and their bearing on evolution, phylogeny, classification and identification are now textbook knowledge.

16S rDNA Analysis

Though the importance of rDNA sequencing, alignment, and data analysis is covered extensively in chapter 1 (Ludwig, 1999), the main points are repeated here. The primary structure of the rDNA molecules spans an enormous geological period, almost 3.6 Gy. Why especially has this molecule been selected for phylogenetic analysis? Both the gene and its product are ubiquitous and have functional constancy, common ancestry, genetic stability, appropriate size, and independently evolving domains within the molecule.

Sequence analysis of rRNA or other phylogenetically meaningful genes (Schleifer and Ludwig, 1989) has become a rapid standard technique, and the sequences generated have a very low error rate. The restriction in the use of this molecule as a phylogenetic marker springs from certain intrinsic properties: considering the billions of years that have passed since their origin, the number of informative positions within sequences is small. To maintain function, a reasonable percentage of the positions must remain invariant or be highly conserved, and many of the remaining characters cannot be changed independently. As a consequence, the majority of evolutionary events will remain undiscovered. Another restriction is that most prokaryotic organisms have 2–14 copies of the multigenic rRNA operon in the genome (Farrelly et al., 1995). PCR amplification will mask possible intracistronic microheterogeneities, which consequently may obscure elucidation of small differences between closely related organisms. Sequence analysis of individually cloned operons will unravel these heterogeneities but these few changes must be regarded as "noise" and hence without phylogenetic implications.

Higher order structures of rDNA and rRNA molecules facilitate sequence alignment which can easily be done manually. Several algorithms are available for phylogenetic analyses (Ludwig, 1999). Once a phylogenetic tree or a dendrogram is generated, the taxonomist must judge whether the branching order of the phylogenetic tree is reliable. Numerous factors can influence the topology of a branching pattern, which is a dynamic construct that changes with any new sequence included, or region selected, for analysis. Nevertheless, the topology of trees, which are generated on the basis of genes subjected to the same fate in evolution (e.g., common horizontal or vertical gene transfer), are rather stable and robust constructs. Results of comparative analyses of other conservative molecules responsible for central functions such as the β–subunit of ATP synthases, elongation factors, phosphoglycerate kinase, and DNA-directed polymerase demonstrate this point. Thus, trees based on rDNAs and rRNAs reflect not only the evolution of these molecules but also, most likely, the evolution of a major portion of the genome. In principle, the primary structure of the most widely analyzed 16S rRNA gene must be regarded as a miniaturized version of a major part of the genome, and, though due to its size of only 1,540 bases, as having much less power to resolve.

DNA-DNA Hybridization

This method was the first molecular approach used routinely for measuring degrees of relatedness and the first phylogenetic one to be generally accepted for improving bacterial clas-

sification. It is still the most rapid and inexpensive of all phylogenetic methods for measuring an average nucleotide similarity of the entire genome; however, it gives no indication of which genes contribute to or detract from the similarity. Also, this technique reveals why neighboring species show somewhat lower similarity values. Though unclear and unsatisfactory, the information found in a genome, containing a range of highly to less conserved genes, has the advantages over information obtained by comparison of individual genes or gene products only. Several hybridization techniques have been thoroughly tested to determine the influence of various experimental parameters and compared to determine reproducibility and limitations (Grimont et al., 1980; Huss et al., 1983; Baumann et al., 1983; De Ley, 1970; De Ley, 1970; Schleifer and Stackebrandt, 1983). DNA-DNA pairing studies, comparing the same strains by different techniques, were in good agreement (see Schleifer and Stackebrandt, 1983 for examples). Some novel techniques and variations of established methods have been introduced such as hybridization in microdilution wells (Ezaki et al., 1989; Hara et al., 1991; Kaznowski, 1995), the random-primed labeling and signal amplification system (Amersham Life Science, Piscataway, NJ), or detection of double-stranded, digoxigenin-(DIG) labeled DNA with anti-DIG antibodies conjugated with alkaline phosphatase (Lind and Ursing, 1986; Ziemke et al., 1998). Some of these novel methods have not been compared with the established ones (S1-, renaturation- and filter methods), but others such as the microplate technique (Ezaki et al., 1989) and the renaturation technique correlated very well (Goris et al., 1999).

Relationships are usually expressed in terms of DNA similarities. It should be noted that because the underlying processes of renaturation are still unknown, the expression "DNA homology" should not be used in connection with DNA reassociation techniques. Wayne and colleagues (1987) recommended use of a second parameter, the Tm(e) value (Tm(e) is the melting point of DNA formed originally by the reassociation process of two single stranded DNA molecules; (e) stands for eluted labeled single stranded DNA which is released by heating up the double stranded DNA), especially in those cases where, under optimal hybridization conditions, DNA similarities fail to discriminate between fine details in relationships. The inverse linear correlation between Tm(e) and DNA similarity makes determination of both parameters somewhat redundant (Grimont et al., 1980; Baumann et al., 1983), and hence Tm(e) values is usually not included in DNA-DNA reassociation studies.

Greater reproducibility and small sampling error (Sneath, 1989) are obvious advantages of DNA hybridization. The disadvantages are the unavailability of equipment, i.e., a thermocontrolled spectrophotometer, and lack of DNA in sufficient quantity and purity, as exists with many archaea and lithoautotrophic bacteria. The limited resolving power of DNA hybridization has been recognized from the very first experiments. It has been calculated that for reassociation under optimal hybridization conditions (25°C below the Tm(e) of the DNA), the two DNA strands must exhibit at least 80% sequence complementarity. Depending on the sequence similarity of the reassociating single strands, a difference of about 20% is then spread between 0% (no hybridization) and 100% (as defined by maximal reassociation obtained with the homologous DNA strands). It is therefore obvious that a given DNA homology value does not reflect the actual degree of sequence similarity of the primary DNA structure. As measured with experimentally introduced mispairings, thermal stabilities have been estimated to decrease from 1 to 2.2% for each percent mispairing (Bautz and Bautz, 1964; Britten and Kohne, 1968; Ullman and McCarthy, 1973). Although these experiments have been performed on short stretches and not on complete genomes, one can nevertheless argue that organisms that share 70% DNA similarities share at least 96% DNA sequence identity (Johnson, 1973). If the number of bases in the genome of E. coli is approximately 4×10^6, then 4% differences or 1.6×10^5 nucleotides are different (not taking into account the possibility that genome rearrangement is a source of decrease in DNA similarity). This divergence could easily account for the significant differences in phenotype observed between strains of some species, e.g., *E. coli* (Brenner, 1991).

Correlation of Individual Phylogenetic Parameters

It is generally accepted that if two organisms have highly similar DNA, they are closely related genetically. The parameters measured by different methods have shown excellent agreement when closely related organisms are compared. However, when parameters from more distantly related organisms are measured, the data are difficult to reconcile (Huss et al., 1983; Grimont et al., 1980). Numerous studies have shown that phenotypic and genetic similarities agree only if the borderline of 70% similarity is obtained under optimal hybridization conditions. Therefore, this borderline has been recommended for species differentiation (Wayne et al., 1987). Values from 30 to 70% reflect a moderate degree of relationship, whereas values become

increasingly unreliable (and taxonomic conclusions should be avoided) once these values fall below the 30% level. One has, however, to consider that this recommendation was derived mainly from experience of working with numerous strains of enterobacterial species (Steigerwalt et al., 1976; Brenner, 1991). Thus, transferring the situation found for a phylogenetically very shallow group of mainly eukaryote-associated organisms to two ancient, highly structured, and enormously diverse prokaryotic domains grossly underestimates the different mechanisms as well as the mode and tempo by which organisms evolve. But then one has to remember that the delimitation value (of 70%) is artificial and used to structure the bacterial world at the level of species.

For highly related organisms, there is very good congruence in general between DNA-DNA and DNA-rRNA hybridizations (De Smedt and De Ley, 1977; De Vos and de Ley, 1983; Johnson and Francis, 1975). The Tm(e)s for strains of species exhibiting more than 60% DNA homology differ by less than 2°C. However, the DNA-rRNA hybridization technique has been superseded first by 16S rDNA cataloguing and then by sequence analysis of 16S rDNA. Values on DNA-rRNA hybridization are found in the literature before 1995. Likewise, similarity coefficients of the 16S rRNA cataloguing approach (S_{AB} values), the predecessor technique of 16S rDNA similarity determination, are found in the literature before 1990. For the same reasons as for total 16S rDNA, correlation between DNA pairing values and S_{AB} values is only marginal (Stackebrandt, 1992). Only of historic value are the correlation blots between rRNA homology and S_{AB} values (Schleifer and Stackebrandt, 1983) and the correlation between S_{AB} values and actual almost complete 16S rRNA sequence homologies (Woese, 1987).

The correlation blot, determined for the two most widely used approaches for discovering prokaryotic phylogeny, justified continuing the use of the DNA-DNA reassociation technique. Let us assume the unlikely case that the plot showed linear correlation between intraspecies DNA similarities of above 70% and 16S rDNA sequence similarities above 97.5%. The DNA hybridization method would have disappeared overnight. Unfortunately (for those who are using the reassociation technique) the situation is different. The 16S rDNA is not a miniaturized mirror image of the genome but is too constrained by its function to change as quickly as less conserved molecules. As a consequence, there is a curvilinear relation between the two parameters (DNA-DNA reassociation and rDNA similarities; Amann et al., 1992; Fox et al., 1992; Stackebrandt and Goebel, 1994). Each approach is strong in those relationship areas that other methods are weak in. Sequence analysis has proven to be a reliable way to distinguish the phylogeny of organisms of different domains (with 55–60% similarity) from moderately related species (around 97% similarity). Above 97% 16S rDNA hybridization values can be as low as 55% or as high as 100%. Several organisms, which are known to share 99.8% or even 100% rDNA similarity, belong to different species because the DNA reassociation values are below the 70% threshold value. Even if one considers that the DNA reassociation values originated from different laboratories using different reassociation methods, the evidence is strong enough to state that the sensitivity of DNA-DNA reassociation is significantly greater than that of 16S rDNA sequencing. When a 16S rDNA similarity value of less than 97% was found to correspond to a DNA-DNA reassociation value of not more than 60%, Stackebrandt and Goebel (1994) recommended that DNA pairing studies did not have to be performed at this and lower levels of sequence similarity. These levels indicated that the strains concerned are not members of the same species.

The correlation blot of 16S rDNA and DNA-DNA similarity values obtained for some species described in 1998 and their nearest phylogenetic neighbors (Fig. 1) demonstrates that the recom-

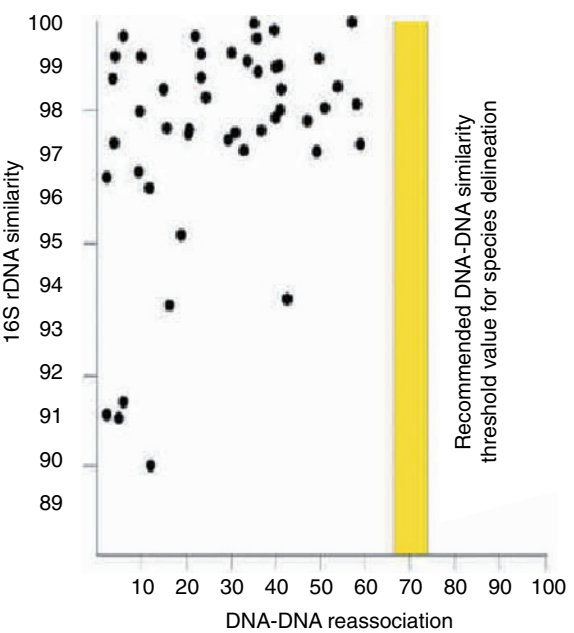

Fig. 1. Correlation blot between 16S rDNA and DNA-DNA reassociation similarities, indicating for several phylogenetically highly related species (as determined by 16S rDNA values of >98%) that DNA-DNA similarities are clearly below the threshold value of 70% recommended for species delineation. Values are taken from volume 48 of the *International Journal of Systematic Bacteriology* (1998).

mendation of Wayne et al. (1987) has been accepted by taxonomists. Except for a single case, all strains of a species share higher than 70% DNA-DNA similarity, while they share less than 70% DNA-DNA similarity with their nearest neighboring species. The one exception refers to *Pelistoga europaea*, which have four genomovars that are related at the 40% DNA similarity level. The authors (Vandamme et al., 1998), however, clearly state that the lack of phenotypic evidence presently excludes the description of four individual species, a step that may be necessary once these differentiating characteristics become available.

Comparison of Phylogenetic Patterns of 16S rDNA

The increasing number of dendrograms and phylogenetic trees in the literature reflects the common notion that the 16S rDNA sequencing method is a "traditional" method. Many trees are not comparable as they were generated from partial sequences and different treeing methods (summarized by Felsenstein, 1982, 1988) and are therefore of historic interest only (Stackebrandt, 1988, 1992). Today nearly complete sequences are compared using a few treeing algorithms of proven resolving power and statistical significance (Ludwig, 1999). But some problems remain. First, the parts of the sequence judged to be of less phylogenetic importance are subjectively omitted. Second, computer programs cannot handle the enormous amount of data in a reasonable time without omitting either the number of reference organisms or sequence information. However, one must differentiate between goals; this determines the number of sequences used. For taxonomic studies, e.g., questions about the intrageneric relationships, the number of sequences is mostly restricted to those of type strains and a few others. In this case the complete sequence information, including that of the variable regions, can be compared. The branching pattern obtained will change if this small dataset is embedded in a larger one composed of sequences of members of families, orders, classes, and so on. At each level, information will be lost by either removal of variable regions or trimming of stem and loop structures to the minimum length common to all members of the dataset and by omission of those regions for which ambiguous sequence information is provided. Each of these steps will most likely lead to changes in the branching pattern of any lineage. Thus, the picture created from the inclusion of thousands of sequences in a single dataset is not more than an approximation of the phylogeny. The literature is full of examples that demonstrate changes of phylogenetic relatedness within genera and families through the influence of new entries in the database. Most of the branching patterns are supported by high bootstrap values that for a given tree indicate that the statistical analysis supports the order of lineages. But this statistical analysis is per se no indication that the pattern reflects the natural relationship with a similar degree of confidence.

If an algorithm does not exist that could provide a tree more closely reflecting the evolution of prokaryotes, then we must accept the pattern that appears to be the most plausible one. It may be the one showing the highest degree of topographic similarity to patterns derived from different informative molecules, although it should be noted that these similarities are not per se proof of phylogenetic "truth." The phylogenetic framework referred to as the most convincing one for the bacterial and archaeal kingdoms is today classified as domains Bacteria and Archaea (Woese, 1987; Olsen et al., 1994).

The Prokaryotic Species: A Natural Entity or a Taxonomic Myth

The evolutionary record as a basis for phylogenetic studies can now be found in the extensive database of molecular sequences, which have placed the bacterial world into the framework of the evolutionary process. But this information alone does not help describe a species or how a species has to be defined. Rather, it puts the prokaryotes on the same level with animals and higher plants in the debate about species as a general unit for biodiversity, evolution and taxonomy. Does this mean that biologists can now define the category species as a comparable biological entity for every organism? The problem is that biologists themselves are not clear about the definition of "species," a concept that lacks a theoretical basis (Bachmann, 1998). The concepts of phylogenetic species (Cracraft, 1983), taxonomic species (Staley and Krieg, 1984), biological species (Dobzhansky, 1937; Istock et al., 1996), which disregards asexual reproduction entirely, and ecological species (Istock et al., 1996) have strengths and weaknesses, and each of them stresses different aspects of biology and evolution. In his dictionary of microbial taxonomic usage, Cowan (1968) even states bluntly that the species is not a natural entity. Surprising perhaps to microbiologists, there are some zoologists (Hull, 1997) and botanists (Bachmann, 1998) who suggest the possibility that the species is not an objective basic unit of taxonomy. The nonexistence of species as an objective category and as a product of natural selection, which after sufficient study is identifiable by the taxonomist, has been recognized by microbiologists for more

than 20 years. Bacteriologists in particular follow guidelines and recommendations to provide stability, reproducibility, and coherence in taxonomy—although in the final analysis, species description is still subjective. This concept does not include the role of reproductive isolation, i.e., the barriers to horizontal gene transfer over large phylogenetic distances; it does not even try to explain the mode of speciation. One may be amused by such a naïve approach—but once you decide that a species can not be recognized as a natural entity, the only alternative is a compromise of a working definition. In that, the fundament of a species can be found in genealogical relatedness of its members (Wayne et al., 1987; Stackebrandt and Goebel, 1994). This strategy has facilitated the practice of taxonomy—a strategy also used by protozoologists, mycologists and algologists. As Bachmann (1998) points out, the most useful general species definition would be the one that allowed "the largest number of individual organisms to be unequivocally assigned to species so that some basic conditions are satisfied." These conditions are: 1) strains are assigned to only one species and never to none; 2) all lines of descent within a species are members of that species; 3) members of a species should be phylogenetically related; and 4) the species (so defined) should apply to taxa that coincide more or less with the intuitively recognized species. Most obviously, conditions 1) to 3) are already in effect in bacteriology, while condition 4) has failed significantly in the past because of inability to classify a prokaryotic species by intuition.

There is no recognized concept of a prokaryotic species, though Istock et al. (1996) defines nine different mechanisms for their evolution. Nevertheless, the pragmatic definition is well accepted among bacteriologists. The combination of phylogenetic grouping based on sequence comparisons with taxonomic classification is a very powerful approach. This polyphasic approach is the only accepted strategy, which does not mean that certain components of the approach are not criticized. The process of revision and constant adaptation appears necessary as not only new insights into cell structure and cell function should be incorporated but also the microbiologists' perception. As Staley and Krieg (1984) phrased it: "A classification that is of little use to the microbiologist, no matter how fine a scheme or who devised it, will be ignored or significantly modified." This is true for each level within a hierarchical system, and the history of microbiology is marked by many examples of rejected systems. The higher ranks are almost completely defined by subjective arguments—to the point where the importance of working with taxa above the rank of genera is considered trivial (O'Hara, 1994). The importance of any hierarchical system goes beyond the main function of classification and identification. Based on knowledge available at the time of its establishment, a hierarchical system should explain and increase understanding of the evolution of organisms and their groupings. In bacteriology, time has seen various hierarchical systems and various proposed phylogenetic paths fail because they were not based upon the natural relationships but rather on properties believed to express natural relationships such as morphology (Cohn, 1872; Stanier and van Niel, 1936), pigmentation, physiology (Orla-Jensen, 1909; Margulis, 1981), and cell constituents (Schleifer and Kandler, 1972). Some of these attempts were important contributions in their time because the classification system based upon them actually reflects phylogenetic divergence (e.g., peptidoglycan structure, lipids, fatty acids). This strategy has changed now, as the order of phylogenetic lineages guides the bacteriologists to the two basic units, the genus and species, without the need for a superimposed system. Actually there is no immediate need to work with a hierarchical system, but it is tempting to do so to comprehensively classify similarities, differences and evolutionary traits. Time and the recognition of the semantic character of the macromolecules DNA, RNA and proteins have shown that this basis was lacking (Zuckerkandl and Pauling, 1965). Today, we see the emergence of higher taxa along the phylogenetic structure and, like in systems of plants and animals, taxa of the same rank are not necessarily comparable units or describable in a coherent way. Also, we must be aware that only a small fraction of prokaryotic species are described, and new entries will not only change the description of the higher ranks but may change the composition of taxa as well. However, the advantage of a rational hierarchical structure, i.e., based on the organisms' evolutionary history, makes it highly likely that changes within the system will occur only within ranks of a common genealogical lineage and not, as in the past, affect and possibly change remotely related taxa.

The species definition applied today does not incorporate the modus of grouping entities into named "natural" species. Several factors that contribute to the evolution of the genome have been identified through intensive multilocus enzyme electrophoreses and sequence typing of housekeeping genes (Maiden et al., 1996) and of random amplified polymorphic DNAs and multilocus enzyme electrophoreses (Selander et al., 1994; Istock et al., 1996). Some organisms, e.g., *Neisseria* and *Rhizobium* species, as well as enterobacterial species (Guttmann and Dykhuizen, 1994), are subjected to reticulate events or

panmixis (Maynart-Smith, 1993; Istock et al., 1996) in which clonal relationships, due to mutational events and vertically transmitted accessoric genetic elements, are disturbed by horizontal genetic transfer, e.g., conjugation, phage transduction DNA transformation (Achtman, 1998). Other strains that are mostly endosymbionts and obligate pathogens, such as members of the genera *Bartonella*, *Brucella*, and *Rickettsia*, are mainly clonal because they are subject only rarely to horizontal gene transfer. In some species the recombination is more frequent among strains of different than of the same species (e.g., the enterobacteria), which leads to the homogenization of the gene pool of the interacting organisms (Guttmann and Dykhuizen, 1994). An attempt to formulate a biological species definition for bacteria takes the following observations into account (Dykhuizen and Green, 1991): 1. Phylogenetic trees from different genes from members of a single species should be different (shown for three genes from *E. coli*). 2. Phylogenetic trees from different genes from members of different species should be the same (as shown for two genes from seven species of *Neisseria*). Without questioning the validity of this approach, it is obvious that this strategy is far beyond the capability of routine sequence analysis methods, especially inasmuch as several strains of a single species must be investigated, and although worth discussing, this approach cannot immediately replace the present pragmatic species definition.

The current pragmatic species definition also does not account for the ecological niche, although the source of the isolate is part of a species description. This site is the strain's actual place in the ecosystem provided the strain is dependent on its environment, e.g., the rhizoplane, rhizosphere, and host in endosymbiotic and pathogenic relationships. The terms (marine water, fresh water, mud, sediment, soil, rumen, skin and so on) are too superficial to describe the exact niche from which complex environmental samples are taken. Knowing about the site of speciation and the environmental selection of members in a clonal population may help explain the path of evolution and the mode of speciation, but this information does not help define the level at which a subpopulation may be regarded as an individual species.

The Pragmatic Species: Definition

The definition of a prokaryotic species has a phylogenetic component given by Cracraft (1983) as "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descendents" and a taxonomic component given by Colwell (1970) as "a group of related organisms that is distinguished from similar groups by a constellation of significant genotypic, phenotypic, and ecological characteristics." This definition combines descriptions of geno-, or genomospecies, taxospecies, and phenospecies, which reflect the different concepts of species upheld in the past decades. From a pragmatic point of view, all these facets have been incorporated into a single definition, though the terms are still in use. An "optimal" species is one that simultaneously represents a phylogenetically, phenotypically, and naturally occurring group, but except for many strains of pathogenic species, the species' ecological niche is either not known or the number of isolated strains is too small to identify their original habitat. It should be remembered, that from the broad diversity of prokaryotic organisms, which may reflect a genetic and epigenetic continuum, a single strain is chosen as the type strain. Strains that are sufficiently similar, i.e., by mainly DNA-DNA reassociation used today in prokaryotic taxonomy, are considered members of this species. This concept of selecting species has been described as the arbitrary species concept (Staley, 1997). The combination of arbitrary selection and artificial species delineation is admittedly arguable and open to discussion, especially when taxonomists covering different fields of biology meet. When compared with the phylogenetic diversity of a prokaryotic species, the phylogenetic diversity of *Homo sapiens* and its closest relatives, the higher evolved apes (all species from man to lemurs, comprising about 200 species, which are related by higher than 75% DNA reassociation; Sibley et al., 1990), would be within the 70% threshold value. Obviously, the species definition of prokaryotes cannot be applied to eukaryotic organisms. For prokaryotic organisms the pragmatic approach to the species definition has been extremely useful and its success is measured by its widespread acceptance.

Delineating a Species

In the daily routine a new isolate runs through an identification process, which may be different from laboratory to laboratory and from taxon to taxon. Many scientists, however, are not interested in a fine resolution of relationship, or they are not in a position to go through the laborious identification process. Some initial superficial tests are performed, like determination of colony morphology and pigmentation, shape, spore formation, Gram stain and relationship to oxygen. The aim of the study is to define the identification procedures to follow—but it can be

expected that phylogenetic diversity in many similar-looking organisms remains undetected. The classification process starts when the isolate does not fit the description of one of the 4,200 validly described species.

Let us assume the laboratory is equipped with facilities to generate a 16S rDNA sequence. It is recommended to start any survey with this molecule, as the database of prokaryotic strains is enormous, covering more than 95% of described species. To search for the closest relative using available 16S rDNA sequence data, taxonomists are offered electronic help by the BLAST system (blast.help@ncbi.nlm.hih.gov), the Ribosomal Database Project (http://www.cme.msu.edu/RDP), or updates of the ARB program (http://www.arb-home.de). The taxonomic browser will guide the user to a phylogenetic arrangement of taxa. The search in RDP and ARB will show the phylogenetic distance to the isolates' nearest neighbor(s), but the quality of the search depends upon the completeness of the 16S rDNA database. The sequences available, species analyzed, and whether comparison is based on short stretches or on almost complete sequences, may vary. Once the approximate nearest phylogenetic neighbor has been identified, a search of the public databases for recent entries is recommended.

The result of the analysis will affect future strategy. Let us assume that the 16S rDNA similarity to its nearest neighbor has values higher than 97–98%. Many scientists will be satisfied knowing the approximate phylogenetic position and will not continue the identification process. Others, however, will be eager to determine the more precise affiliation of the isolate. The way to proceed depends upon the number of species in the phylogenetic vicinity of the isolate.

1. In case the isolate falls within the boundaries of a genus, the description of this genus will be a guide to the few key properties needed to place the isolate in this genus. If the species within this genus are separated by distinct phenotypic properties (which one should assume but which is not always the case), these should be sought in the isolate and, if present, the isolate has been identified. If not, DNA-DNA reassociation studies are recommended to determine whether the isolate is the nucleus of a new species. In case DNA similarities are lower than about 70% DNA-DNA reassociation, the isolate should be described thoroughly, providing evidence for the genus characteristics and those properties that distinguish the new species from the established ones.

Examples are found frequently in the literature and a few of them are shown here:

The most widely encountered situation is the description of a new species which shows less than 70% DNA-DNA reassociation with those species to which it is closely related by 16S rDNA similarity: Hybridization values for the type strain of the new species and that of the closest relative usually range between close to 70% and almost zero percent. To give two examples, *Kocuria rhizophila* is separated from *Kocuria varians* by a similarity value of 52.6% (Kovacs et al., 1999), while the DNA-DNA relationship between the type strains of *Methanococcus infernus* and *Methanococcus jannaschii* was less than 10% (Jeanthon et al., 1998).

In those cases where the DNA-DNA reassociation value between an isolate and a described species is ≥70%, this information will usually not be recorded, unless the new strain leads to the description of a subspecies or to an emendation of the species description. Many examples exist in the literature that demonstrate the intraspecies genomic homogeneity.

DNA-DNA reassociation reveals that the type species of two different species are actually strains of the same species: The species *Kocuria erythromyxa* was reclassified as *Kocuria rosea* on the basis of 95% DNA-DNA reassociation and similar phenotypic differences (Schumann et al., 1999).

Species are separated at the threshold value of around 70% DNA-DNA similarities on the basis of differences at the epigenetic level. For example, the separation of the type strains of *Desulfurella acetivorans* from *Desulfurella multipotens*, sharing 69% DNA-DNA similarity, was based on the ability of the latter strain to use butyrate as growth substrate and to grow chemolithoautotrophically on mineral medium containing molecular hydrogen, CO_2 and elemental sulfur (Miroshnichenko et al., 1994).

The recommendations (Wayne et al., 1987) to delineate species in genomic terms at a threshold value of around 70% DNA-DNA reassociation are guidelines but should not be applied as fixed rules. Though the majority of species are actually described as suggested, there are a few exceptions:

One example refers to the lack of any phenotypic differences in the two putative species that are separated by higher than 70% DNA-DNA reassociation. In practice, it would be impossible to affiliate other strains to either species and to distinguish between the two species without performing DNA-DNA reassociation studies (Vandamme et al., 1998). One should, however, consider that the primary structure of a 16S rRNA, like that of other genes, is a linear compilation of phenetic characters, whose composition at a defined position should be treated as a phenetic property. For example, ornithine at position 3 of the peptidoglycan subunit is treated as a different taxonomic marker than the pres-

ence of diaminopimelic acid or lysine at the same position in a different organism. Thus the differences in nucleotides involved in compensatory base exchange in the 16S rDNA nucleotide sequences that occur between two closely related species distinguishable by DNA-DNA reassociation should be accepted as characters with discriminating power. Using this strategy, a new species of *Sulfitobacter mediterraneus* has been described which by the profile of its metabolic properties could not be distinguished from *Sulfitobacter pontiacus* (Pukall et al., 1999). Whether to consider other gene sequences, significant differences in the patterns of whole cell proteins, or restricted or amplified nucleic acids should be decided case by case.

The other examples refer to medically important organisms. Strains of *Escherichia coli* and *Shigella dysenteriae* are extremely closely related and exhibit DNA hybridization values as high as 89% (Brenner, 1973). Nevertheless, for epidemiological purposes the two taxa are not considered strains of the same species but are presently allocated to two different genera. On the other hand, certain strains of *Clostridium botulinum* are remotely related only by 16S rDNA analysis, they share less then 10% DNA-DNA similarity as measured by hybridization, and they are more closely related to other *Clostridium* species than they are related among themselves. However, as all of them express a botulinum toxin, which immediately guides the physician in the therapy of botulism, no attempts have been made to alter the classification of these strains (Fig. 2).

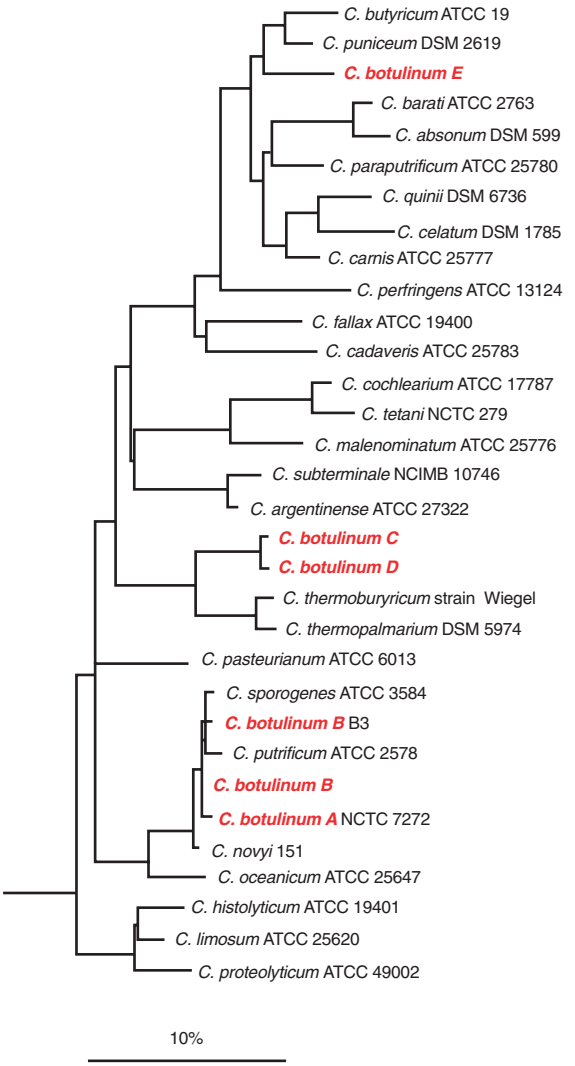

Fig. 2. The phylogenentic relatedness of *Clostridium botulinum* strains exhibiting different serotypes among strains of different *Clostridium* species of group I (Collins et al., 1994). The 16S rDNA dendrogram is a detail of the ARB tree. The scale bar corresponds to 10 nucleotide substitutions per 100 sequence positions.

1. Despite the recommended value of 70% DNA similarity, taxonomists working with some defined prokaryotic groups have altered this value to come to a better correlation between phenotypic and genotypic similarities. Within the family Pasteurellaceae, a DNA-DNA reassociation value of and above 85% describes a species (Mutters et al., 1985). Similar values have been found for the interspecies relatedness of *Blastomonas pertussis*, *Blastomonas parapertussisi* and *Blastomonas bronchiseptica* (Kloos et al., 1981) and between members of the spotted fever group of *Rickettsia* (Walker, 1989). Another well-known example in the literature is the fate of the members of the genus *Brucella* (Moreno, 1997). This genus contains six species (Meyer, 1990), in spite of the fact that DNA-DNA reassociation values separate these species above 98% similarity (Verger et al., 1985). For the workers in the field, the presence of individual DNA restriction patterns, phenotypic and antigenic properties, and, above all, the distinct biological behavior of the species, e.g., host range and pathogenicity, are more indicative than the strict application of the general rule of separating species.

2. In the case of 16S rDNA, if similarity values indicate an approximately equidistant relationship to members of different genera, the diagnostic properties given for these genera must be tested for the isolate. Such highly related genera have been described in the order Actinomycetales, α and γ subclasses of Proteobacteria, and the *Bacillus* line of descent. If the properties match those of one of the genera, the identification process will be restricted to members of this genus and one has to proceed as indicated under 1. If the analysis of the genus-

specific properties reveals no match with any of the genera, it is likely that the isolate represents yet another closely related genus of this genus cluster and the description of the new species and the new genus will go hand in hand. Well-known examples are the closely related species of the genus *Rhizobium*. Nevertheless, at this high level of 16S rDNA relationship, the analysis also will have to include the only technique recommended for the elucidation of intragenus relationships, i.e., the determination of DNA-DNA reassociation values between the species of the new genus and the species of the neighboring genera. Another example has been described for the genera *Blastomonas* and *Erythromonas* in the alpha subclass of Proteobacteria (Yurkov et al., 1997). The type strain of *E. ursincola* shares 99.2% 16S rDNA similarity with the type strain of *B. natatoria*. Based upon the presence of bacteriochlorophyll a in *E. ursincola* and its absence in *B. natatoria*, it was concluded that these organisms represent different genera. The DNA-DNA reassociation value of about 40% for these two strains (Tindall, pers. comm.) supported the taxonomic separateness of the two species. If, however, future studies show that the presence of bacteriochlorophyll a is not a unifying property of *E. ursincola* strains, or if bacteriochlorophyll is found in strains of *B. natatoria*, the taxonomic rationale for having two genera will diminish.

3. If the new isolate shares less than 97% sequence similarity with the nearest phylogenetic neighbor, then as many taxonomists now recommend (Stackebrandt and Goebel, 1994) DNA-DNA reassociation studies are unnecessary, because the latter values will range clearly below the 70% reassociation borderline value recommended for species definition. Indeed, a survey of articles of volume 48 of the *International Journal of Systematic Bacteriology* indicates that for about 30% of all newly described species sharing less than 97.5% 16S rDNA sequence similarity with their closest neighbor, DNA-DNA reassociation has not been determined. In those cases in which DNA-DNA reassociation values were provided, the values are clearly below the 70% threshold (Fig. 1).

Several genes other than the one coding for 16S rDNA have been sequenced recently to test the discriminating power of 16S rRNA genes. Interestingly, in *Shewanella* species, genes coding for gyrB were found to have a less conservative primary structure (Venkateswaran et al., 1998) than those coding for 16S rRNA and thus appear to be better suited for the elucidation of close relationships. In *Pseudomonas*, results of gyrB analysis matched those of 16S rDNA analysis, but only when the highly variable regions were omitted from the latter molecule (Yamamoto and Harayama, 1998; Yamamoto et al., 1999). Other genes used were the chaperonin GroRL (Viale et al., 1994), heat shock protein (hsp65) for strains of the *Mycobacterium avium* complex (Swanson et al., 1997), a gene (sodA) coding for a manganese-dependent superoxide dismutase in streptococci (Poyart et al., 1998), the ompA gene of Rickettsiae (Fournier et al., 1998), the mba gene fragments of *Ureaplasma* (Knox et al., 1998), or the RNAse P in Actinobacteria (Cho et al., 1998).

Increasingly, the spacer regions (ITS) separating the genes coding for the 16S rRNA and the 23S rRNA are used to determine inter- and intraspecies relatedness (Barry et al., 1991; Gü and Stanisich, 1996; Leblond-Bourget et al., 1996). Though this approach is currently used mainly for differentiation but not for the delineation of species, this and other molecular techniques such as chromosomal DNA fingerprinting (RFLP, restriction fragment length polymorphism; AP-PCR, arbitrarily primed PCR; ERIC-PCR, enterobacterial repetitive intergenic consensus PCR), gene fingerprinting (rDNA-RFLP analysis; Vaneechoutte, 1996), or ribotyping (Grimont and Grimont, 1986; Webster et al., 1994) can be useful in revealing the homo- or heterogeneity of strains of a species.

The Phenotypic Circumscription of a Species

In contrast to the rather stringent genomic definition of a "species," the phenotypic characterization of a new species is very variable. The properties to be investigated depend upon those indicated as being specific for the genus and on the set of characters already indicated for discriminating between species described for the genus.

Extensive morphological and ultrastructural characterization must be presented, especially for species of novel genera. Records on enrichment and isolation, motility, colony characterization, optimal growth conditions, growth requirement and substrates, and on base composition of DNA are parts of a set of characterizing features. Analysis of special features is required for certain taxa, such as antigenic characterization for *Leptospira* and mycoplasmas. Many of the properties to be provided for the description of a species are listed either in the descriptions of minimal standards, which are available for species of some genera, or they are compiled in *Bergey's Manual of Systematic Bacteriology*. Information about the phylogenetic position of a putative new type strain facilitates the selection

of such features and guides the search for taxonomically relevant properties.

Delineation of the Genus

The definition of a genus given by Cowan (1968) has not been changed by the input of molecular data. Cowan states that genus is "... one of the basic ranks in the hierarchical systems used in biology, and probably the highest rank with any significance in microbiology. In position between FAMILY and SPECIES, it is best considered as a collection of species with many characters in common; unfortunately no one has indicated the extent of this sharing of characters, and it is purely a matter of personal judgement ... as to what constitutes a genus. Like the SPECIES, the genus is a subjective concept without any foundation in fact."

A significant finding of the analysis of rRNA, rDNA and DNA-DNA reassociation studies was to point out the genetic heterogeneity of many phenotypically defined genera. Since genus and species are those ranks for which proper descriptions are needed most urgently, the new results were both confusing and encouraging (Stackebrandt and Woese, 1984): confusing in a sense in that in many cases, the working basis (the genus) had to be redefined after genera described on traditional grounds were found to be phylogenetically incoherent or after the type species was found to actually be a member of a different genus. Examples have been described for *Methanobacterium*, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Clostridium*, *Streptococcus*, *Flavobacterium*, *Bacteroides*, *Arthrobacter*, *Micrococcus*, *Brevibacterium*, *Nocardia* and several genera of phototrophic organisms, but it should be stressed that almost each genus was involved in the reclassification process to a varying degree. Some examples are listed in Table 4.

The results of the reclassification process, i.e. the elimination of misclassified strains, not only resulted in the description of genomically and phenotypically homogeneous genera, but also led to the reduction of species number per genus (Table 4).

A new genus has to be described when a strain or a strain cluster is shown to branch outside the radiation of a validly described genus and the isolated phylogenetic position is accompanied by distinct phenotypic properties not found among the neighboring genera. On the other hand, the placement of a new taxon with a novel pattern of phenetic properties within the radiation of a genus may point towards its taxonomic heterogeneity, which consequently may lead to the dissection of the genus. The decision about which phenotypic properties to use for the circumscription of a novel genus is up to the taxonomist but depends to some extent upon the description of the neighboring genera. The genus-specific characteristics must be present in each species of the genus. The following is a short list of examples of highly to moderately related genera and their discriminating properties:

Chemotaxonomic properties: In the order Actinomycetales for example the high degree of chemical diversity in the peptidoglycan, fatty acids, polar lipids, menaquinones, whole cell sugars or teichoic acid offers superb diversity at the epigenetic level to delineate genera. The correlation with phylogenetic analysis is so high that the finding of a new combination of such patterns indicates a new genus (Embley and Stackebrandt, 1994).

Morphological, chemotaxonomic and growth properties: The main basis for the division of the former genus *Bacillus* into eight genera has been the extensive phylogenetic analysis of its species (Stackebrandt et al., 1986; Ash et al., 1991; Rainey et al., 1993). The separation into several genera is based mainly upon the chemical structure of peptidoglycan, cell shape, spore shape, anaerobic growth, optimum pH, growth in 10% NaCl, and cellular fatty acids. As compared with the actinomycete genera, the importance of chemotaxonomic properties is low. The most species-rich genus, *Bacillus* (>60 species) itself, is heterogeneous with respect to amino acid composition of peptidoglycan, spore shape, anaerobic growth, presence of swollen sporangium and other features, and it can be expected that this genus will be subject of even further dissection.

Biochemical properties: The genus *Rhizobium* has been dissected into *Sinorhizobium*, *Azorhizobium*, and *Mezorhizobium* and these genera, together with *Allorhizobium*, *Mycoplana*, *Phyllobacterium*, *Agrobacterium*, *Bartonella bacilliformis* and *Blastobacter aggregatus* constitute a highly related group of mainly plant-associated bacteria (>92% 16S rDNA similarity). Phylogenetic analysis indicates that members of *Rhizobium* do not form a coherent genus but some members are more closely related to *Allorhizobium* (de Lajudie et al., 1998). The discriminative features between *Rhizobium* species and members of morphologically similar genera are predominantly results of carbon assimilation tests.

Morphological, physiological and growth properties: The number of genera within the family Chromatiaceae has recently been significantly enlarged, following the dissection of *Cromatium* into six genera and the reclassification of two *Thiocapsa* species as the type species of two novel genera (Imhoff et al., 1998b). This move was due to the phylogenetic heterogeneity of the genera involved though no novel phenotypic data were found that would clearly support the

Table 4. Some examples for the dissection of phenetically defined genera by transfer of species into phylogenetically coherent genera.

Phenetic definition	Number of species: Before	Number of species: Transferred reclassification	Emerging phylogenetically coherent genera	Higher order affiliation
Bacillus	124	36	*Bacillus*	
		3	*Alicyclobacillus*	
		19	*Paenibacillus*	
		10	*Brevibacillus*	Bacillaceae
		3	*Aneurinibacillus*	
		1	*Virgibacillus*	
Bacteroides	65	40	*Bacteroides*	Bacteroidaceae
		1	*Ruminobacter*	Succinivibrionaceae, γ-subclass of Proteobacteria
		7	*Porphyromonas*	Bacteroidaceae
		21	*Prevotella*	Bacteroidaceae
		1	*Anaerorhabdus*	Cytophagales
		1	*Megamonas*	not known
		1	*Rikenella*	Cytophagales
		1	*Mitsuokella*	Sporomusa subbranch of Clostridium group
		1	*Dichelobacter*	γ-subclass of Proteobacteria
		1	*Fibrobacter*	Fibrobacter line of descent
		1	*Sebaldella*	Fusobacterium line of descent
		1	*Dialister*	Sporomusa subbranch of Clostridium group
		1	*Campylobacter*	ε-subclass of Proteobacteria
		1	*Capnocytophaga*	Cytophagales
		1	*Tissierella*	Clostridium group
Brevibacterium	26	16	*Brevibacterium*	Brevibacteriaceae, Actinomycetales
		4	*Curtobacterium*	Microbacteriaceae, Actinomycetales
		3	*Corynebacterium*	Corynebacteriaceae, Actinomycetales
		2	*Cellulomonas*	Cellulomonadaceae, Actinomycetales
		4	*Microbacterium*	Microbacteriaceae, Actinomycetales
		1	*Arthrobacter*	Micrococcaceae, Actinomycetales
		1	*Desemzia*	Camobacterium group, Enterococcaceae
		1	*Exiguobacterium*	Bacillaceae
Clostridium	143	14	*Clostridium*	Clostridium subline of Gram-positive bacteria, Clostridiaceae
		1	*Oxalophagus*	Bacillaceae
		1	*Paenibacillus*	Bacillaceae
		1	*Eubacterium*	Clostridiaceae
		1	*Syntrophospora*	Clostridiaceae
		1	*Oxobacter*	Clostridiaceae
		2	*Moorella*	Clostridiaceae
		2	*Thermoanaerobacter*	Clostridiaceae
		2	*Thermoanaerobacterium*	Bacillaceae
		1	*Caloramator*	Bacillaceae
		1	*Filifactor*	Clostridiaceae
		1	*Sporohalobacter*	Haloanaerobiales
Flavobacterium	40	21	*Flavobacterium*	Flavobacteriaceae
		1	*Empedobacter*	Flavobacteriaceae
		2	*Sphingomonas*	Zymononas-group, α-subclass of Proteobacteria
		2	*Microbacterium*	Microbacteriaceae, Actinomycetales
		1	*Halomonas*	Halomonadaceae, γ-subclass of Proteobacteria
		1	*Myroides*	Flavobacteriaceae
		1	*Planococcus*	Bacillaceae
		1	*Cytophaga*	Cytophagales
		1	*Vogesella*	β-subclass of Proteobacteria
		1	*Telluria*	β-subclass of Proteobacteria
		1	*Marinobacter*	γ-subclass of Proteobacteria

reclassification process. Consequently, the traditional taxonomic markers (motility, presence of gas vesicles, morphology, salinity and temperature optimum, vitamin requirement, base composition of DNA, and chemoautotrophy) were reassessed and new patterns of differential characteristics were proposed to describe the 14 genera of Chromatiaceae.

End products of carbohydrate fermentation, morphology and chemotaxonomy: The genus *Bacteroides* has been a dumping ground for many phylogenetically misclassified strains, the extent of which was only unraveled by 16S rDNA analysis. As Table 3 shows, 12 new genera have been established for former *Bacteroides* species, some of which are related to members of the Proteobacteria.

The availability of a most comprehensive phylogenetic framework, covering the majority of described species, leads to the recognition of genus boundaries and, consequently, to the establishment of phylogenetically homogeneous genera. Genera in which species are described following their phylogenetic analyses are also mostly homogeneous, e.g. genera *Campylobacter*, *Helicobacter* and *Arcobacter* (Fig. 3). Nevertheless, in some areas of the phylogenetic tree the reclassification process did not parallel the progress in the recognition of relatedness. The prime examples are the deeply branching lineages of the *Clostridium-Bacillus* subline of Gram-positive bacteria, e.g., *Clostridium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, and species of other genera that are phylogenetically heterogeneous. Another example is the family Pasteurellaceae, in which members of the genera *Pasteurella*, *Haemophilus* and *Actinobacillus* are not yet reclassified to match their phylogenetic relatedness (Fig. 4). The phylogenetic branching clearly indicates the inappropriateness of some classical taxonomic properties to define genera, but novel discriminating characteristics to circumscribe the new emerging genera have not yet been found. The features identified as being of little taxonomic value are the combination of: 1) Gram-positive staining, rod-shaped morphology, spore formation, anaerobic metabolism lacking sulfur reduction (*Clostridium*); and 2) Gram-positive staining behavior, morphology, lack of spore formation and anaerobic metabolism (*Peptococcus*, *Ruminococcus*, *Eubacterium*). The situation is complicated even more by the branching of the spherical, spore-forming *Sarcina* species within the radiation of the

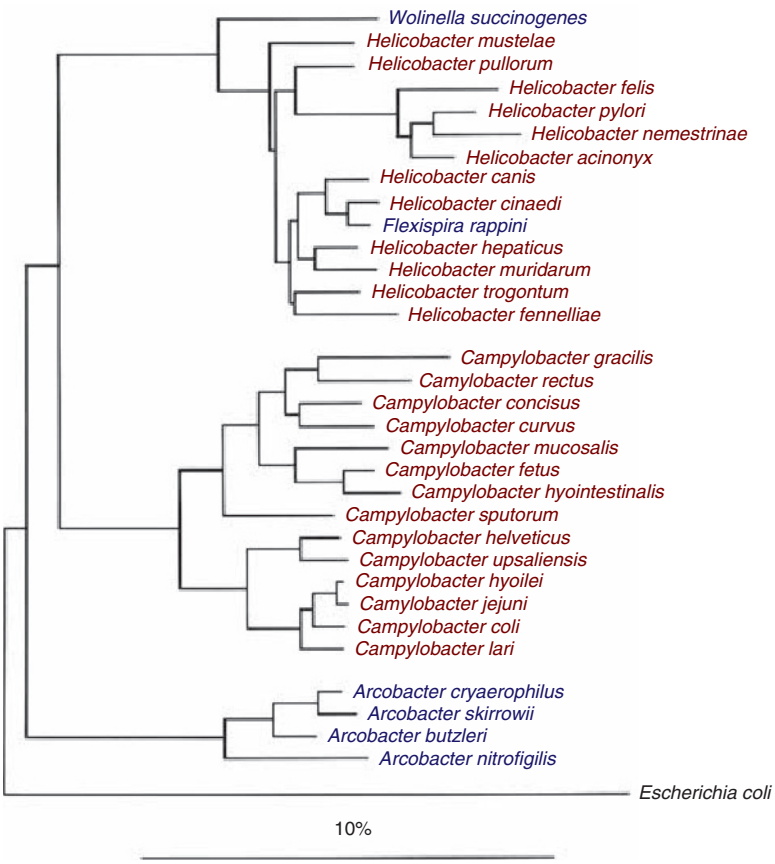

Fig. 3. 16S rDNA dendrogram of the *Campylobacter-Helicobacter-Arcobacter* line of descent, classified as the ε-subclass of Proteobacteria. The intrageneric structure of the genera is taxonomically coherent. The scale bar corresponds to 10 nucleotide substitutions per 100 sequence positions. The dendrogram was generated by the neighbor-joining algorithm (Felsenstein, 1993).

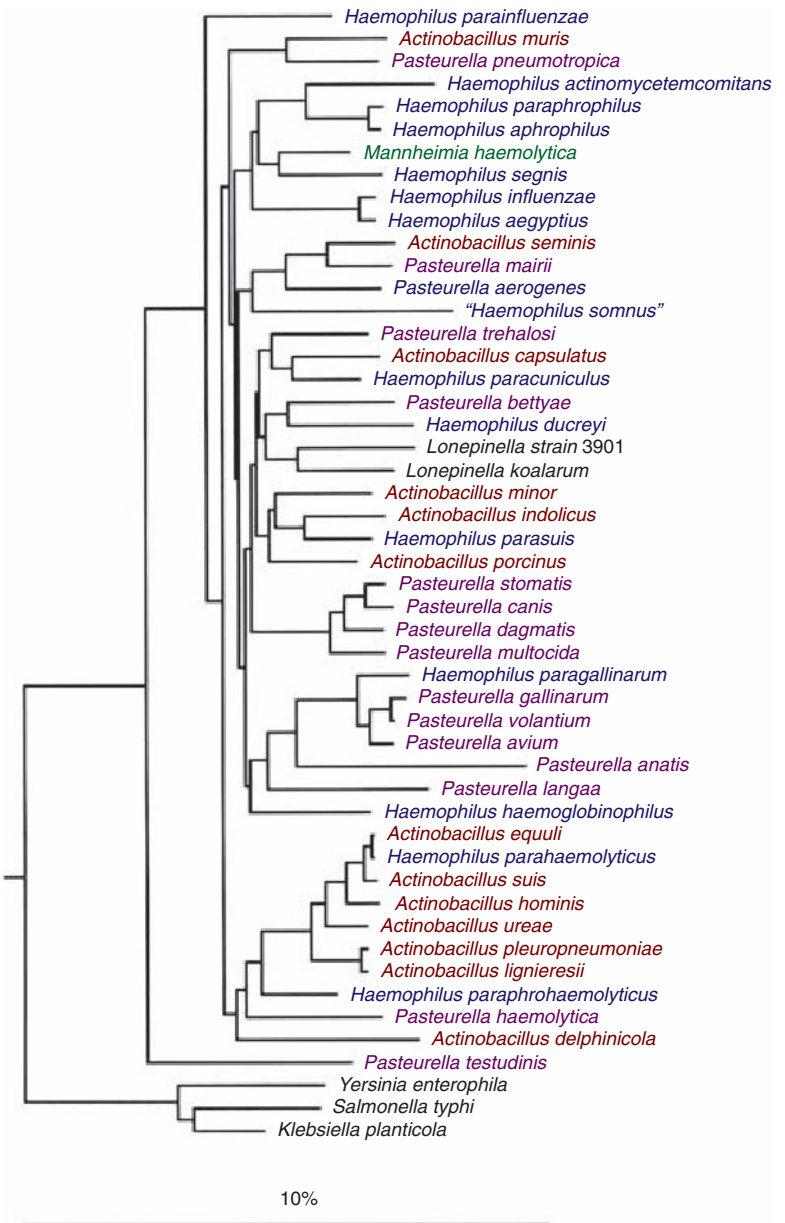

Fig. 4. 16S rDNA dendrogram of the Pasteurellaceae, a member of the γ-subclass of Proteobacteria. Due to the lack of discriminating phenotypic characteristics the intrageneric structure of the genera is taxonomically very incoherent. The scale bar corresponds to 10 nucleotide substitutions per 100 sequence positions. The dendrogram was generated by the neighbor-joining algorithm (Felsenstein, 1993).

Clostridium cluster I (according to Collins et al., 1994) that contains the type species and through the intermixing of Gram-negative species (Cluster IX) with Gram-positive *Clostridium*-type organisms (Schleifer et al., 1990; Stackebrandt and Rainey, 1997). As most of the genera defined by Gram-negative species are phylogenetically coherent taxa, such as *Sporomusa*, *Selenomonas* and *Pectinatus*, the misclassified non-type species of *Clostridium*, *Eubacterium* and some other genera must be reclassified, provided phenotypic properties are available that would support the reclassification. This information, however, is missing for most of the lineages prone to reclassification, except for a few examples in which new genera have been described, e.g., *Moorella*, *Filifactor*, *Thermoanaerobacter* and *Thermoanaerobium*.

Genera May Have Different Phylogenetic Depth

Individual genera that are phylogenetically coherent and have been properly described phenotypically may vary significantly in their phylogenetic depth, i.e., the relative time that passed after the two most unrelated members of a genus separated from a common ancestor. It has been noted (Stackebrandt, 1992) that in contrast to phylogenetically defined taxa, those based on

phenotypic description in the past have no depth per se, as nonmolecular properties are not able to measure relative evolutionary time. Genera can either have few or many species, but as long as secondary or tertiary semantides are analyzed, nothing can be said about when the common ancestor of the respective taxon evolved. The definition of a genus assumes that the phenotypic properties of its species are uniformly distributed among the species while the underlying structure of those genes used for phylogenetic analysis (and probably of other genes as well) may vary dramatically. Actually, for 16S rRNA genes, the degree of sequence variation between members of a genus may range from small (for instance, above 97% for *Micrococcus*), to moderate (92% for *Streptococcus*), to significant (about 79% for *Spirochaeta*) (97, 92, and 79% are all sequence similarity values.) Except for some recently evolved eukaryote-associated symbionts, it is still impossible to correlate sequence divergence of a given gene with a time scale, but it is possible to determine the relative age of a taxon from a comparison of 16S rDNA similarity values of the most unrelated species of a genus. For practical reasons, three categories named "age groups" have been described (Stackebrandt, 1988). These "groups" are hypothetical entities not clearly delineated in the hierarchical tree.

Members of the first "age group" were assumed to have evolved during the anaerobic phase of evolution. Species of such genera are in most cases well separated, which explains why DNA-DNA hybridization fails to relate them. Representative genera are found in the several families of methanogenic bacteria, as well as in *Bacteroides*, *Spirochaeta*, and *Clostridium* (senso strictu, i.e., cluster I) and their several phylogenetically related lineages, which need to be reclassified or have already been reclassified. The subjective selection of genus-specific phenotypic traits has not permitted determination of the actual phylogenetic depth of these taxa. It is of interest to note that certain phenotypic traits of taxonomic significance, including morphology, spore formation, proteins of the photosynthetic apparatus, and biochemical properties, appear to have remained constant over billions of years. The dramatic differences between the molecular and the phenotypic level appear to be rooted in mechanisms that cause a disjunction between the evolution of genotype and phenotype. This problem has been discussed by Nanney (1984), who suggested that the highly conserved morphological characteristics of eukaryotic species are the result of a compounding of molecular properties that may themselves be very divergent. As a consequence, divergent structure-forming components may interact in a way that conserves the resulting morphological structure, as well as biochemical pathways and physiological properties, in the case of prokaryotes.

Members of the second "age group" are those genera whose ancestors evolved during the transition period when the earth passed from an anaerobic to an aerobic environment. Descendants of this group are either facultatively anaerobic or aerobic. Most species are moderately related, but groups of closely related species exist. Their existence may be viewed as a more recent speciation event, causing strains of certain species to evolve faster than others. The reasons for this are not known, but changes in the evolutionary rate caused by changes in the environment may play a dominant role. Examples are found in *Bacillus*, *Streptococcus*, *Lactobacillus*, and *Corynebacterium*.

Members of genera belonging to the third "age group" probably evolved during the aerobic phase of evolution. Genera are very shallow phylogenetic taxa, since even the most distant species are still highly related. Most of these genera can be separated easily from closely related taxa by a combination of chemotaxonomic markers. The presence of different phenotypes in closely related genera is an indication of rapid evolution at the overall DNA level. This is seen not only in many actinomycete genera, e.g., *Streptomyces*, *Actinomadura*, *Staphylococcus*, *Listeria*, but also in many Gram-negative genera, e.g., *Vibrio*, *Shewanella*, *Rhizobium*, *Hyphomicrobium* and most genera of the Enterobacteriaceae. Genes coding for ribosomal RNA, on the other hand, are so conserved in their primary structure that changes at the level of the overall chromosome do not manifest within a short period. It is therefore obvious that DNA-DNA reassociation studies will reflect the actual relatedness between the species most closely, whereas comparison of complete rDNA sequences often fails to reliably determine the intrageneric structure of these taxa.

The presence of groups with varying phylogenetic depth explains why initial attempts have failed to delineate taxa by a purely phylogenetic—and inflexible—approach. The following example demonstrates this impracticability. For the methanogenic bacteria (Balch et al., 1979), a lower range of S_{AB} values of 0.55 to 0.65 was set for species differentiation (S_{AB} values are now replaced by rDNA similarity values, and S_{AB} values of 0.55 to 0.65 correlate with about 88 to 91 % similarities). If this same range were applied to the bacterial genus *Staphylococcus*, all of its members would have to be reduced to a single species. Application of the phylogenetic genus definition to the methanogens on the branching pattern of the order actinomycetes would have

even more dramatic consequences in that all genera would be united in a single genus (Fox and Stackebrandt, 1987). Using operational definitions as the sole criteria for taxon delineation, most of the existing phenotypically well defined and phylogenetically coherent genera would have to be considered invalid. Individual genera would have to be dissected to form several new genera with identical properties (that were once used to combine its members), or different genera, whose members exhibit such a wide variety of phenotypes that the classification would be of little practical value, would have to be united. The effects of the different delineation strategies on the classification of actinomycetes have been demonstrated schematically (Fox and Stackebrandt, 1987).

It must, however, be remembered that the delineation of a genus in bacteriology does not have to follow the historical record at all but depends upon the availability of phenotypic data coupled with the opinion of the taxonomists (Cowan, 1968). The availability of a phylogenetic framework has initiated a trend that led to the description of genera as phylogenetically shallower than genera based solely upon phenotypic properties. This is most dramatically seen in the dissection of some former species-rich genera, e.g., *Micrococcus*, *Brevibacterium*, *Pseudomonas*, and *Bacteroides* that has led to the description of many monospecific genera.

Classification Is a Dynamic Process (Stackebrandt, 1991)

Microbiologists are aware that the available phylogenetic branching patterns, although sensational and revolutionary because of their new potential, only very incompletely reflect the actual situation in nature. Phylogenetic reconstructions are based on inferred homologies but, unless witnessed by the evolutionary history of taxa, i.e., by fossil data, cannot be considered definitive (Rothschild et al., 1986). Furthermore, the tree mirrors the presence of certain categories, e.g., the spirochaetes, the planctomycetes, and the thermotogas, that may be self-defining since they are emerging constantly, no matter which molecule and method are used. However, the isolated position of these groups, well defined by genotype and phenotype today, may disappear tomorrow when more organisms are investigated. Thus, whenever new information—either within established taxa or in neighboring groups—requires corrections, flexibility is called for and changes have to be made for the benefit of a better agreement between phylogeny and taxonomy. The main advantages of the phylogenetic system lie in its stability: only the rank (either vertical or horizontal) but not its place within the hierarchical structure will be changed—as happened in past systems (a comparison of *Bergey's Manual of Determinative Bacteriology* from the first through eighth edition is instructive).

Even the most convincing tree is always in a dynamic state; this forces taxonomists to stay flexible to adjust not only established ranks but also nomenclature according to new insights. One problem still remains: the original advantage of the tree—its objectivity (in so far as is possible)—is weakened by subjective (variable emphasis on characters) clustering of organisms. As in previous decades, the most practicable system (or parts thereof) will succeed against competing systems with less persuasive arguments. The ultimate goal is to establish a hierarchical system where all taxa show phylogenetic coherency and, at least for ranks below the family level, a great deal of phenotypic coherency as well. On the other hand, sufficient differences need to be known to distinguish taxa from each other by stable and easily determined characters. While phylogenetic coherency is easy to define, the term "phenotypic coherency" varies according to the taxonomist. Again, practical considerations must come before petty splitting or lumping.

Profound knowledge about the phylogenetic clustering of members of the taxa in search is prerequisite. An optimal survey would work with coded, unnamed organisms to judge the resulting branching pattern without prejudice. The study should include the type strain of the type species and, if any information is already available, the type strain of the most unrelated representatives of the taxon. Depending on numbers of strains investigated, degree of relationships, and cost effectiveness, studies will include DNA-DNA pairing. The resulting pattern depicts the relative branching order, that, depending on the size of the database and the selection of reference organism, will immediately yield information about the phylogenetic homogeneity of a group of isolates. In the second step, the branching pattern is superimposed with phenotypic data to delineate clusters of organisms which are phylogenetically coherent and easy to recognize by phenotypic characters. This is prerequisite not only for identification, but also to decide which of the several possible branching patterns best reflects phylogeny most closely. It should be mentioned in this context that in the presence of varying evolutionary rates, species with the greatest nucleic acid sequence similarity are not necessarily the most closely related; while programs that optimize branch length take care of this problem, numerical phenetic analyses in fact

would cluster these species as neighbors. The combination of taxon-describing characters will not be predictable in most cases and the search will have to be extended to features not previously considered of taxonomic significance. Still many phylogenetically coherent taxa exist for which appropriate characters have not been found as yet.

The Higher Taxa

Analysis of sequences of rDNA and genes coding for proteins are routine some 10 years after the introduction of the PCR technology and the automated sequencing process. Large-scale genome sequencing projects are underway and the availability of an enormous number of sequences of homologous genes will one day allow a much more precise measurement of the branching order of lineages. However, with new lineages emerging from pure culture studies and analysis of complex microbial communities, the main topic of discussion is presently not the definition of higher taxa but very early evolutionary events, such as those that led to the formation of the eukaryotic cell, and the data discussed are sequence analyses of genes such as hsp70 (chaperone protein), glutamate dehydrogenase, glutamine synthase, aspartate aminotransferase and others (Gupta and Golding, 1993; Gogarten et al., 1989). Lateral gene transfer effects must not be neglected when phylogenetic trees based upon heat shock proteins (hsp70) are interpreted (Gribaldo et al., 1999; Philippe et al., 1999). The question whether or not the Gram-positive bacteria are more closely related to the Archaea than to the Gram-negative bacteria (Gupta and Golding, 1996) is a problem that can not be resolved on the basis of a few signature stretches of the gene and the amino acid sequence derived therefrom. The problem awaits a better understanding of the fate of the molecule in early evolution and greater ability to interpret the sequence data from a single gene in context of the overall biology of the organism.

According to Woese and colleagues (Woese et al., 1990), extant organisms are grouped within one of three major lines of descent, for which the domain has been proposed. The term "taxon domain" has replaced the term "primary kingdom" (Woese and Fox, 1977) originally given for the three main lines of descent which, based upon 16S rRNA analyses, are as unrelated to each other as each of them is related to the eukaryotic line. With the higher resolution provided by complete sequences of small-subunit rRNAs and other homologous marker molecules, this picture has been confirmed. The archaeal lineage is clearly separated from the other prokaryotic lineage, the Bacteria, forming a sister group of the Eucarya.

The main issue discussed at the end of the 1970s was whether the tripartition of extant species is accompanied by phenotypic properties that would support the phylogenetic finding. These properties were believed to exist because molecular analyses revealed the presence of evolutionary ancient groupings. While characters shared between members of two of the three domains are of no use for placing strains in the phylogenetically correct kingdom, certain characters are indeed exclusive for a particular primary domain and hence of diagnostic value. However, one can not exclude the possibility that nonhomologous phenotypes occur among members of different domains that mirror common evolutionary origin. On the other hand, homologous properties may actually be found exclusively among members of two domains, but this distribution is not due to common ancestry but to horizontal gene transfer.

Besides the structure and nucleotide composition of the 16S rRNA, several epigenetic properties were unique to Archaea and supported the validity of the description of this domain. Above all they were the lack of a typical peptidoglycan (replaced by a pseudomurein, a proteinaceous wall, or a heteropolysaccharide; Kandler, 1982), the presence of ether-linked isoprene units, and the complex modification pattern of ribosomal RNAs. In addition, certain archaeal taxa exhibit unique properties, which are absent in other taxa of this domain and in any bacterial or eukaryal taxon. The most well recognized ones are the coenzymes involved in methanogenesis; the energy-generating bacteriorhodopsin, halorhodopsin, and other sensory rhodopsins contained in the purple membranes of halophiles; survival under hyperthermophilic conditions; and singular physiological features such as the presence of a modified Entner-Doudoroff pathway. Other features will be discovered through the comparative analyses of fully sequenced archaeal genomes.

Since publication of the last edition of the Prokaryotes (Balows et al., 1991), new main lines of descent have been shown to exist in the domains Archaea and Bacteria, and most lineages have been significantly extended by studies on pure cultures and analysis of environmental samples. Ranks above the genus level have been described for several of these main lineages and for some of their sub-branches. What has been stated about the unreliability of standardized sequence similarity values of a single molecule to define a genus is also true for all higher ranks from family to kingdom. Consequently, a coherent hierarchical system of prokaryote taxa does not exist. While for one lineage a fully hierarchical

structure has been provided (Stackebrandt et al., 1997), there is no consensus about the level of the highest rank of a lineage. Lineages that are approximately equivalent in phylogenetic depth (i.e., the 16S rDNA similarity value that separates the most remotely related members of that lineage) are called kingdom (within the domain Archaea), phylum (some of the lineages originally defined by Woese et al., 1985, e.g., *Chloroflexus*, *Chlorobium*, *Deinococcus*, *Thermus*, *Bacteroides*, *Clostridium-Bacillus*), class (Actinobacteria, Proteobacteria), or order (Aquificales, Thermotogales, Spirochaetales, Verrucomicrobiales, Chlamydiales, Planctomycetales).

The phylogenetically defined higher taxa stand side by side with higher taxa established in the pre-phylogeny era. Some taxa were found to match the phylogenetic circumscription, e.g., the class Mollicutes, the orders Spirochaetales, Chlamydiales, and Myxobacteriales and the family Enterobacteriaceae, while others had to be emended or redefined on the basis of 16S rDNA sequence data to fit into a phylogeny-based hierarchical system, e.g., Micrococcaceae and Pseudomonadaceae. Yet other higher taxa such as the Bacillales and Clostridiales are still awaiting a formal revision, as phylogenetic evidence strongly points towards their dissection.

The Archaea

Concerning the phylogeny-based description of higher taxa, the domain Archaea has received considerable attention, primarily because of research activity in the laboratories of Ralph Wolfe and Carl Woese at the University of Illinois at Urbana-Champaign. Shortly after archaebacteria (as these organisms were named in 1979) were recognized, Balch et al. (1979) provided a comprehensive hierarchical system based on the assumption that the rate of 16S rRNA evolution in these organisms was similar. Consequently, species, genera, families and orders were delineated by ranges of S_{AB} values of 16S rRNA,which were 0.55–0.65, 0.46–0.51, 0.34–0.36 and 0.22–0.28, respectively. Later it became obvious that different bacterial species not only evolve a different mode and at a different rate (Woese et al., 1985c), but also their rRNA and rDNA may have different G+C content (Woese et al., 1991; Rainey et al., 1993; Liesack et al., 1992). The result was artificial misplacement of bacterial species in the phylogenetic dendrogram. The suggestion not to use a rather inflexible range of phylogenetic distances for the delineation of any taxon (Fox and Stackebrandt, 1987; Stackebrandt, 1992) has been accepted and applied to more recent descriptions of higher taxa in the domain (Burggraf et al., 1997).

When higher taxa were proposed for the second archaeal kingdom, the Crenarchaeota, only a few species had been described. It is therefore not surprising that the phenetic descriptions of the orders Thermoproteales (Zillig et al., 1981), Sulfolobales (Stetter, 1989), as well as those of the families Thermoproteaceae (Zillig et al., 1981) and Desulfurococcaceae (Zillig et al., 1982), Pyrodictiaceae and Thermofilaceae (Burggraf et al., 1997), are virtually the same as the genera they encompass. The inclusion of more organisms in a higher taxon, e.g., Thermoproteales and Thermoproteaceae (Burggraf et al., 1997) broadens the phenetic description.

The Bacteria

A similar situation is encountered among the deeply branching lineages of the domain Bacteria. Encouraged by the ease at which higher taxa were described for archaeal lineages, orders and families were almost simultaneously described with the recognition of a new genus or a cluster of neighboring genera. Examples are the order Aquificales, embracing the family Aquifexaceae, and the genera *Aquifex*, *Calderobacterium*, *Hydrogenobacter*, and *Thermocrinis*, as well as the order Thermotogales, embracing the family Thermotogaceae and several genera, e.g., *Thermotoga*, *Geotoga* and *Petrotoga*.

These examples demonstrate that the description of a higher taxon for a phylogenetic lineage is facilitated by the small number of organisms and by the isolated position of the lineages. The situation is different in the four main bacterial lineages, which show a complex phylogenetic structure: the Gram-positive bacteria (division Firmicutes), the class Proteobacteria, the Bacteroidaceae-Cytophagales line of descent, and the cyanobacteria phylum. Within the cyanobacterial lineage, higher taxa have been described on the basis of morphology and, within the Prochlorophytales, of pigment composition. Analysis of 16S rDNA clearly demonstrates that the present affiliation of genera into the orders Chroococcales, Nostocales, Oscillatoriales, Pleurocapsales and Stigonematales is not always supported from a phylogenetic point of view.

One of the most unexpected relationships that emerged from the analyses of rRNA, rDNA, and certain genes coding for proteins was the specific grouping of the *Bacteroides* and *Cytophaga-Flavobacterium* lines of descent. No higher taxon has been proposed as yet for the lineage comprising the phylogenetic assemblage of genera but, with the exception of the phylogenetically coherent family Sphingobacteriaceae (Steyn et al.,

1998), organisms are related to the traditional higher taxa Cytophagales and Bacteroidaceae.

The Higher Taxa of the Gram-Positive Bacteria

The Gram-positive bacteria constitute the division Firmicutes (Gibbons and Murray, 1978), which, with the exception of members of *Deinococcus*, appears to form a phylogenetically coherent taxon. The common ancestry of the two major sublines, however, one embracing the actinomycetes, the other containing the clostridia, bacilli and their relatives, has not been convincingly demonstrated by rDNA analyses. If, for the time being, it is assumed that these organisms indeed share a common ancestry, then the deep separation between organisms with a DNA base composition of less than about 50 mol% G+C (the *Clostridium-Bacillus* lineage) and those with a higher G+C content (the Actinobacteria) may facilitate resolution of their hierarchical structure.

A hierarchical classification system has been described for the actinomycetes and their relatives (Stackebrandt et al., 1997). The rationale for doing so was based on the fact that nearly all type strains of validly described species were characterized phylogenetically and that the genera constituted phylogenetically and phenetically coherent taxa. The decision to classify phylogenetically neighboring genera into families, neighboring families into suborders, and to continue up to the level of the class Actinobacteria was done irrespective of phenetic properties on which higher classification of these genera was based in the past. Rather than focusing on characteristics such as morphological, physiological and chemotaxonomic traits, which, except for the presence of mycolic acids, have no or only restricted phylogenetic meaning above the genus level, ranks were defined on the basis of emerging phylogenetic clusters and the presence of taxon-specific rDNA signature nucleotides. As a consequence the actinomycete proper has been classified into 5 subclasses, 6 orders, 10 suborders, and 35 families. Each taxon is characterized by a set of 16S rDNA signature nucleotides which were present in all or at least in the vast majority of members of a taxon at the time of its description. This way of circumscribing taxa is similar to the traditional, phenotype-based classification in that new members may have properties that differ to some extent from those of other members of the taxon. Consequently, the deviation may lead to an emendation of the taxon or, where the deviation is significant, to a dissection of the taxon. Any deviation from the signature pattern of a new member of a taxon may cause the taxonomist to revise the description of this taxon. In contrast to the past, lumping or splitting of taxa will not dramatically change the classification system, although the transfer of a taxon into the neighboring taxon of the same level may occur in those cases where the branch points of taxa in the phylogenetic tree are not well resolved. Within the second major lineage of the Firmicutes, the situation is more complicated. This phylogenetic cluster contains several higher taxa described by phenetic characteristics, e.g., the orders Bacillales and Clostridiales as well as the families Bacillaceae, Clostridiaceae, and Peptococcaceae, but the phylogenetic structure of such taxa does not correlate at all with the phylogenetic suprageneric classification. The situation is even more complicated by the present inability to define a genus *Clostridium*, needed to start a comprehensive phylogenetic classification process. The only higher taxon phylogenetically described so far within the *Clostridium* lineage is the order Haloanaerobiales, containing the families Haloanaerobiaceae (Oren et al., 1984) and Halobacteroidaceae containing Gram-negative, fermentative, halophilic and anaerobic bacteria (Rainey et al., 1995). The phenetic order Bacillales, developed from an ancestor of the Clostridiales, embraces the phylogenetically and phenetically coherent class Mycoplasmatales and the families Streptococcaceae and Lactobacillaceae, next to other higher taxa to be described. It is obvious that any attempt to create a fully comprehensive phylogeny-based hierarchical classification system for the *Clostridium-Bacillus* lineage has to await a thorough reclassification of the majority of genera contained in the lineage. This process has started with the dissection of the genus *Bacillus* and the establishment of new genera but will continue at a slower pace than in other lineages because of the significant degree of discord between phenetic classification and the phylogenetic position of species.

The Higher Taxa of the Proteobacteria

Members of the Proteobacteria are distributed into five subclasses. From the beginning of the molecular era of taxonomy, many of the Gram-negative taxa were investigated in parallel by 16S rRNA cataloging and determination of 16S rDNA similarities (Fox et al., 1977; Palleroni et al., 1973), and the agreement of the branching patterns was convincing. Almost complete 16S rDNA sequences have been generated for these and other organisms now seen to be members of the Proteobacteria. The phylogenetic clustering of organisms of quite different phenotypes initially surprised traditional microbiologists and gave the first indication that conventional classification does not reflect natural relationships.

Phototrophic bacteria were found to be neighbors of nitrite-oxidizing and carbon-monoxide-oxidizing forms, and organisms associated with eukaryotic cells were more closely related than originally believed (e.g., *Agrobacterium*, *Rhizobium*, *Brucella*, *Rochalimea*). But most unexpectedly, almost all of the more general characters used so far in classification lost their significance as taxon-describing features, e.g., photosynthesis (Stackebrandt et al., 1988a), carbon monoxide oxidation (Auling et al., 1988), methane- and methanol oxidation (Bowman et al., 1993), as well as all kinds of cell shapes, such as helical (Woese et al., 1982), budding (Stackebrandt et al., 1988b), or prosthecate (Schlesner et al., 1989). Stimulated by the phylogenetic data, supporting evidence has subsequently become available from chemotaxonomic studies. Among these criteria are polyamine patterns (Busse and Auling, 1988), ubiquinone types (Urakami et al., 1989), fatty acid compositions (Urakami and Komagata, 1987; Sittig and Schlesner, 1993), chemical compositions of lipid A, and the core region of lipopolysaccharides (Weckesser and Mayer, 1988).

The process of transforming phylogenetic evidence into the description of higher taxa is slow. This is not only true for a formal description of the subclasses but especially for the groupings within the subclasses. The reasons are obvious: 1) emphasis is placed on the generation of phylogenetically coherent genera; 2) neighboring genera may differ from each other significantly in phenotypic properties, which excludes the provision of phenetically coherent higher taxa; 3) the phylogenetic distances separating groups of genera from each other are small and the order is most likely distorted by new sequence entries; and 4) many new proteobacterial genera are presently described that may result in emendations and changes in the order of higher taxa.

The richest family structure is present in the γ-subclass in which a large number of genera, which are not necessarily phylogenetically coherent (e.g., *Pasteurella*, *Haemophilus*, *Serratia*, *Enterobacter*), are members of phylogenetically coherent families such as *Pasteurellaceae* and *Enterobacteriaceae*. Other well-defined families are Vibrionaceae, Aeromonadaceae, Legionellaceae and other families shown in Fig. 5. The only family that seems to be phylogenetically incoherent is Methylococcaceae. While the majority of genera of this family are positioned as shown in Fig. 4, the genus *Methylococcus* branches adjacent to the family Chromatiaceae. The other subclasses are less formally structured. Among the α-subclass, the Acetobacterceae, Rhizobiaceae, and the Rickettsiales constitute phylogenetically rather homogeneous taxa, while in the β-subclass, which appears as a

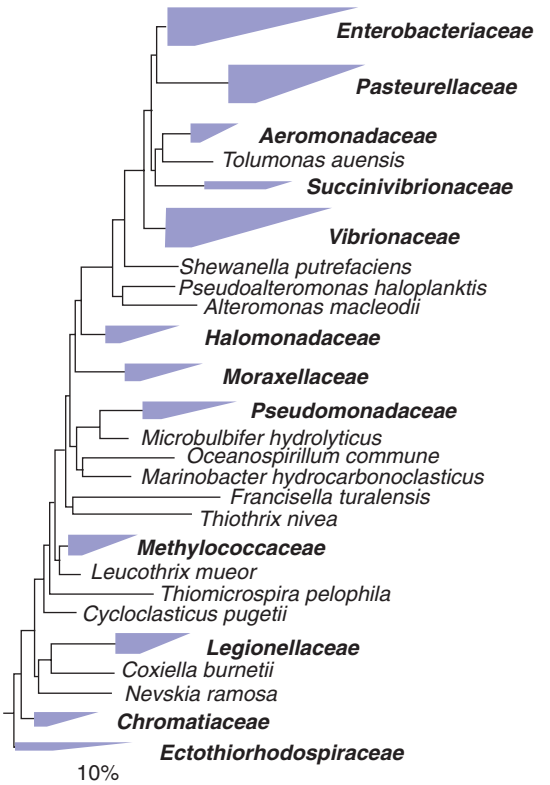

Fig. 5. 16S rDNA dendrogram of the γ-subclass of Proteobacteria, showing the position of phylogenetically coherent families. The 16S rDNA dendrogram is a detail of the (ARB tree). The scale bar corresponds to 10 nucleotide substitutions per 100 sequence positions.

subgroup of the γ-subclass, this refers to *Comamonadaceae* and *Neisseriaceae*. Within the δ-subclass the order *Myxobacterales* provides one of the rare examples in bacteriology in which the taxonomic structure as derived from phenotypic characterization, i.e., complex and highly ordered morphologic processes, is indeed valid indication of phylogenetic structure (Spröer et al., 1999).

Application of the Polyphasic Approach to Classification

The following is an example of the polyphasic approach to systematics (Fig. 6). First, it is assumed that the organisms abbreviated A through H have no taxonomic history but present novel isolates. In reality this is not the case because they represent well-known actinomycete genera and species (Stackebrandt et al., 1997). Analysis of 16S rDNA data leads to the placement of their sequences within the radiation of members of the class Actinobacteria, forming a sister-branch of the family Pseudonocardiaceae. As the phylogenetic depth of the new

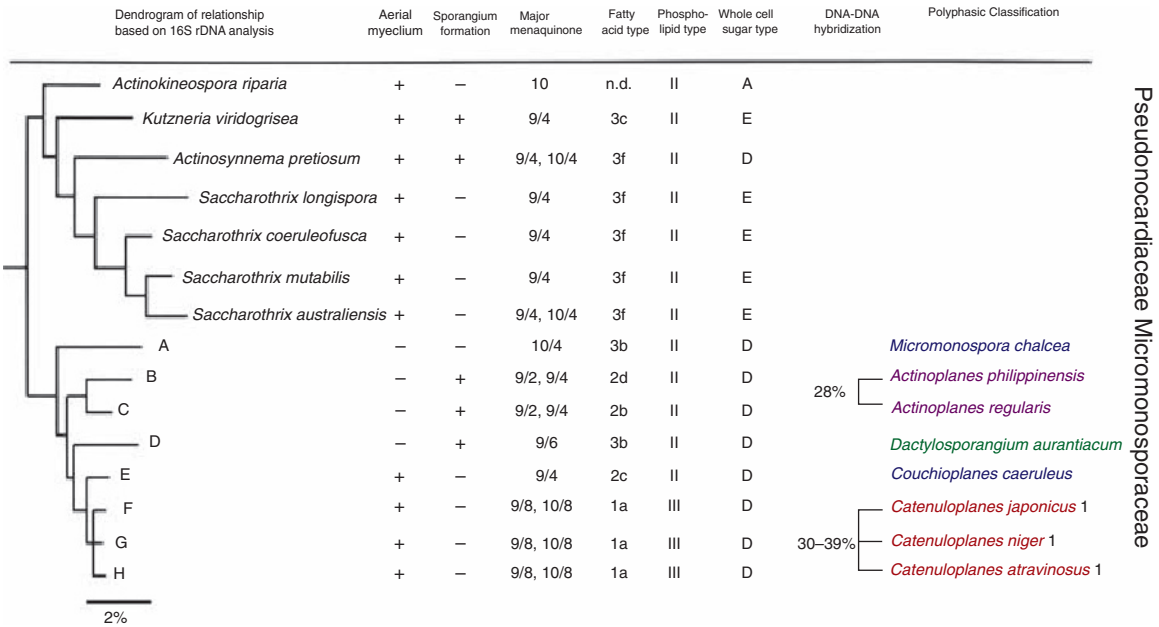

Fig. 6. Example of a polyphasic approach to bacterial taxonomy. The present classification is based on phylogenetic coherence of genera delineated from each other on the basis of morphological and chemotaxonomic properties. The selection of suitable properties depends upon the taxon under investigation. For clarity, the information on fatty acids, phospholipids, and whole cell sugars is abbreviated (*Bergey's Manual of Systematic Bacteriology*, Wilkins & Wiley, Baltimore, 1986). [1], data from Tamura et al., 1993, 1995.

lineages is comparable to that of the genus *Saccharothrix*, lineages A through H could constitute a novel genus. However, as the rate of evolution is different in members of different genera, the newly emerged phylogenetic cluster might well embrace two or more genera. Hence, the provision of a phylogenetic dendrogram alone does not a priori permit conclusions about the rank of taxa. According to the polyphasic approach, one would try to allocate as many genetically stable characters as possible to the isolates A to H to find characters that are unique to one phylogenetic cluster but different from neighboring clusters. As some characters may be shared by different genera, it is the presence of a unique pattern of characters that decides if a rank is delineated from its neighbors. Figure 6 lists some of the morphological and chemotaxonomic characters used in the polyphasic classification of actinomycetes. As derived from the example of the Pseudonocardiaceae, each genus is characterized by a unique set of morphological and chemical properties. Species of the genus *Saccharothrix* share the genus-specific pattern (but they can be distinguished from each other by physiological reactions). Patterns obtained for the (hypothetically) new organisms are novel for the actinomycetes genera and indicate five subgroups worthy of genus rank. To determine whether the isolates with identical phenetic patterns constitute individual species, DNA-DNA reassociation experiments must be performed. Isolates B and C (Stackebrandt et al., 1983), as well as isolates F, G and H (Yokota et al., 1993) represent individual species, as in each case the reassociation values are below 70%. Consequently the isolates can be classified into five new genera containing 8 new species which can be phenotypically separated (not shown).

The affiliation of the new monophyletic genera to a family on the basis of phenotypic data is not appropriate because of the great morphological and chemical diversity that would not exclude identification of other actinomycete genera as members of this family. As discussed (Stackebrandt et al., 1997), the presence of a pattern of 16S rDNA signature nucleotides common to all members of *Micromonospora* and related genera and different from those defined for other actinomycete families circumscribes the family Micromonosporaceae at the genomic level. Another set of signature nucleotides common to all monophyletic families led to the description of the order Actinomycetales, yet another set to the definition of the six actinobacterial orders of the class Actinobacteria.

Literature Cited

Achtman, M. 1998. Microevolution during epidemic spread of Neisseria meningitidis. Electrophoresis 19:593–596.

Amann, R. I., Lin, C., Key, R., Montgomery, L., and Stahl, D. A. 1992. Diversity among Fibrobacter strains: Towards a phylogenetic classification. System. Appl. Microbiol. 15:23–31.

Ash, C., Farrow, J. A. E., Wallbanks, S., and Collins, M. D. 1991. Phylogenetic heterogeneity of the genus Bacillus revealed by comparative analysis of small-subunit ribosomal RNA sequences. Lett. Appl. Microbiol. 13:202–206.

Auling, G., Busse, J., Hahn, M., Hennecke, H., Kroppenstedt, R., Probst, A., and Stackebrandt, E. 1988. Phylogenetic heterogeneity and chemotaxonomic properties of certain Gram-negative aerobic carboxydobacteria. System. Appl. Microbiol. 10:264–272.

Bachmann, K. 1998. Species as units of diversity: an outdated concept. Theor. Biosci. 117:213–230.

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., and Wolfe, R. S. 1979. Methanogens reevaluation of a unique biological group. Micribiol. Rev. 43:260–296.

Barry, T., Colleran, G., Glennon, M., Dunican, L. K., and Gannon, F. 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods Appl. 1:51–56.

Baumann, P., Baumann, L., Woolkalis, M. J., and Bang, S. S. 1983. Evolutionary relationships in Vibrio and Photobacterium a basis for a natural classification. Ann. Rev. Microbiol. 37:369–398.

Bautz, E. K. F., and Bautz, F. A. 1964. The influence of noncomplementary bases on the stability of ordered polynucleotides. Proc. Natl. Acad. Sci. USA 52:1476–1481.

Bowman, J. P., Sly, L. I., Nichols, P. D., and Hayward, A. C. 1993. Revised taxonomy of the methanotrophs: description of Methylobacter gen. nov., emendation of Methylococcus, validation of Methylosinus, and Methylocystis species, and a proposal that the family Methylococcaceae includes only the group I methanothrophs. Int. J. Syst. Bacteriol. 43:735–753.

Brenner, D. J. 1973. DNA reassociation in the taxonomy of enteric bacteria. Int. J. Syst. Bacteriol. 23:298–307.

Britten, R. J., and Kohne, D. E. 1968. Repeated sequences in DNA. Science 161:529–540.

Buchanan, R. E. 1916. Studies in the nomenclature and classification of the bacteria. I. The problem of bacterial nomenclature. J. Bact. 1:591–596.

Buchanan, R. E. 1918. Studies in the nomenclature and classification of the bacteria. V. Subgroups and genera of the Bacteriaceae. J. Bact. 3:27–61.

Burggraf, S., Huber, H., and Stetter, K. O. 1997. Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. Int. J. Syst. Bacteriol. 47:657–660.

Busse, J., and Auling, G. 1988. Polyamin pattern as a chemotaxonomic marker within the *Proteobacteria*. System. Appl. Microbiol. 11:1–8.

Cho, M., Yoon, J.-H., Kim, S.-B., and Park, Y.-H. 1998. Application of the ribonuclease P (RNase P) RNA gene sequence for phylogenetic analysis of the genus Saccharomonospora. Int. J. Syst. Bacteriol. 48:1223–1230.

Cohn, F. 1872. Untersuchungen über Bacterien. Beitr. Biol. Pfl. 2:127–224.

Cohn, F. 1875. Untersuchungen über Bacterien II. Beitr. Biol. Pfl. 3:141–207.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H., and Farrow, J. A. E. 1994. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44:812–826.

Colwell, R. R. 1970. Polyphasic taxonomy of the genus Vibrio: numerica taxonomy of Vibrio cholerae, Vibrio parahaemolyticus, and related Vibrio species. J. Bacteriol. 104:410–433.

Cowan, S. T. 1968. A dictionary of microbial taxonomic usage. Oliver & Boyd. Edinburgh.

Cracraft, J. 1983. Species concept and speciation analysis. Johnson, R. F. Current Ornithology. Plenum Press. New York, 159–187.

Darwin, C. 1859. On the origin of species. Murray. London.

de Lajudie, P., Laurent-Fulele, E., Willems, A., Torck, U., Coopman, R., Collins, M. D., Kersters, K., Dreyfuß, B., and Gillis, M. 1998. Allorhizobium undicola gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate Neptunia natans in Senegal. Int. J. Syst. Bacteriol. 48:1277–1290.

De Ley, J. 1970. Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. J. Bacteriol. 101:738–754.

De Smedt, J., and De Ley, J. 1977. Intra- and intergeneric similarities of Agrobacterium ribosomal ribonucleic acid cistrons. Int. J. Syst. Bacteriol. 27:222–240.

De Vos, P., and De Ley, J. 1983. Intra- and intergeneric similarities of Pseudomonas and Xanthomonas ribosomal ribonucleic acid cistrons. Int. J. Syst. bacteriol. 33:487–509.

Dobzhansky, T. 1937. Genetics and the origin of species. Columbia University Press. New York.

Dykhuizen, D. E., and Green, L. 1991. Recombination in Escherichia coli and the definition of biological species. J. Bacteriol. 173:7257–7268.

Embley, T. M., and Stackebrandt, E. 1994. The molecular phylogeny and systematics of the actinomycetes. Ann. Rev. Microbiol. 48:257–289.

Ezaki, T., Hashimoto, Y., and Yabuuchi, E. 1989. Fluorimetric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39:224–229.

Farrelly, V., Rainey, F. A., and Stackebrandt, E. 1995. Effect of genomicsize and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Envir. Microbiol. 61:2798–2801.

Felsenstein, J. 1982. Numerical methods for inferring evolutionary trees. Quart. Rev. Biol. 57:379–404.

Felsenstein, J. 1988. Phylogenies from molecular sequence interference and reliability. Ann. Rev. Genet. 22:521–565.

Felsenstein, J. 1993. PHYLIP (phylogenetic inference package) version 3.5.1. Department of Genetics, University of Washington, Seattle, http://evolution.genetics.washington.edu.

Fournier, P.-E., Roux, V., and Raoult, D. 1988. Phylogenetic analysis of spotted fever group rickettsiae by study of the outer surface protein rOmpA. Int. J. Syst. Bacteriol. 48:839–849.

Fowler, V. J., Widdel, F., Pfennig, N., and Stackebrandt, E. 1986. Phylogenetic relationships of sulfate- and sulfur-reducing eubacteria. System. Appl. Microbiol. 8:32–41.

Fox, G. E., Pechman, K. J., and Woese, C. R. 1977. Comparative cataloging of 16S ribosomal ribonucleic acid:

molecular approach to prokaryotic systematics. Int. J. Syst. Bacteriol. 27:44–57.

Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., Dyer, T. A., Wolfe, R. S., Balch, W. E., Tanner, R., Magrum, L., Zablen, L.-B., Blakemore, R., Gupta, R., Lewis, B. J., Stahl, D. A., Luehrsen, R., Chen, K. N., and Woese, C. R. 1980. The phylogeny of prokaryotes. Science 209:457–463.

Fox, G. E., and Stackebrandt, E. 1987. The application of 16S rRNA cataloguing and 5S rRNA sequencing in bacterial systematics. Colwell, R. and Grigorova, R. Methods in Microbiology, vol. 19. Academic Press. London, 405–458.

Fox, G. E., Wisotzkey, J. D., and Jurtshuk, Jr., P. 1992. How close is close: 16S RNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42:166–170.

Gibbons, N. E., and Murray, R. G. E. 1978. Proposals concerning the higher taxa of bacteria. Int. J. Syst. Bacteriol. 28:1–6.

Gibson, J., Ludwig, W., Stackebrandt, E., and Woese, C. R. 1985. The phylogeny of the green photosynthetic bacteria: absence of a close relationship between Chlorobium and Chloroflexus. Syst. Appl. Microbiol. 6:152–156.

Gogarten, J. P., Kibak, H., Dittrich, P., Taiz, L., Bowman, E. J., Bowman, B. J., Manolson, M. F., Poole, R. J., Date, T., Oshima, T., Konishi, J., Denda, K., and Yoshida, M. 1989. Evolution of the vacuolar H+-ATPase: implications for the origin of eukaryotes. Proc. Natl. Acad. Sci. USA 86:6661–6665.

Goris, J., Suzuki, K., De Vos, P., Nakase, T., and Kersters, K. 1999. Evaluation of a microplate DNA-DNA hybridization method compared to the initial renaturation method. Can. J. Microbiol. 44:1148–1153.

Gribaldo, S., Lumia, V., Creti, R., Conway de Macario, E., Sanangelantoni, A., and Cammarano, P. 1999. Discontinuous occurrence of the hsp70 (dnaK) gene among Archaea and sequence features of HSP70 suggest a novel outlook on phylogenies inferred from this protein. J. Bacteriol. 181:434–443.

Grimont, P. A. D., Popoff, M. Y., Grimont, F., Coynault, C., and Lemelin, M. 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. Curr. Microbiol. 4:325–330.

Grimont, F., and Grimont, P. A. D. 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann. Inst. Pasteur/Microbiol. (Paris) 137B:165–175.

Gupta, R. S., and Golding, G. B. 1993. Evolution of HSP70 gene and its implications regarding relationships between Archaebacteria, Eubacteria and Eukaryotes. J. Mol. Evol. 37:573–582.

Gupta, R. S., and Golding, G. B. 1996. The origin of the eukaryotic cell. Trends Biochem. Sci. 21:166–171.

Gürtler, V., and Stanisich V. A. 1996. New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. Microbiology (Reading) 142:3–16.

Guttman, D. S., and Dykhuizen, D. E. 1994. Clonal divergence in Escherichia coli as a result of recombination, not mutation. Science 266:1380–1383.

Hara, T., Shimoda, T., Nonaka, K., and Ogata, S. 1991. Colorimetric detection of DNA-DNA hybridization in microdilution wells for taxonomic application on bacteria strains. J. Ferm. Bioeng. 72:122–124.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. 1994. Bergy's manual of determinative bacteriology, 9th edition. Williams & Wilkins. Baltimore,

Hull, D. 1976. Are species really individuals? Syst. Zool. 25:174–191.

Huss, V. A. R., Festl, H., and Schleifer, K. H. 1983. Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst. Appl. Microbiol. 4:184–192.

Imhoff, J. F., Petri, R., and Süling, J. 1998. Reclassification fo species of the spiral-shaped phototrophic purple non-sulfur bacteria of the γProteobacteria: description of the genera Phaeospirillum gen. nov., Rhodovibrio gen. nov., Rhodothalassium gen. nov. and Roseospira gen nov., as well as transfer of Rhodospirillum fulvum to Phaeospirillum fulvum comb. nov., of Rhodospirillum molischianum to Phaeospirillum molischianum comb. nov., of Rhodospirillum salinarum to Rhodovibrion salinarum comb. nov., of Rhodospirillum sodomense to Rhodovibrio sodomensis comb. nov., of Rhodospirillum salexigens to Rhodothalassium salexigens comb. nov. and of Rhodospirillum medosalinum to Roseospira mediosalina. Int. J. Syst. Bacteriol 48:793–798.

Imhoff, J. F., Süling, J., and Petri, R. 1998a. Phylogenetic relationships among the Chromatiaceae, their taxomonic reclassification and description of the new genera Allochromatium, Halochromatium, Isochromatium, Marichromatium, Thiococcus, Thiohalocapsa and Thermochromatium. Int. J. Syst. Bacteriol. 48:1129–1143.

Istock, C. A., Bell, J. A., Ferguson, N., and Istock, N. L. 1996. Bacterial species and evolution: theoretical and practical perspectives. J. Indus. Microbiol. 17:137–150.

Jeanthon, C., L'Haridon, S., Reysenbach, A.-L., Vernet, M., Messner, P., Sleytr, U. B., and Prieur, D. 1998. Methanococcus infernus sp. nov., a novel hyperthermophilic lithotrophic methanogen isolated froma deep-sea hydrothermal vent. Int. J. Syst. Bacteriol. 48:913–919.

Johnson, J. L. 1973. The use of nucleic acid homologies in the taxonomy of anaerobic bacteria. Int. J. Syst. Bacteriol. 23:308–315.

Johnson, J. L., and Francis, B. S. 1975. Taxonomy of the clostridia: ribosomal ribonucleic acid homologies among the species. J. Gen. Microbiol. 88:229–244.

Kandler, O. 1982. Cell wall structures and their phylogenetic implications. Zbl. Bakt. Hyg., I. Abt. Orig, C 3:149–160.

Kandler, O., and König, H. 1985. Cell envelopes of archaebacteria. Woese, C. R. and Wolfe, R. S. The Bacteria, vol. 8, Archaebacteria. Academic Press. New York, 413–458.

Kaznowski, A. 1995. A method of colorimetric DNA-DNA hybridization in microplates with covalently immobilized DNA for identification of Aeromonas ssp. Med. Microbiol. Lett. 4:362–369.

Kloos, W. E., Mohapatra, N., Dobrogosz, J., Ezell, J. W., and Manclark, C. R. 1981. Deoxyribonucleotide sequence relatioships among Bordetella species. Int. J. Syst. Bacteriol. 31:173–176.

Kluyver, A. J., and van Niel, C. B. 1936. Prospects for a natural system of classification of bacteria. Zbl. Bakt. Abt. 2 94:369–403.

Knox, C. L., Giffard, P., and Timms, P. 1998. The phylogeny of Ureaplasma urealyticum based on the mba gene fagment. Int. J. Syst. Bacteriol. 48:1323–1331.

Kovács, G., Burghardt, J., Pradella, S., Schumann, P., Stackebrandt, E., and Màrialigeti, K. 1999. Kocuria palustris sp. nov. and Kocuria rhizophila sp. nov, isolated from the

rhizoplane of the narrow-leaved cattail (Typha angustifolia). Int. J. Syst. Bacteriol. 49:167–173.

Leblond-Bourget, N., Philippe, H., and Decaris, B. 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific Bifidobacterium phylogeny. Int. J. Syst. Bacteriol. 46:102–111.

Liesack, W., Söller, R., Stewart, T., Haas, H., Giovannoni, S., and Stackebrandt, E. 1992. The influence of tachytelically evolving gene sequences on the topology of phylogenetic trees—intrafamily relationships and the phylogenetic position of Planctomycetaceae as revealed by comparative analysis of 16S ribosomal RNA sequences. Syst. Appl. Microbiol. 15:357–362.

Linnaeus, C. 1753. Species plantarum. Holmiae.

Ludwig, W. 1999. The phylogeny of prokaryotes: a living tree. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. and Stackebrandt, E. The prokaryotes. Springer. New York.

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russel, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M., and Spratt, B. G. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic organisms. Proc. Natl. Acad. Sci. USA 95:3140–3145.

Margulis, L. 1981. Symbiosis in cell evolution. W.H. Freeman and Company. San Francisco.

Maynart-Smith, J., Smith, N. H., O'Rourke, M., and Spratt, B. G. 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA 90:4384–4388.

Meyer, M. E. 1990. Current concepts in the taxonomy of the genus Brucella. Nielsen, K and Duncan, B. Animal brucellosis. CRC Press. Boca Raton, 1–17.

Migula, W. 1900. System der Bakterien. Gustav Fischer. Jena.

Miroshnichenko, M. L., Gongadze, G. A., Lysenko, A. M., and Bonch-Osmolovskaya, E. A. 1994. Desulfurella multipotens sp.nov., a new sulfur-respiring thermophilic eubacterium from Raoul Island (Kermadec archipelago, New Zealand). Arch. Microbiol. 161:88–93.

Moreno, E. 1997. In search of a bacterial species definition. Rev. Biol. Trop 45:753–771.

Mutters, R., Ihm, P., Pohl, S., Fredericksen, W., and Mannheim, W. 1985. Reclassification of the genus Pasteurella Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposal for the new species Pasteurella dagmatis, Pasteurella canis, Pasteurella stomatis, Pasteurella anatis, and Pasteurella langaa. Int. J. Syst. Bacteriol. 35:309–322.

Nanney, D. 1984. The molecular diversity and evolutionary antiquity of the Tetrahymena pyriformis species complex. Dryl, S., Kazubski, L., Kuznicki, L. and Ploszaj, J. Progress in Protozoology. Acta Protozoologica. Warsaw.

O'Hara, R. J. 1994. Evolutionary history and the species problem. Am. Zoologist 34:12–22.

Olsen, G. J., Woese, C. R., and Overbeek. 1994. The winds of (evolutionary) changes: breathing new life into microbiology. J. bacteriol. 176:1–6.

Oren, A., Paster, B., and Woese, C. R. 1984. Haloanaerobiaceae: a new family of moderately halophilic, obligatory anaerobic bacteria. System. Appl. Microbiol. 5:71–80.

Orla-Jensen, S. 1909. Die Hauptlinien des natürlichen Bakteriensystems. Zbl. Bakt. Abt. 2 22:305–346.

Palleroni, N. J., Kunisawa, R., and Doudoroff, M. 1973. Nucleic acid homologies in the genus Pseudomonas. Int. J. Syst. Bacteriol. 23:333–339.

Philippe, H., Budin, K., and Moreira, D. 1991. Horizontal transfers confuse the prokaryotic phylogeny based on the HSP76 protein family. Mol. Microbiol. 31:1007–1012.

Poyart, C., Quesne, G., Coulon, S., Berche, P., and Trieu Cuot, P. 1998. Identification of streptococci to species level by sequencing the gene encoding the manganese dependent superoxide dismutase. J. Clin. Microbiol. 36:41–47.

Prévot, A. R. 1938. Études de systématique bactérienne. Ann. Inst. Pasteur 60:285–307.

Pringsheim, E. G. 1923. Zur Kritik der Bakteriensystematik. Lotus 71:357–377.

Pukall, R., Buntefuß, D., Frühling, A., Rohde, M., Kroppenstedt, R., Burghardt, J., Lebaron, P., Bernard, L., and Stackebrandt, E. 1999. Sulfitobacter mediterraneus sp. nov., a new sulfite-oxidizing Gram-negative bacterium of the alpha subclass of Proteobacteria. Int. J. Syst. Bacteriol. 49 pt. 2:513–519.

Rainey, F. A., Ward, N. L., Morgan, H. W., Toalster, R., and Stackebrandt, E. 1993. Phylogenetic analysis of anaerobic thermophilic bacteria: an aid for their reclassification. J. Bacteriol. 175:4772–4779.

Rainey, F. A., Fritze, D., and Stackebrandt, E. 1994. The phylogenetic diversity of thermophilic members of the genus Bacillus as revealed by 16S rDNA analysis. FEMS Microbiol. Lett. 115:205–212.

Rainey, F. A., Zhilina, T. N., Boulygina, E. S., Stackebrandt, E., Tourova, T. P., and Zavarzin, G. A. 1995. The taxonomic status of the fermentative anaerobic bacteria: description of *Haloanaerobiales* ord. nov., Halobacteroidaceae fam. nov., Orenia gen. nov and further taxonomic rearrangements at the genus and species level. Anaerobe 1:185–199.

Rothschild, L. J., Ragan, A., Coleman, A. W., Heywood, P., and Gerbi, S. A. 1986. Are rRNA sequence comparisons the Rosetta stone of phylogeny? Cell 47:1–1.

Schleifer, K.-H., and Kandler, O. 1972. The peptidoglycan types of bacterial cell walls and their taxonomic implications. Bact. Rev. 36:407–477.

Schleifer, K.-H., and Stackebrandt, E. 1983. Molecular systematics of prokaryotes. Ann. Rev. Microbiol. 37:143–187.

Schleifer, K.-H., and Ludwig, W. 1989. Phylogenetic relationships among bacteria. In: Fernholm, B., Bremer, K. and Jörnwall, H. (eds.), The hierachy of life. Elsevier Science Publisher. Amsterdam, 103–117.

Schleifer, K.-H., Leuteritz, M., Weiss, N., Ludwig, W., Kirchhof, G., and Seidel-Rüfer, H. 1990. Taxonomic study of anaerobic, Gram-negative, rod-shaped bacteria from breweries: emended description of Pectinatus cerevisiiphilus and description of Pectinatus frisingensis sp. nov., Selenomonas lacticifex sp. nov., Zymophilus raffinosivorans gen. nov., sp. nov., and Zymophilus paucivorans sp. nov. Int. J. Syst. Bacteriol. 40:19–27.

Schlesner, H., Kath, T., Fischer, A., and Stackebrandt, E. 1989. Studies on the phylogenetic position of Prosthecomicrobium pneumaticum, P. enhydrum, Ancalomicrobium adetum, and various Prosthecomicrobium-like bacteria. System. Appl. Microbiol. 12:150–155.

Schumann, P., Spröer, C., Burghardt, J., Kovacs, G., and Stackebrandt, E. 1999. Reclassification of the species Kocuria erxthromyxa (Brooks and Murray 1981) as Kocuria rosea (Flügge, 1886). Int. J. Syst. bacteriol 49:393–396.

Seewaldt, E., Schleifer, K.-H., Bock, E., and Stackerbrandt, E. 1982. The close phylogenetic relationship of Nitro-

bacter and Rhodopseudomonas palustris. Arch. Microbiol. 131:287–290.

Selander, R. K., Li, J., Boyd, F., Wang, F.-S., and Nelson, K. 1994. DNA sequence analysis of the genetic structure of populations of Salmonella enterica and Escherichia coli. Priest, F. G., Ramos-Cormenzana, A. and Tindall, B. J. Bacterial diversity and systematics. Plenum Press. New York, 17–50.

Shah, H. N., and Collins, M. D. 1989. Proposal to restrict the genus Bacteroides (Castellani and Chalmers) to Bacteroides fragilis and closely related species. Int. J. Syst. Bacteriol. 39:85–87.

Sibley, C. G., Comstock, J. A., and Ahlquist, J. E. 1990. DNA hybridization evidence of homoid phylogeny: a reanalysis of the data. J. Mol. Evol. 30:202–236.

Sittig, M., and Schlesner, H. 1993. Chemotaxonomic investigation of various prosthecate and/or budding bacteria. System. Appl. Microbiol. 16:92–103.

Sneath, P. A. H. 1989. Analysis and interpretation of sequence data for bacterial systematics: the view of a numerical taxonomist. Syst. Appl. Microbiol. 12:15–31.

Spröer, C., Reichenbach, H., and Stackebrandt, E. 1999. The correlation between morphological and phylogenetic classification of myxobacteria. Int. J. Syst. Bacteriol. 49:1255–1262.

Stackebrandt, E., Wunner-Füssel, Fowler, V. J., and Schleifer, K.-H. 1981. Deoxyribonucleic acid homologies and ribosomal ribonucleic acid similarities among sporeforming members of the order *Actinomycetales*. Int. J. Syst. Bacteriol. 31:420–431.

Stackebrandt, E., and Woese, C. R. 1984. The phylogeny of prokaryotes. Microbiol. Sci. 1:117–122.

Stackebrandt, E., Embley, M., and Weckesser, J. 1988. Phylogenetic, evolutionary, and taxonomic aspects of phototrophic eubacteria. Olson, J. M., Ormerod, J. G., Amesz, J., Stackebrandt, E. and Trüper, H. G. Green photosynthetic bacteria. Plenum Press. New York, 201–215.

Stackebrandt, E., Fischer, A., Roggentin, T., Wehmeyer, U., Bomar, D., and Smida, J. 1988. A phylogenetic survey of budding, and/or prosthecate, non-phototrophic eubacteria: membership of Hyphomicrobium, Hyphomonas, Pedomicrobium, Filomicrobium, Caulobacter and Dichotomicrobium to the alpha subdivision of purple non-sulfur bacteria. Arch. Microbiol. 149:547–556.

Stackebrandt, E. 1988. Phylogenetic relationships vs. phenotypic diversity: how to achieve a phylogenetic classification system of the eubacteria. Can. J. Microbiol. 34:552–556.

Stackebrandt, E. 1992. Unifying phylogeny and phenotypic properties. Balows, A., Trüper, H. G., Dworkin, M., Harder, W. and Schleifer, K.-H. The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Springer. New York, 19–47.

Stackebrandt, E., and Goebel, B. M. 1994. A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846–849.

Stackebrandt, E., and Rainey, F. A. 1997. Phylogenetic relationships. Rood, I. J., McClane, B. A., Songer, J. B. and Titball, R. W.The clostridia: molecular biology and pathogenesis. Academic Press. New York, 3-19.

Stackebrandt, E., Rainey, F. A., and Ward-Rainey, N. L. 1997. Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. Int. J. Syst. Bacteriol. 47:479–491.

Stackebrandt, E., Tindall, B., Ludwig, W., and Goodfellow, M. 1999. Prokaryotic diversity and systematics. Lengeler, J., Drews, G. and Schlegel, H. G. Biology of the Prokaryotes. Thieme. Stuttgart, 675–720.

Staley, J. T. 1977. Biodiversity: are microbial species threatened? Curr. Pos. Biotech. 8:340–345.

Staley, J. T., and Krieg, N. R. 1984. Bacterial classification I. Classification of prokaryotic organisms: an overview. Williams, S. T., Sharpe, M. E. and Holt, J. G. Bergey's manual of systematic bacteriology. Williams & Wilkins. Baltimore, 2999–2302.

Stanier, R. Y., and van Niel, C. B. 1941. The main outlines of bacterial classification. J. Bact. 42:437–466.

Steigerwalt, A. G., Fanning, G. R., Fife-Ashbury, M. A., and Brenner, D. J. 1976. DNA relatedness among species of Enterobacter and Serratia. Can. J. Microbiol. 22:121–137.

Stetter, K. O. 1989. Order III. *Sulfolobales* ord. nov. Staley, T., Bryant, M. P., Pfennig, N. and Holt, J. G. Bergey's manual of systematic bacteriology, vol. 3. Williams & Wilkins. Baltimore, 2250–2253.

Steyn, P. L., Segers, P., Vancanneyt, M., Sandra, P., Kersters, K., and Joubert, J. J. 1998. Classification of heparinolytic bacteria into a new genus Pedobacter, comprising four species: Pedobacter heparinus comb. nov., Pedobacter piscium comb. nov., Pedobacter africanus sp. nov., and Pedobacter saltans sp. nov. Proposal of the family Sphingobacteriaceae fam. nov. Int. J. Syst. Bacteriol. 48:165–177.

Swanson, D. S., Kapur, V., Stockbauer, K., Pan, X., Frothingham, R., and Musser, J. M. 1997. Subspecific differentiation of Mycobacterium avium complex strains by automated sequencing of a region of the genes (hsp65) encoding a 65-kilodalton heat shock protein. Int. J. Syst. Bacteriol. 47:414–419.

Tamura, A., Hasegawa, T., and Huang, L. H. 1993. Catenuloplanes japonicus gen. nov., nom.rev., a new genus of the order *Actinomycetales*. Int. J. Syst. Bacteriol. 43:805–812.

Tamura, T., Yokota, A., Huang, L. H., Hasegawa, T., and Hatano, K. 1995. Five new species of the genus Catenuloplanes: Catenuloplanes niger sp. nov., Catenuloplanes indicus sp. nov., Catenuloplanes atrovinosus sp. nov., Catenuloplanes castaneus sp. nov., and Catenuloplanes nepalensis sp. nov. Int. J. Syst. Bacteriol. 45:858–860.

Ullman, J. S., and McCarthy, B. J. 1973. The relationship between mismatched base pairs and the thermal stability of DNA duplexes. Biochim. Biophys. Acta 294:416–424.

Urakami, T., and Komagata, K. 1987. Cellular fatty acid composition with special reference to the existance of hydroxy fatty acids in Gram-negative methanole-, methane, and methylamine-utilizing bacteria. J. Gen. Appl. Microbiol. 33:135–165.

Urakami, T., Tamaoka, J., Suzuki, K.-I., and Komagata, K. 1989. Acidomonas gen. nov., incorporating Acetobacter methanolicus as Acidomonas methanolica comb. nov. Int. J. Syst. Bacteriol 39:50–55.

Vandamme, P., Segers, P., Ryll, M., Hommez, J., Vancanneyt, M., Coopman, R., De Baere, R., Van de Peer, Y., Kersters, K., De Wachter, R., and Hinz, K. H. 1998. Pelistega europaea gen. nov., sp. nov., a bacterium associated withb respiratory disease in pigeons: taxonomic structure and phylogenetic allocation. Int. J. Syst. Bacteriol. 48:431–440.

Vaneechoutte, M. 1996. DNA fingerprinting techniques for microorganisms. Molec. Biotech. 6:115–143.

Venkateswaran, K., Dollhopf, M. E., Aller, R., Stackebrandt, E., and Nealson, K. H. 1998. Shewanella amazonensis sp. nov., a novel metal-reducing facultative anaerobe from Amazonian shelf muds. Int. J. Syst. Bacteriol. 48:965–972.

Verger, F. F., Grimont, P. A. D., Grimont, F., and Grayon, M. 1985. Brucella, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. 35:292–295.

Viale, A. M., Arakaki, K., Soncini, F. C., and Ferreyra, R. G. 1994. Evolutionary relationships among eubacterial groups as inferred from GroRL (chaperonin) sequence comparison. Int. J. Syst. Bacteriol. 44:527–533.

Walker, D. H. 1989. Rocky Mountain spotted fever: a new disease in need of microbiological concern. Clin. Microbiol. Rev. 2:227–240.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, L., Moore, L. H., Moore, W. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., and Trüper, H. G. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463–464.

Webster, J. A., Bannerman, T. L., Hubner, R. J., Ballard, D. N., Cole, E. M., Bruce, J. L., Fiedler, F., Schubert, K., and Kloos, W. E. 1994. Identification of the Staphylococcus sciuri species group with EcoRI fragments containing rRNA sequences and description of Staphylococcus vitulus sp. nov. Int. J. Syst. Bacteriol. 44:454–460.

Weckesser, J., and Mayer, H. 1988. Different lipid A types in lipopolysaccharides of phototrophic and related nonphototrophic bacteria. FEMS Microbiol. Rev. 54:143–154.

Woese, C. R., and Fox, G. E. 1977. The concept of cellular evolution. J. Mol. Evol. 10:1–1.

Woese, C. R., Blanz, P., Hespell, R. B., and Hahn, C. M. 1982. Phylogenetic relationships among various helical bacteria. Curr. Microbiol. 7:119–124.

Woese, C. R., Debrunner-Vossbrinck, B., Oyaizu, H., Stackebrandt, E., and Ludwig, W. 1985. Gram-positive bacteria: possible photosynthetic ancestry. Science 229:762–765.

Woese, C. R., Stackebrandt, E., Macke, T., and Fox, G. E. 1985. A phylogenetic definition of the major eubacterial taxa. System. Appl. Microbiol. 6:143–151.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Woese, C. R., Kandler, O., and Wheelis, M. L. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucaryas. Proc. Natl. Acad. Sci. USA 87:4576–4579.

Woese, C. R., Achenbach-Richter, L., Rouviere, P., and Mandelco, L. 1991. Archaeal phylogeny: reexamination of the phylogenetic position of Archaeoglobus fulgidus in the light of certain composition-introduced artifacts. System. Appl. Microbiol. 14:364–371.

Yamamoto, S., and Harayama, S. 1998. Phylogenetic relationships of Pseudomonas putida strains deduced from the nucleotide sequences of gyrB, rpoD and 16S rRNA genes. Int. J. Syst. Bacteriol. 48:813–819.

Yamamoto, S., Bouvet, J. M., and Harayama, S. 1999. Phylogenetic structure of the genus Acinetobacter based on gyrB sequences: comparison with the grouping by DNA-DNA hybridization. Int. J. Syst. Bacteriol. 49:87–95.

Yurkov, V., Stackebrandt, E., Buss, O., Vermeglio, A., Gorlenko, V., and Beatty, J. T. 1997. Reorganization of the genus Erythromicrobium: description of "Erythromicrobium sibiricum" as Sandaracinobacter sibiricus gen. nov., sp. nov., and of "Erythromicrobium ursincola" as Erythromonas ursincola gem. nov., sp. nov. Int. J. Syst. Bacteriol. 47:1172–1178.

Ziemke, F., Höfle, M. G., Lalucat, J., and Rosello-Mora, R. 1998. Reclassification of Owen's genomic group II as Shewanella baltica sp. nov. Int. J. Syst. Bacteriol. 48:179–186.

Zillig, W., Stetter, K. O., Schäfer, W., Janekovic, D., Wunderl, S., Holz, I., and Palm, P. 1981. *Thermoproteales*: a novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from Icelandic solfataras. Zentralbl. Bakteriol. Microbiol. Hyg.1 Abt. Orig. C2 3:205–227.

Zillig, W., Stetter, K. O., Prangishvilli, D., Schäfer, W., Wunderl, S., Trent, J., Janekovic, D., Holz, I., and Palm, P. 1982. Desulfurococcaceae, the second family of the extremely thermophilic, anaerobic, sulfur-respiring Thermoproteales. Zentralbl. Bakteriol. Microbiol. Hyg. Abt. Orig. C 2:304–317.

Zuckerkandl, E., and Pauling, L. 1965. Molecules as documents of evolutionary history. J. Theor. Biol. 8:357–366.

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CHAPTER 1.4

Prokaryote Characterization and Identification

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The aim of characterization (in the present context) is to obtain a complete collection of data describing the properties of a prokaryotic pure culture, i.e., to develop a description. The aim of "identification" is to equate the properties of a pure culture with those of a well-characterized and accepted species. When identification in this sense cannot be accomplished, the aim of identification must shift to characterization of a new species, i.e., to a new description.

It is clear that the amount of data required for the identification of an isolate with an established species is usually lower than the amount of data collected for characterization. And it is also clear that the final aim of characterization, as mentioned above, is never reached because continuing progress in scientific and technological methods allows the study of an ever-increasing number of characters or properties of a species. Although this continuous progress results in a higher reliability in identification, the practical aim of identification is to base it upon the smallest possible number of characteristics. Therefore, identification in many cases (especially with pathogenic organisms) is a compromise between accuracy and speed. The selected characteristics used for identification usually are weighted and are those that have proved to be significant in distinguishing one organism from another.

Prokaryote Systematics

Definitions

We may define systematics as the scientific study of organisms with the ultimate object of characterizing and arranging them in an orderly manner. Systematics also might be defined as "the study of organismal diversity and interrelationships." Following Cowan (1968), systematics includes taxonomy (with its subdivisions, see below) and includes aspects of ecology, biochemistry, genetics, pathology, molecular biology, and microscopy.

Taxonomy is often used as a synonym for systematics. Stanier et al. (1986) define taxonomy as "the art of biological classification," while Simpson (1961) defines it as the theoretical study of classification, including its bases, principles, and rules. Cowan (1968) states that taxonomy consists of:

1. *Classification*, which means the orderly arrangement of units into groups.
2. *Nomenclature*, which means the labeling of units defined by classification.
3. *Identification* of unknowns with the units defined by classification and labeled by nomenclature (i.e., identification is the practical application of the arts of classification and nomenclature).

Classification is often confused with identification, because identification may be understood either transitively (identification of unknowns with units defined by classification) or intransitively (to describe the identity of a species as such and use it as a basis for classification). Thus Stanier et al. (1986) consider the two functions of taxonomy to be: 1) to identify and describe the basic taxonomic units or species; and 2) to devise an appropriate way of arranging and cataloging these units. The interrelations between these items are depicted in a flow diagram in Fig. 1.

Nomenclature of Bacteria

Since most microbiologists are not primarily involved in taxonomic work, a short introduction to the International Code of Nomenclature of Bacteria will be presented in the following discussion.

The 1975 revision of the International Code of Nomenclature of Bacteria (Lapage et al., 1975) became valid 1 January 1976. It consists of 7 General Considerations, 9 Principles, 65 Rules (some being supplemented by Recommendations which do not have the force of Rules), 10 Appendices, the Statutes of the International Committee on Systematic Bacteriology, and the Statutes of the International Union of Microbiological Societies (IUMS).

The International Code of Nomenclature of Bacteria is designed to guarantee the correct names of taxa. It contains rules about the rank of taxa; naming of taxa; nomenclatural types and their designation; priority and publication of names; citation of authors and names; changes in names of taxa as a result of transference, union, or change in rank; rules about illegitimate names

This chapter was taken unchanged from the second edition.

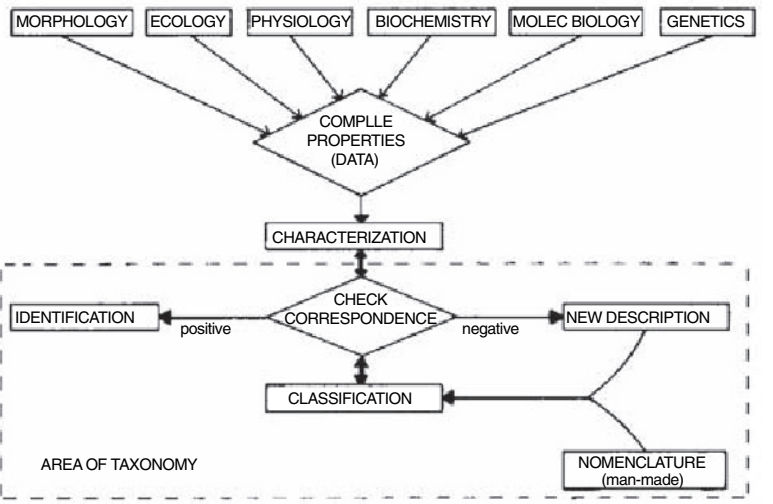

Fig. 1. Information flow diagram indicating the relationships between the characterization and the classification of a bacterial strain. The two important working steps (indicated by diamond-shaped boxes) are compiling properties and checking for correspondence between characterization and classification.

and epithets; replacement, rejection, and conservation of names and epithets. Also, a number of rules and Appendix 9 pertain to the correct usage of Latin orthography.

Appendix 4 of the International Code of Nomenclature of Bacteria is especially important because it contains the conserved and rejected names of bacterial taxa. The Opinions of the Judicial Commission of the International Committee on Systematic Bacteriology are contained in Appendix 5. These opinions have the same force as the rules. New Opinions are published in the *International Journal of Systematic Bacteriology.*

The Rules of the International Code of Nomenclature of Bacteria are designed to make effective the Principles. They are internationally accepted and must be obeyed in bacterial nomenclature, even retrospectively. Bacterial nomenclature is not completely independent; for practical reasons, bacteria may not bear names that are validly used for algae, fungi, protozoa, or viruses (Principle 2).

In the Code, the word "taxon" (plural "taxa") is used for any taxonomic group of organisms (General Consideration 7). The correct name of a taxon (Principle 6) is based upon:

1. Valid publication. Since 1976, this means publication of the name in the *International Journal of Systematic Bacteriology.* The publication of the name in this journal must be accompanied by a description of the taxon or by a reference to a previous effectively published (see below) description of the taxon (Appendix 7). A type must be designated for the new taxon or combination (Rule 27).

2. Legitimacy. Names must be in accordance with the Rules.

a) Priority of publication. Each taxon above species (up to and including order) can bear only one correct name, the earliest that is in accordance with the Rules of the International Code of Nomenclature of Bacteria (Rule 23a). The name of a species is a binary combination of generic name and specific epithet (Rule 12a). Within a genus, a species can only bear one correct epithet, the earliest given in accordance with the Rules of the International Code of Nomenclature of Bacteria (see also below: *Approved Lists of Bacterial Names*).

b) Effective publication. "Effective" publication means to make printed material generally available, by sale or distribution, to the scientific community, for the purpose of providing a permanent record (Rule 25). New names presented at meetings, in minutes or abstracts of meetings, in collection catalogs, newsletters, newspapers, nonscientific periodicals, patents, or distributed on microfilms or microcards, etc., are not considered effectively published by the International Code of Nomenclature of Bacteria.

A taxon consists of one or more elements. For each named taxon of the various taxonomic categories (Table 1), there shall be designated a nomenclatural "type" that is the element of the taxon to which the name is permanently associated.

The taxonomic categories form a hierarchy which is depicted in Table 1. In general, little use is made of subcategories, such as "suborder" and "subfamily," or of the category "tribe."

The basis of the taxonomic hierarchy is essentially the species. The definition of a prokaryote species has often given rise to discussions and arguments among biologists. Cowan (1968) humorously summarized a species as "a group of organisms defined more or less subjectively by the criteria chosen by the taxonomist to show to best advantage as far as possible and putting into practice his individual concept of what a species is."

Table 1. Taxonomic categories, examples, and types.

Taxonomic category	Latin suffix	Example[a]	Type (definition)	Type (example)
Division[a]	Not fixed	Gracilicutes		
Class		Photobacteria	One of the contained orders	
Subclass		Anoxyphotobacteria		
Order	-ales	Rhodospirillales	Genus, on whose name the name of the higher taxon is based	*Rhodospirillum*
Suborder	-ineae	Rhodospirillineae		
Family	-aceae	Rhodospirillaceae		
Subfamily	-oideae	Rhodospirilloideae		
Tribe	-eae	Rhodospirilleae		
Subtribe	-inae	Rhodospirillinae		
Genus	Not fixed	*Rhodospirillum*	Designated species	*R. rubrum*
Subgenus		—		
Species		*R. rubrum*	Designated strain	Strain ATCC 11170
Subspecies		—		

The examples given are classical and should be changed during further development of a phylogeny-based taxonomy, as follows: Above order: proposal of Gibbons and Murray (1978); the class was changed to Proteobacteria by Stackebrandt et al. (1988). Below subclass: from Pfennig and Trüper (1971), where subcategories were not mentioned.

The textbook written by Stanier et al. (1986) states: "... a species consists of an assemblage of individuals (or, in microorganisms, of clonal populations) that share a high degree of phenotypic similarity, coupled with an appreciable dissimilarity from other assemblages of the same general kind."

The modern phylogenetic definition of a species (Wayne et al., 1987) is based on DNA-DNA hybridization procedures and generally would include all strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔTm. Both values must be considered. Phenotypic characteristics should agree with this definition and would be allowed to override the phylogenetic concept of species only in a few exceptional cases.

It is recommended that a distinct genospecies (i.e., a species discernible only by nucleic acid comparisons) that cannot be differentiated from another genospecies on the basis of any known phenotypic property not be named until some phenotypic differentiating property is found (Wayne et al., 1987).

When a new species is described, the type must be indicated. Whenever possible the type should be a pure culture designated as type strain. For a species described from impure cultures, a description, a preserved specimen, or an illustration may serve as the type (Rule 18a). If such an organism is brought into pure culture later on, it may be designated as the type strain and replace the original type (Rule 18h).

Before publication of the name of a new species, a culture of the type strain should be deposited in at least one of the permanently established culture collections from which it is readily available. The strain number given by the culture collection should be quoted in the published description (Recommendation 30a).

Subspecies designations can be used for genetically close organisms that diverge in phenotype. There is some evidence, based on frequency distribution of ΔTm values in DNA hybridization, that the subspecies concept is phylogenetically valid and can be distinguished from the infrasubspecific variety concept, which is based solely upon selected "utility" attributes, but not demonstrable by DNA reassociation (Wayne et al., 1987).

Appendix 10 of the International Code of Nomenclature of Bacteria deals with infrasubspecific subdivisions. An infrasubspecific taxon is one strain or a set of strains showing the same or similar properties and treated as a taxonomic group. An infrasubspecific term is used to refer to the kind of taxa below subspecies.

The terms commonly used for such subdivisions usually contain the suffixes -type, -var, or -form. Appendix 10 recommends the use of the suffix -var in order to avoid confusion with the strict use of the term "type" for the nomenclatural type (Rule 15). The following terms are recommended (with the terms that should be avoided in parentheses): biovar (biotype, physiological type); chemovar; chemoform (chemotype); cultivar; forma specialis (special form); morphovar (morphotype); pathovar (pathotype); phagovar (phagotype, lysotype); phase; serovar (serotype); state.

"Biovar" pertains to biochemical or physiological properties, "chemovar" to production of a particular chemical, "chemoform" to chemical constitution, "cultivar" to special cultivation properties, "forma specialis" to a parasitic, symbiotic, or commensal bacterium distinguished primarily by adaptation to a particular host or habitat (it should be named preferably by the scientific name of the host in the genitive). "Morphovar" pertains to morphological characteris-

tics, "pathovar" to pathogenic reactions in one or more hosts, "phagovar" to reactions to bacteriophage, "serovar" to antigenic characteristics. The term "phase" should be restricted to well-defined stages of naturally occurring alternating variations. "State" is used for colonial variants—e.g., rough, smooth, mucoid—which may be defined antigenically and then considered as serovars.

An alphabetical compilation of all names that have been used in bacteriology (except for the cyanobacteria), including the evaluation of their legitimacy and valid and effective publication, is contained in the Index Bergeyana (Buchanan, Holt, and Lessel, 1966), with three addenda prepared up to 1969 (Hatt and Zvirbulis, 1967; Zvirbulis and Hatt, 1969a, 1969b). For the language of taxonomy and nomenclature, we recommend the *Dictionary of Microbial Taxonomy* (Cowan, 1978), where helpful and sometimes humorous instructions are provided.

Approved Lists of Bacterial Names

Since 1 January 1980, priority of bacterial names is—by international agreement—based upon the *Approved Lists of Bacterial Names* (Skerman, McGowan, and Sneath, 1980). Names that were not included in the *Approved Lists* lost standing in bacterial nomenclature, but are, however, available for reuse (Rule 24a).

Since 1 January 1980, valid publication of new names and new nomenclatural combinations can only be obtained by publication in the *International Journal of Systematic Bacteriology*, either of an original article or in the "Validation Lists" regularly appearing in that journal.

These lists constitute validation of the publication of new names and new combinations that were previously effectively published outside the *International Journal of Systematic Bacteriology.* Up to the end of 1989, 31 such validation lists have been published in the *International Journal of Systematic Bacteriology.*

Towards a Modern Taxonomy

Prokaryote taxonomy began when Ferdinand Cohn (1872) grouped the bacteria—according to their overall morphological appearance—into cocci, short rods, elongate rods, and spirals. Cohn (1875) also perceived the high degree of relationship between the "common bacteria" and the cyanobacteria (then called blue-green algae) and grouped them together as the Schizophyta.

Although the purely morphological approach soon proved to be insufficient, morphology and, increasingly, ultrastructure, still play an important role in identification.

A new era in bacterial taxonomy, dominated by physiology, began with the classification system of Orla-Jensen (1909). Numerous physiological properties of bacterial cultures were determined for characterization and predominantly used for identification. Later, enzymes were studied and metabolic pathways elucidated.

Orla-Jensen 1909 and later Kluyver and van Niel (1936) as well as Stanier and van Niel (1941) proposed taxonomic systems based on the presumed evolutionary relationships of bacteria. Van Niel (1946) himself, however, pointed out the shortcomings of the deductive phylogenetic approach to bacterial classification. The shortcomings were mainly seen in the lack of suitable parameters that clearly allow differentiation between "relationship" and mere "resemblance," not to mention the general lack of paleontological evidence. Stanier (1971) pointed out that the question of relatedness can be probed at two slightly different levels: 1) the strictly genetic level where general organization and base sequence homologies of the deoxyribonucleic acids of different organisms are compared; and 2) the epigenetic level (a term coined by Stanier), where properties are compared that are expressed at the level of translation (or, less commonly, of transcription).

Since then the study of microbial phylogeny has attracted increasing attention, and the taxonomy of prokaryotes undoubtedly will eventually reflect phylogeny. The work of C. R. Woese (for details, see Prokaryote Systematics: The Evolution of a Science in this Volume) has especially been a "milestone" in this respect. It has been recommended (Wayne et al., 1987) that DNA-DNA hybridization should be the standard arbiter for the designation of species (see also above).

Phylogenetic relationships have been assessed at all taxonomic levels by analysis of rRNA and/or the gene that encodes it. Current analyses involve the application of DNA/rRNA hybridization and comparative sequencing of 5S rRNA, 16S rRNA and 23S rRNA. Other macromolecules besides rRNAs, e.g. elongation factor Tu and β-subunit of ATP-synthase, can also be used as phylogenetic markers (Schleifer and Ludwig, 1989). Data obtained from comparative sequence analyses provide the best basis at the present time for phylogenetic relationships among all bacteria. However, differences in evolutionary rates in various groups of organisms and other considerations mentioned below prevent the use of phylogenetic parameters alone in delineating taxa. Therefore, the integrated use of phylogenetic and phenotypic characteristics, called polyphasic taxonomy (Colwell, 1970) is necessary for the delineation of taxa at all levels from kingdom to genus (Murray et al., 1990).

For such analyses to be accurate and universally applicable, it is essential to start with a typical strain which is designated the type strain of the type species for the genus or genera under study. To determine the phylogenetic range of a genus, one must use type strains of the two most divergent species, which should be selected by DNA-DNA hybridization or other appropriate phylogenetic measure, and/or by consideration of phenotypic characteristics. It is frequently essential and always advantageous to use additional, authentic, well-characterized reference strains when they are available.

Because a polyphasic approach, including phenotypic and phylogenetic data, is essential for designating taxa, descriptions of new genera should, whenever possible, include either sequencing or hybridization data. The optimal methods for determining phylogenetic relationships above the species level are DNA-rRNA hybridization and 16S rRNA sequencing of 1,000 or more bases. 23S rRNA may provide greater sensitivity for some applications but need not be done routinely. Sequencing of 5S rRNA can also be useful (Erdman et al., 1985) but because of the small size of the macromolecule, less information is provided. When comparing results to published sequences, scientists should be aware of data from strains other than the type. In the absence of a type strain sequence, a strain authenticated to be at the same species level to the type strain by DNA-DNA relatedness should be satisfactory (Wayne et al., 1987).

All sequences from which phylogenetic and taxonomic conclusions have been derived should be published or made available through data banks. This must be done not only for the organisms under investigation, but also for the reference strains used in tree construction. Failure to do so has resulted in the inability to test the proposed phylogenetic conclusions and prevents building a database available to all scientists. Restriction of sequence information to small fragments allows recognition of taxa and may be useful for identification, but is of questionable value in deriving phylogenetic conclusions.

Phylogenetic relations have been assessed at all taxonomic levels by analysis of rRNA or rRNA genes. Chemotaxonomic markers and genetic transformation are unevenly distributed but rarely give information on the hierarchic rank of the taxa studied. There is, however, good evidence in bacterial systematics of congruence between the distribution of specific chemical markers and the relative position of species in phylogenetic trees. The use of chemotaxonomic markers can be expected to help in delimiting groups of related species (Murray et al., 1990).

As a taxonomic consequence, the first step in the identification of bacteria is to try to assign organisms to genera. Therefore, the greatest clarity in circumscription and utility in the choice of characters must be accorded to the generic level. It is completely impracticable to define genera solely on the basis of phylogenetic data. Genera need to be characterized using phenotypic properties even if the choice of phenotypic markers might change as a result of the development of better tests. A degree of flexibility is necessary in the definition of genera. In cases where there is disparity between phylogenetic and phenotypic data, priority should be provisionally given to the latter. In such instances, further detailed comparative studies of the phenotype should be encouraged to resolve the apparent disparity so that classification reflects phylogenetic relationships (Murray et al., 1990).

Numerical Taxonomy

A well-established means for the assessment and evaluation of phenotypic data is numerical taxonomy (Goodfellow, 1977; Sneath, 1971; Sneath and Sokal, 1973; Sokal and Sneath, 1963; Hill, 1974). For numerical studies, the results are tabulated in a table of t organisms versus n characters. The term OTU (operational taxonomic unit) is used for an individual strain. The characters should be independent and should come about equally from the various different categories of properties (morphology, physiology, biochemistry, serology, etc.). For statistical reasons, the total number of characters should be above 60. Whereas in conventional taxonomy so-called characteristic tests and differentiating media are in use (with special importance for identification), numerical taxonomy principally allots the same weight to each character, because there is no logical alternative that would allow independence from the personal opinion of the scientist. This principle was introduced into (plant) taxonomy by Adanson (1763).

The number of common characters is considered as a quantitative measure for the taxonomic relationship (not phylogenetic "relatedness"). As an important consequence, not all members (species) of a group must have one special property in common. Similarities are quantitatively expressed by the matching coefficient of Sokal and Michener (Skerman, 1967), usually expressed in percent:

$$S_{SM} = \frac{\text{sum of positive and negative matches}}{\text{total number of tests}}$$

The similarities between each OTU and every other OTU under study are set out in tables in the form of a triangular similarity matrix (also called a Sneath diagram). By forming linkage groups and performing cluster analyses, the

numerical taxonomist finally arrives at a rearranged matrix that depicts linkage clusters. Another way to express relations between OTUs after an analysis is by the use of dendrograms. Kaneko (1979) introduced the correlative similarity coefficient as a new criterion for forming dendrograms. Other coefficients for numerical taxonomy have been evaluated by Austin and Colwell (1977). Detailed information about the use of computer analysis in numerical taxonomy can be obtained from Sneath (1972, 1977, 1979a, 1979b, 1979c, 1980a, 1980b, 1980c). Computer-aided identification systems have also been described, e.g., by Edwards (1978), Kellogg (1979), Schindler, Duben, and Lysenko (1979), Beers and Lockhard (1962), Gyllenberg (1965), Holmes and Hill (1985), Lapage et al. (1970, 1973).

Assessment of Morphological and Ultrastructural Characters

Microscopic Characters

Cell Shape The shape of cells, although of general importance in bacteriology, plays a special role in the identification of stalked, budding, or sheathed bacteria, as well as within the physiological group of the phototrophic bacteria, especially the cyanobacteria. In medical microbiology, cell shape is especially important for the identification of fusiform bacteria, coryneform bacteria, spirochetes, spirilla, and vibrios. Where conspicuous cell aggregates are formed, they are a very useful tool for identification, as is the formation of fruiting bodies by the myxobacteria (see The Myxobacteria in Volume 7) and the formation of mycelia by actinomycetes (see Introduction to the Taxonomy of Actinobacteria and The Families Dietziaceae, Gordoniaceae, and Tsukamurellaceae Nocardiaceae in Volume 3). Cocci occur as diplococci, sarcina packets, chains (*Streptococcus*), and clumps (*Staphylococcus*). In the Chromatiaceae and Chlorobiaceae, there exist such typical forms as *Thiopedia* (platelets), *Thiocapsa* (tetrads), and *Thiodictyon* and *Pelodictyon* (nets).

The disease "Vincent's angina" is diagnosed in many laboratories only by microscopic demonstration of a marked overgrowth of the microflora normally present in the oral cavity and pharynx by fusiform and spirochetal bacteria. The variability in size and shape can be a help in identifying a species; cells of a club-shaped *Corynebacterium diphtheriae* are pleomorphic, whereas cells of diphtheroids are more uniform in shape. The demonstration of a large number of Gram-positive cocci in feces may indicate an enterocolitis caused by *Staphylococcus aureus*, and the appearance of Gram-negative diplococci in cerebrospinal fluid suggests a meningitis with *Neisseria menigitidis* as the etiological agent.

Cell Size The diameter (thickness) of cells is used to differentiate between species in genera such as *Chromatium* and *Beggiatoa*. Due to the reproductive patterns of bacteria, in most cases the diameter of a cell is a more stable property than its length.

Cellular Motility Motility of cells may not be a generally stable character, although it is important in the identification of the various groups of gliding bacteria: e.g., The Myxobacteria in Volume 7, flexibacteria The Order Cytophagales in Volume 7, The Family Chloroflexaceae in Volume 7, gliding Cyanobacteria—Ecology, Physiology, and Molecular Genetics in Volume 4, and Beggiatoaceae (The Genus *Beggiatoa* and *Thioploca* in Volume 6).

Since great variability in motility occurs within the Enterobacteriaceae, motility is of comparatively little importance there (see New Members of the Family Enterobacteriaceae in Volume 6).

In medical microbiology, direct inspection for motility is used in a few cases. Venereal lesions can be examined by very experienced personnel for the typical movement of *Treponema pallidum*. Most members of the *Proteus* group are very actively motile. Starting from one colony on nutrient agar, these strains spread over the surface of the agar plate, a phenomenon known as swarming.

Care should be taken not to confuse the terms "immotile" and "nonmotile." A species (or strain) may be called nonmotile only if it is permanently so and has no means of locomotion (flagella or gliding) at all. On the other hand, "immotile" is a temporary status of a cell.

Flagellation The type of flagellation, i.e., position and number of flagella, is often used in the definition of genera and even higher taxa. However, flagellation is not always a constant characteristic. For example, although it is a generally accepted opinion that pseudomonads and vibrios possess polar flagella, certain marine *Vibrio* species (formerly in the genus *Beneckea*) have polar flagella only in liquid media; on solid media, they develop lateral flagellation (Allen and Baumann, 1971; Reichelt and Baumann, 1973). The peritrichously flagellated hydrogen oxidizing bacterium, *Alcaligenes eutrophus* (formerly *Hydrogenomonas*), is closely related to a genus that also contains polarly flagellated, hydrogen oxidizing bacteria, such as *Pseudomonas facilis* (formerly *Hydrogenomonas*).

These examples may demonstrate the relative value of type of flagellation for the identification

of bacteria. For further details, we recommend the review by Rhodes (1965) on flagellation as a criterion for the classification of bacteria.

Sporulation The presence of spores or the ability to form spores under limited nutrient conditions is of great importance since positive endospore formation aids in rapid identification.

The only endosporeforming bacteria that have clinical importance belong to the genera *Bacillus* and *Clostridium*. The ability to form endospores has, therefore, a great distinguishing value. Since sporulation of human pathogens is not common in original clinical material or in media used for the isolation of the bacteria, subculture in special sporeforming media is often helpful for demonstrating the spores. The endospores can be detected by a specific spore stain, but usually the Gram stain or direct examination with a phase contrast microscope is sufficient because the endospores are highly refractive and do not take up dyes of the Gram stain. Not only the presence but also the position of the spore within the cell may be of great diagnostic value, e.g., for the identification of *Clostridium tetani* and *C. botulinum*.

For the identification of actinomycetes, the shape and color of their conidiospores are of great importance.

Cellular Inclusions Microscopically visible cellular inclusions other than spores may be used in identification procedures for certain bacteria. Globules of elemental sulfur inside the cells are used in differentiation of phototrophic sulfur bacteria (The Family Ectothiorhodospiraceae in Volume 6). A protein crystal will help to identify *Bacillus thuringiensis*. The highly refractile gas vacuoles found in phototrophic and other bacteria are usually specific taxonomic criteria.

The demonstration of polymetaphosphate (volutin granules) may be a help for the presumptive identification of *Corynebacterium diphtheriae* and for distinguishing *C. diphtheriae* from diphtheroids. In clinical specimens and in organisms grown on media without potassium tellurite, these inclusions were found typically in most cells of *C. diphtheriae*, morphovar *mitis*, as polar bodies, whereas diphtheroids produce these granules usually only in a few cells or not at all. Because the basophilic volutin granules appear reddish when stained with toluidine blue, such storage materials are also called metachromatic granules. Common procedures for the staining of volutin granules in cells of corynebacteria are Albert's and Neisser's stain: after staining, the polymetaphosphate bodies appear blue-black and the cytoplasm appears light brown.

Color The color of single cells studied under the microscope may reveal whether a purple sulfur bacterium contains the conspicuous purple-red carotenoid okenone, thus allowing quick allocation in the family Chromatiaceae.

The color of cell suspensions is of far greater importance. In many cases of pigment-containing bacteria, an experienced microbiologist will be able to judge from typical colors without recording absorption spectra of the respective suspensions. For safe identification, however, an absorption spectrum is indispensable, e.g., for the identification of phototrophic bacteria (see The Phototrophic Way of Life in Volume 2).

Whereas the color of single cells or cell suspensions may not always give the decisive clue, the color of colonies—either in deep agar-shake cultures or on agar surfaces—may help in identification. This is the case with *Serratia marcescens* within the Enterobacteriaceae, with *Halobacterium* (to distinguish it from other extremely halophilic bacteria), and with the phototrophic bacteria.

Colonial Morphology The morphology of a bacterial colony will vary greatly with the temperature, pH, atmosphere, age, and medium on which it is grown. Therefore, colonial morphology is merely a helpful character in identification when standardized media and growth conditions are used. In this case, the colonial pigmentation, surface, size, shape, production of slime, elevation, odor, opacity, swarming behavior, consistency, and changing color of the medium are important parameters for the characterization of new organisms. In some cases these parameters are also of importance for the identification of clinical isolates. For example, tiny, opaque to transparent colonies of about 0.5 mm in diameter on blood agar with a clear zone around the colonies (beta hemolysis), sliding entirely across the agar when touched with a wire loop, are likely to be *Streptococcus pyogenes*. In contrast, colonies of *Streptococcus pneumoniae* on blood agar are mucoid, about 1 mm in diameter, surrounded by a zone of discoloration (alpha hemolysis), and often umbilicated, which means that the autolyzed flat center of the colony is surrounded by an elevated rim.

Often, species such as *Staphylococcus aureus* can be preliminarily identified because of the golden-yellow pigmentation of the butyrous colonies, which are 2–3 mm in diameter and often surrounded by a small zone of β hemolysis. Strain variation may occur in colonial morphology. Wild-type strains of *Shigella sonnei* form convex colonies with a circular shape and a smooth surface, whereas colonies of mutants of *S. sonnei* that have lost the ability to synthesize

specific components of the outer cell wall are flat with a rough surface and an irregular shape. In addition to staining behavior and biochemical activity, the colonial appearance is an important characteristic for the separation of *C. diphtheriae* into the morphovars *gravis*, *mitis*, and *intermedius*. Agar-dissolving bacteria are quickly identified by the holes they form in agar surfaces. Liquefaction of gelatinous solid media is used in medical diagnostic bacteriology, as well as for the identification of *Rhodocyclus gelatinosus*.

Ultrastructural Characters

The fine structure of cells studied by electron microscopy provides several characters that have turned out to be useful in identification. The decision of whether a cell is Gram positive or negative may be derived from electron micrographs, but other cell wall layers (Glauert and Thornley, 1969: the "topography" of cell walls) and very typical intracytoplasmic membrane systems also become visible. The latter are especially important for the identification of phototrophic (cyanobacteria and purple bacteria), methylotrophic, and chemolithoautotrophic (thiobacilli and nitrifiers) bacteria. Special organelles, the chlorosomes (Staehelin et al., 1978), characterize the Chlorobiaceae, and the phycobilisomes characterize the cyanobacteria. Carboxysomes, a polymerized form of the enzyme ribulose-bisphosphate carboxylase, appear as conspicuous polyhedral bodies in autotrophic bacteria only (Shively, 1974).

Gram-Staining Behavior

The ultra-structural and chemical differences between Gram-negative and Gram-positive bacterial cell walls are well substantiated. The Gram stain remains an indispensable method, especially in clinical and food microbiology, though there are examples of staining variability due to age and other factors. Some species react Gram-positive in the exponential growth phase, but become Gram-negative in the stationary phase. The methanogenic bacteria, none of which so far contains a peptidoglycan cell wall, in part stain Gram-negative, in part Gram-positive (Kandler and Hippe, 1977; Kandler and König, 1978). In the halobacteria, which also lack peptidoglycan, while *Halobacterium* reacts Gram-negative, *Halococcus* reacts Gram-positive.

Chemical Composition of Cell Constituents

Our increasing knowledge of the chemical composition of cell constituents has considerably improved the classification and identification of prokaryotes. Chemotaxonomic methods are now widely used, in particular, for those groups of prokaryotes where morphological and physiological characters have largely failed or have not been sufficient to provide a satisfactory classification. Simple and rapid qualitative analyses of cell walls or even of cells can be used to discriminate among groups of bacteria. However, when applying chemotaxonomic markers, microbiologists should be aware that sometimes one criterion, such as identical DNA base ratio or, less frequently, identical cell wall or lipid composition, can occur in quite different organisms. If this occurs, it is more prudent to rely on chemotaxonomic differences to exclude an organism from a taxon than to use a similarity as the sole criterion for inclusion.

DNA Base Composition

The usefulness and the taxonomic implications of the GC content of the DNA base have been reviewed by Mandel (1969). The DNA composition is one of the required characteristics of the minimum list of data for a description of a new species. By far the most used technique is the determination of the thermal denaturation temperature, but the determination of buoyant density in CsCl can also be used. Other methods, including chromatographic determinations and spectrophotometric approximations from absorbancy ratio, play only a minor role (Schleifer and Stackebrandt, 1983; Johnson, 1986).

As already pointed out by Mandel (1969), the GC content is only an exclusionary determinant in the classification of bacteria, in that two strains differing in more than 5 mol% should not be allocated to the same species, whereas on the other hand, a similar DNA base composition does not necessarily imply that the two strains are closely related.

Phylogenetic studies on the primary structure of the 16S rRNA (Woese, 1987) have demonstrated that the GC content of the DNA is not a phylogenetic marker per se. Only in the classification of Gram-positive eubacteria does the GC content allow the allocation of a strain into one of the two major lines of descent, one of them embracing high GC-content organisms, the other embracing those with low GC values. The situation is different among the Gram-negative eubacteria, which embrace several major lines of descent, to which organisms cannot be allocated according to their gross GC content.

Organisms whose DNA base compositions differ by more than 10 mol% ordinarily should not be considered members of the same genus. However, this raises the question of whether or not taxonomists should set fixed boundaries for the

range in the GC content for taxa above the species level. There are examples of phylogenetically coherent genera such as *Lactobacillus* and *Spirochaeta* with a GC content differing by more than 10 mol%. In practice, however, DNA base composition, in combination with other chemotaxonomic markers, has proved to be a valuable character in resolving taxonomic problems and in supporting results from phylogenetic studies.

Membrane Composition

Besides representing a selective barrier, the cytoplasmic membrane assumes many functions attributable to specialized organelles in eukaryotic cells. Prokaryotes show a greater variety in their membrane composition than eukaryotes do. The composition of the membrane is an important character for the identification of prokaryotes.

Polar Ester Lipids of Eubacteria Polar lipids are amphipathic molecules and can be extracted readily from bacterial cells with chloroform-methanol mixtures. Phospholipids are the most common polar lipids. They are derivatives of phosphatidic acid (phosphoglycerides) and, in most eubacteria, are quite uniform with regard to their overall pattern. Therefore, they are not of great value as chemotaxonomic markers. Glycolipids are not as widespread as phospholipids and are found predominantly, although not exclusively, in Gram-positive bacteria (Shaw, 1975). Diglycosyl diglyceride is usually the major glycolipid. Members of the same genus normally contain the same glycolipid, e.g., α-diglucosyl diglyceride is found in streptococci, whereas galactosylglucosyl diglyceride is present in lactobacilli. The presence of diacylinositol mannoside appears to be typical for members of the genus *Propionibacterium*. However, the same glycolipid may also occur in quite distantly related organisms. For example, *Acholeplasma laidlawii* contains the same diglucosyl diglyceride as found in some streptococci.

Fatty Acids Fatty acid composition of lipids can be helpful in the classification of eubacteria. The majority of all fatty acids are found in the membrane as acyl substitutes of glycerol, sugar, or amino compounds. Gram-negative eubacteria also contain fatty acids in their cell wall as constituents of lipopolysaccharides, phospholipids, and lipoprotein. The fatty acyl ester lipids of eukaryotic and prokaryotic membranes are replaced by ether lipids in archae-bacteria.

Most fatty acids of eubacteria are in the range of C_{12} to C_{20}. There are branched fatty acids, hydroxy fatty acids, cyclopropane fatty acids, and saturated and unsaturated ones. They may have even-numbered or odd-numbered carbon chains. Differences in the fatty acid composition can be used for the rapid identification of various eubacteria (Tornabene, 1985; Miller, 1982). Fatty acid patterns can be determined rather easily and quickly, and automatic identification is even possible (Eerola and Lehtonen, 1988). However, the bacteria have to be cultivated under carefully controlled conditions since fatty acid patterns may alter in response to exogenous and endogenous parameters, such as growth temperature, composition pH of medium, or age of the culture.

Hopanoids Prokaryotes do not normally synthesize sterols. There are only two genuine exceptions, namely *Methylococcus capsulatus* and *Nannocystis exedens* (Tornabene, 1985). However, in recent years, hopanoids and other polyterpenoids have been discovered in prokaryotes (Ourisson et al., 1987), which can be considered as sterol surrogates. Hopanoids have been found in various eubacteria. For instance, they are present in most cyanobacteria, methylotrophs, Rhodospirillaceae, acetic acid bacteria, and various other Gram-negative and Gram-positive eubacteria. They have not been found in archaebacteria, purple sulfur bacteria, or Enterobacteriaceae. Although archaebacteria do not contain hopanoids, they have phytanyl and bisphytanyl ethers that are also polyterpenoids.

Ether Lipids There are two different types of ether lipids: plasmalogens, which contain a vinyl ether bound to glycerol, and alkyl ethers, which represent the condensation of fatty alcohols to glycerol. Plasmalogens are found in anaerobic eubacteria. Membranes of archaebacteria contain alkyl glycerol ether lipids (Goldfine and Langworthy, 1988). The alkyl chains usually consist of phytanyl residues. Diethers containing two C_{20} phytanyl chains are characteristic membrane components of all archaebacteria. Tetraethers containing two C_{40} phytanyl chains are present in thermophilic and many of the methanogenic archaebacteria, but not in halobacteria.

Aliphatic Hydrocarbons The occurrence of aliphatic hydrocarbons can be used to separate micrococci from staphylococci. Micrococci contain long-chain (C_{22}–C_{32}), methyl-branched monoolefins which are also helpful in differentiating species of this genus. Such hydrocarbons can also be found in some coryneform bacteria and in *Pseudomonas maltophilia*, but not in any other pseudomonad (Tornabene, 1985).

Isoprenoid Quinones Isoprenoid quinones play an important role in electron transport and active transport. Different bacteria not only syn-

thesize different quinone types (ubiquinone, menaquinone, demethylmeraquinones), but in particular the length and the degree of saturation of polyprenyl side chains are of considerable value in classification (Collins and Jones, 1981). The cyanobacteria contain neither ubiquinones nor menaquinones but phylloquinones and plastoquinones, which are normally associated with green plants. Most strictly aerobic, Gram-negative eubacteria produce only ubiquinones, whereas facultatively anaerobic, Gram-negative eubacteria contain in addition menaquinones and/or demethylmenaquinones. Aerobic and facultatively anaerobic, Gram-positive eubacteria produce only menaquinones. Strictly anaerobic eubacteria lack isoprenoid quinones or contain only menaquinones. Menaquinones are also found in archaebacteria.

Bacteriochlorophylls The occurrence of different bacteriochlorophylls can be helpful in the classification of phototrophic bacteria (Oelze, 1985). The majority of the species of the Rhodospirillaceae, Ectothiorhodospiraceae, and Chromatiaceae contain bchl *a* only. However, some of them possess bchl *b* instead.

In Chlorobiaceae and Chloroflexaceae, bchl *c, d,* and *e* are the main pigments. They are always accompanied by small amounts of bchl *a.* Bchl *a* is also found in *Erythrobacter*, whereas *Heliobacterium chlorum* contains bchl *g.*

Cytochromes Bacterial cytochromes are involved in a wide variety of redox processes such as aerobic and anaerobic respiration and photosynthetic electron transfer. Most cytochromes are associated with the cytoplasmic membrane. However, there are some *c*-type cytochromes and various oxygenases located within either the cytoplasm or the periplasmic space. Cytochrome patterns of bacteria can be used as a valuable chemotaxonomic character since, unlike the mitochondrial respiratory chains of eukaryotes, bacterial respiratory chains contain a greater variation of different cytochromes (Jones and Poole, 1985). Conventional difference spectrophotometry, preferably at low temperatures, is sufficient to determine the cytochrome pattern (Faller et al., 1980). It has been reported that the cytochrome pattern $bcaa_3$ and *o* occurs predominantly in Gram-positive bacteria and the pattern *bcdo* and a_1 is often found in Gram-negative bacteria (Jones and Poole, 1985). However, other studies have revealed that cytochrome *d* is also present in some Gram-positive bacteria, in particular in micrococci and coryneform bacteria (Faller et al., 1980; Faller and Schleifer, 1981). Cytochrome *c* is often absent in both Gram-negative and Gram-positive bacteria, and enterobacteria can be easily separated from pseudomonads since the former do not contain cytochrome *c* and are therefore oxidase negative.

When cytochrome patterns are used for taxonomic studies, it has to be taken into account that growth conditions (nutrition, growth phase) can influence quantitatively and, to a lesser extent, qualitatively the cytochrome content of a bacterial culture (Faller et al., 1980; Faller and Schleifer, 1981). To assess the cytochrome-synthesizing capacity of a strain, cells from both the exponential and the stationary growth phase (as a minimum) have to be examined.

Cell Wall Ultrastructure and Composition

Most prokaryotic cells are surrounded by a cell wall that maintains their shape and preserves the plasma membrane against osmotic disruption. These cell walls consist of several classes of unique heteropolymers whose existence helps not only to distinguish prokaryotic from eukaryotic organisms but also to separate the eubacteria into distinct groups, such as the Gram-positive and the Gram-negative bacteria. The phylogenetic difference between Gram-positive and Gram-negative eubacteria is well established (Woese, 1987). The Gram-positive eubacteria are distinguished from the Gram-negative ones by their ability to retain iodine-fixed crystal violet in the presence of alcohol. However, the Gram reaction is quantitative rather than qualitative, and some eubacteria are Gram-variable. A positive Gram reaction may depend on the length of alcohol treatment and/or on the growth phase. A clear separation of Gram-positive and Gram-negative bacteria can be obtained by the differences in the ultra-structure and chemical composition of their cell walls.

In the electron microscope, the cell wall profiles of these eubacteria are quite distinct (Beveridge, 1981). The cell wall of Gram-positive eubacteria reveals in profile one thick (30–80 nm) and more or less homogeneous layer, whereas Gram-negative eubacteria have a thinner, distinctly layered cell wall with an outer membrane resembling the typical trilaminar cytoplasmic membrane in profile. The polymers found in the cell walls of these two groups of bacteria are chemically quite different. The walls of Gram-negative cells are mainly composed of lipopolysaccharide, phospholipid, protein, lipoprotein, and relatively little peptidoglycan (usually less than 10% of the total cell wall). The Gram-positive cells contain as major components peptidoglycan (usually more than 30% of the total cell wall), polysaccharides or teichoic acid (or both), or teichuronic acid. Thus, in contrast to the Gram-negative, the Gram-positive eubacteria contain hardly any lipids in their cell

walls. There is, however, one exception: acid-fast bacteria. They are resistant to decolorization with acidic ethanol after staining with fuchsin (Ziehl-Nielsen staining). These acid-fast bacteria (*Mycobacterium*, *Norcardia*, and *Corynebacterium sensu stricto*) are Gram-positive eubacteria which contain large amounts of lipids in their cell walls—in particular, mycolic acids (Minnikin and Goodfellow, 1980). The results of a study by Goren et al. (1978) indicate that the lipid barrier of the cell wall mycolyl-arabinogalactan hinders the penetration of the bleaching acid.

Peptidoglycan (murein, mucopeptide) is the only cell wall polymer found in both Gram-positive and Gram-negative eubacteria. It is also present in cell walls of cyanobacteria (Weckesser et al., 1979). The fine structure and the chemical composition of the cell walls of cyanobacteria are similar to those of Gram-negative eubacteria. However, in cyanobacteria, the peptidoglycan layer is thicker and its degree of cross-linkage is higher than in Gram-negative eubacteria (Woitzik et al., 1988). Moreover, polysaccharides can be covalently linked to the peptidoglycan of unicellular cyanobacteria. There are only a few prokaryotes, such as the mycoplasma group (Mollicutes) and the Archaebacteria, which lack peptidoglycan.

Enterobacterial Common Antigen Enterobacterial common antigen (ECA) is a surface antigen typical for all members of the family Enterobacteriaceae (Kuhn et al., 1988). It is located in the outer membrane and can easily be detected in nonencapsulated strains by passive hemagglutination or colony immunoblotting (Meier-Dieter et al., 1989). It is composed of a linear polysaccharide chain containing three different amino sugars. The sugar chain can be linked to a diglyceride through phosphodiester linkage or, in a few rough mutants, it can also be bound to the lipopolysaccharide. Unlike other common antigens, e.g., the outer membrane proteins, it is restricted to the Enterobacteriaceae and shows strong cross-reaction between all species studied so far. Common antigens may also exist for Bacteroidaceae (Marx et al., 1982) and *Pseudomonas aeruginosa* (Sawada et al., 1985).

Lipopolysaccharides Lipopolysaccharides are known to contain the endotoxic principle of Gram-negative eubacteria and are the chemical basis for the O antigens, also called surface antigens (Wilkinson, 1977). The side chain carries the determinants of O-antigenic specificity and is the most important one of the three cell-surface antigens for the serological classification of Enterobacteriaceae at an infrasubspecific level. The other two antigens are H (bacterial flagellum) and K (capsule). The O-specific side chains consist of up to 30 repeating units, each containing three to six sugar residues. The variety, substitution, and linkage of these sugars are responsible for the antigenic diversity (Jann and Westphal, 1975). Besides common hexoses, also 6-deoxyhexoses, 3,6-dideoxyhexoses, and *O*-methyl sugars are found as constituents of the cell surface antigens. *O*-methylated sugars are characteristic for lipopolysaccharides from purple bacteria and cyanobacteria (Weckesser et al., 1979).

The core region of the lipopolysaccharide is composed of a short, acidic heterooligosaccharide that in most Enterobacteriaceae consists of phosphorylated 2-keto-3-deoxyoctonate (KDO), phosphorylated L-glycero-D-mannoheptose, D-glucose, D-galactose, and *N*-acetylglucosamine. The composition of the core structure is much more conserved within the Gram-negative bacteria than the O-specific chains (Wilkinson, 1977). It is essentially the same in all S-form lipopolysaccharides from *Salmonella*. In general, KDO and aldoheptose are the most distinctive components of bacterial lipopolysaccharides. Although KDO is a generally reliable marker for most lipopolysaccharides, the absence of such a sugar in some of them and the detection of a KDO-polymer in *Escherichia coli* (Taylor, 1974) indicate some limitations to its use as a diagnostic tool. Moreover, the determination of small amounts of KDO with the usual thiobarbituric acid method is rather difficult (Hofstad, 1974).

Lipid A carries the endotoxic activity of lipopolysaccharide and is the most conservative structural part (Lüderitz et al., 1978). The backbone of lipid A consists of a β-1,6-linked D-glucosamine disaccharide which carries phosphate residues in positions 1 and 4. Long-chain fatty acids, which make up the bulk of lipid A fractions, are linked to both the amino and hydroxyl groups of glucosamine. The *N*-acyl residue is characteristically a 3-hydroxyalkanoic acid, e.g., β-hydroxymyristic acid in enterobacterial lipopolysaccharides. However, some Gram-negative bacteria contain a form of lipid A which exhibits a different backbone composition. Thus, in the lipid A of *Pseudomonas diminuta*, *Rhodopseudomonas viridis*, and *R. palustris*, glucosamine is replaced by 2,3-diamino-2,3-dideoxy-D-glucose. Phosphate is absent in the lipid A of some bacteria and cyanobacteria (Weckesser et al., 1979). Some types of lipid A contain, in addition to glucosamine, other saccharide components.

Chemotyping (qualitative composition of the polysaccharide moiety) and serotyping of the O-specific chains should be, as in the case of Enterobacteriaceae, suitable for further differentiation within a species (infrasubspecific taxon). However, the complete serological cross-

reaction of O antigens of certain strains of *Salmonella* and *E. coli* suggests a need for greater diagnostic caution. The structure of the polysaccharide core can be used in some cases to separate at the genus level (*Salmonella*, *Shigella*). On the other hand, cores of different structure are found within the genus *Escherichia* (Nikaido, 1970). A limited number of studies with Rhodospirillaceae indicate that lipid A may be a valuable chemotaxonomic marker (Weckesser et al., 1979).

Cell Wall Polysaccharides of Gram-Positive Eubacteria In addition to peptidoglycan, Gram-positive bacteria contain polysaccharides and/or teichoic acids in their cell walls.

Polysaccharides In contrast to the thoroughly studied lipopolysaccharides, little is known about the cell wall polysaccharides of Gram-positive eubacteria. The only cell wall polysaccharides which have been intensively studied are those of streptococci. They form the basis for the serological grouping of streptococci. Supraspecific, specific, and infrasubspecific taxa can be distinguished by the serological specificity of these cell wall polysaccharides (Schleifer and Kilpper-Bälz, 1987).

Other well-characterized polysaccharides are arabinogalactan and arabinomannan, which are linked to mycolic acids and found in acid fast and related bacteria (Lederer et al., 1975). The presence of arabinose, galactose, and *meso*-diaminopimelic acid in a cell wall hydrolysate is characteristic for the *Corynebacterium-Mycobacterium-Nocardia* group of bacteria (Keddie and Bousfield, 1980).

Teichoic Acids Cell wall teichoic acids are covalently linked to peptidoglycan. They usually consist of polyols (glycerol, ribitol, mannitol) which in most cases are connected through phosphodiester bridges and substituted by sugars, amino sugars, or D-alanine residues (Archibald, 1974; Anderton and Wilkinson, 1985). There are also teichoic acids lacking polyols. In rare cases, *N*-acetylamino sugar residues are linked through phosphodiester bridges and form an integral part of the polymer chain (Endl et al., 1983). Cell wall teichoic acids are found only in a limited number of Gram-positive eubacteria, such as various actinomycetes, bacilli, brevibacteria, certain lactobacilli, listeria, and staphylococci.

The chemical composition, structure, and serological specificity of this cell wall polymer have proven to be of value in the classification of Gram-positive eubacteria. A well-known example consists of the wall teichoic acids of staphylococci (Endl et al., 1983, 1984). The presence of teichoic acid is a characteristic marker in the separation of staphylococci from micrococci which lack this polymer. Cell wall teichoic acids are also a valuable tool in the classification of *Brevibacterium linens* and related strains (Fiedler et al., 1981) of certain lactobacilli (Sharpe, 1970; Knox and Wicken, 1973) and actinomycetes (Naumova, 1988).

Mycolic Acids Mycolic acids are high-molecular-weight, 3-hydroxy acids with a long alkyl branch in position 2. They are restricted to members of the genera *Caseobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus*. Differences in their structure have proved to be a valuable chemotaxonomic marker in the classification and identification of these taxa (Minnikin and Goodfellow, 1980; Dobson et al., 1985).

Peptidoglycan Peptidoglycan (sometimes called murein) is a heteropolymer consisting of glycan strands that are cross-linked through short peptides. The glycan strand is made up of alternating β-1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, a derivative of glucosamine and unique constituent of peptidoglycan. The peptide moiety is linked to *N*-acetylmuramic acid and contains both L- and D-amino acids. The peptidoglycan of Gram-negative eubacteria is remarkably uniform (Schleifer and Kandler, 1972) and only a few exceptions are known (Schleifer and Stackebrandt, 1983).

Gram-positive eubacteria contain a multilayered peptidoglycan and reveal, in contrast to Gram-negative organisms, a great variation in the composition and structural arrangement of their peptidoglycans. Minor variations can be found in the glycan strand. In mycobacteria and nocardia, the *N*-acetyl group of muramic acid is oxidized to *N*-glycolyl (Lederer et al., 1975). A relatively simple procedure for detecting the presence of *N*-glycolylation of peptidoglycans has been described by Uchida and Aida (1977).

The greatest variation occurs within the peptide moiety of the peptidoglycan. Both the peptide subunit (stem peptide) and the interpeptide bridges, which cross-link the peptide subunits, can vary in their structure and composition. Based on the mode of cross-linkage, two main groups of cross-linkage, A and B, can be distinguished (Schleifer and Kandler, 1972).

A given peptidoglycan structure is a fairly stable character and fulfils the most important prerequisites of a useful taxonomic marker (Schleifer and Kandler, 1972). No single-step mutations are known so far which lead to an altered peptidoglycan structure. Phenotypic variations are also rather limited and can be easily controlled (Schleifer et al., 1976).

The determination of the primary structure of the peptidoglycan (peptidoglycan type) provides the full content of taxonomically useful information and has revealed differences between organisms which could not be detected by simple qualitative cell wall analyses (Keddie and Bousfield, 1980). However, in many cases where the peptidoglycan structure is already known, simple cell wall analysis provides a powerful tool for the primary stage of identification. In some groups of Gram-positive bacteria, such as the actinomycetes and the coryneform bacteria, the determination of the cell wall composition is usually a prerequisite for a reliable classification and identification (Keddie and Bousfield, 1980).

Cell Envelope Polymers in Archaebacteria With the exception of mycoplasma, *Planctomyces*, and *Isophaera*, the absence of peptidoglycan is a characteristic feature that distinguishes archaebacteria from eubacteria. Archaebacteria exhibit at least five different morphological types of cell envelopes. Type I shows a more or less homogeneous electron-dense layer, 10 to 200 nm in width, and consists mainly of pseudomurein (Methanobacteriales), a nonsulfated (*Methanosarcina*), or a sulfated acidic heteropolysaccharide (*Halococcus*). Most of the Gram-positive archaebacteria show this type of cell envelope (Kandler and König, 1985). Type 2 is found only in the Gram-positive *Methanothermus fervidus*. The rigid cell wall is composed of pseudomurein covered with a surface layer of protein (Stetter et al., 1981). Type 3 is widely distributed among Gram-negative archaebacteria, such as all thermoacidophiles and many methanogens and halophiles. In contrast to Gram-negative eubacteria, neither a specific sacculus nor an outer membrane is present. The cell envelope consists of only one surface layer (the S layer) composed of protein or glycoprotein subunits (Sleytr and Messner, 1983). Type 4 is a rather complex cell envelope and is found only in *Methanospirillum*. The individual cell is surrounded by an electron-dense layer (10 nm in width) probably of proteinaceous nature, and several cells are held together by a sheath consisting of protein fibrils (Sprott and McKeller, 1980). Type 5 lacks any kind of cell envelope and is found in *Thermoplasma* (Darland et al., 1970) and *Methanoplasma* (Rose and Pirt, 1981). The differences in the cell envelope of archaebacteria can be used as a chemotaxonomic tool for their identification and classification (Kandler and König, 1985).

Other Cellular Constituents

Electrophoretic Protein Patterns Whole-cell protein patterns, reviewed by Jackmann (1987) and Kersters (1985), can be used for classification and identification. The method requires considerable standardization and pattern correction in order to obtain optimum results (Albritton et al., 1988). It shows similar discrimination to DNA-DNA hybridization. More distant relationships can be measured by analysis of ribosomal proteins (Böck, 1985). In the latter example, a two-dimensional separation was applied. It is superior to one-dimensional separation since there are less superposition effects, and fewer corrections are necessary.

Electrophoretic Enzyme Patterns Multilocus enzyme electrophoresis has long been a standard method in systematics and population genetics of eukaryotes and has also been applied to prokaryotes (Selander et al., 1986). Water-soluble cellular enzymes are separated by electrophoresis and detected by enzyme-specific staining methods. Differences in the amino acid sequence can yield mobility variants of an enzyme (electromorphs) that can be directly equated with alleles at the corresponding structural gene locus. The disadvantage of this method is that numerous gels are required in order to score sufficient different enzymes in a polymorphic population.

Polyamines Polyamines function as polycations under physiological conditions and are widely distributed among prokaryotes. They stabilize nucleic acids and membranes and influence many enzymatic reactions. They represent a valuable chemotaxonomic marker in methanogenic bacteria and the Proteobacteria. A distinct polyamine pattern could be found for each of the four families of methanogenic bacteria (Scherer and Kneifel, 1983). The unusual polyamine 2-hydroxyputresceine occurs as a specific component within members of the beta subclass of the Proteobacteria, whereas in the alpha subclass, a triamine, such as spermidine or symhomospermidine, was found as the characteristic component (Busse and Auling, 1988).

Nucleic Acid Probes

Nucleic acid probes can be used for the rapid detection and identification of bacteria. They consist of fragments of single-stranded nucleic acids (mostly DNA) that bind to complementary target nucleic acids, either DNA or RNA. The lengths of the fragments range from 15 to 10,000 nucleotides. The longer ones are isolated from the nucleic acid of the target organism, the shorter ones are synthesized in the laboratory.

The nucleic acid probe technique can be divided into four steps: 1) An appropriate probe is designed and labelled with a detector group. 2) The target nucleic acid is extracted from the organism or from a specimen containing this organism. 3) The target nucleic acid is hybridized with the specific probe. 4) The amount of hybrid formed is determined. The different steps are discussed below.

Isolation or Synthesis of Specific Nucleic Acid Probes

Probes can be designed by several different approaches. The simplest way is the application of whole cell DNA. Bacterial cells are blotted onto membrane filters or microdilution plates, lysed, and hybridized with labelled genomic DNA of reference strains (Morotomi et al., 1988; Ezaki et al., 1989). The method is simple, quick, and useful for a large number of strains, but it lacks specificity and closely related species cannot be distinguished. The latter may be achieved by isolating and cloning species-specific DNA fragments (Schmidhuber et al., 1988). Unfortunately, the genetic stability of such random DNA fragments is not guaranteed. To distinguish virulent strains from avirulent ones or to detect strains or species with distinct metabolic activities, probes should be used which are complementary to genes coding for these properties (Moseley et al., 1982; Liebl et al., 1987).

Probes reacting with 16S or 23S rRNA or their genes are of special interest. These rRNA molecules are ubiquitous and contain regions of various degree of conservation (Woese, 1987; Höpfl et al., 1989). Therefore, information on the nucleotide sequence of these molecules can be used to design probes of various specificity ranging from subspecies to kingdom level. It is even possible to design a probe that will react with any cellular life form. These probes are ideal for distinguishing between phylogenetically related and unrelated groups of organisms. The use of probes with different specificity allows rapid identification of an isolate (Regensburger et al., 1988). A one-step identification may be possible by the use of "reversed hybridization," i.e., labelled target nucleic acid is hybridized to a panel of unlabelled immobilized DNA probes (Dattagupta et al., 1989).

Extraction of Target Nucleic Acid

Target nucleic acids have to be isolated from organisms or specimens before hybridization to nucleic acid probes can be performed. Release of nucleic acids can readily be achieved by treatment with detergent, alkali, lysozyme, or a combination of these reagents. The lysis of Gram-positive bacteria is more difficult. Other chemical methods, e.g., treatment with hot alkali or phenol, or even physical methods, e.g., sonication or shaking with glass beads in a ball mill, must be used to achieve sufficient lysis of cells. For colony hybridization of Gram-negative cells, the colonies are fixed to nylon or nitrocellulose membranes and lysed by simple alkali treatment (Sambrook et al., 1989).

Hybridization of Target Nucleic Acid with Nucleic Acid Probe

Specific hybridization consists of two steps: first, the binding (hybridization) of the probe to the target nucleic acid and second, the separation of specific hybrids from nonspecifically bound or unbound probe. Hybridization can be performed on solid supports or in solution. For solid-phase hybridization, denatured target nucleic acid is immobilized on nitrocellulose or nylon membranes. Unbound or nonspecifically bound probe can be readily removed from the membrane-bound hybrid by washing. The rate of hybridization in solution is considerably higher than for solid-phase hybridization, and the target nucleic acid is completely accessible for the probe. The only disadvantage of the hybridization in solution is the more complicated separation of the hybrids from the unbound probe.

A special kind of solid phase hybridization is in situ hybridization. One method uses in situ lysis of bacterial cells grown on or transferred to membrane filters (colony hybridization). It is a convenient and rapid method to detect and identify distinct groups of bacteria in mixed microbial populations (Festl et al., 1986). Another method involves single-cell hybridization with radioactively or fluorescently labelled oligonucleotide probes directed against 16S rRNA. Fixed whole cells can be made permeable to oligonucleotide probes, thus allowing a phylogenetically based identification (Giovannoni et al., 1988; DeLong et al., 1989). This technique can also be applied for a species-specific detection of bacteria in natural samples (Amann et al., 1990).

Detection of Hybrids

The nucleic acid probe has to be labelled prior to hybridization in order to detect the formed hybrids. The label can be covalently bound to the probe (direct labelling), or an unlabelled detector group can be covalently linked to the probe and detected by a labelled binding protein (indirect labelling). Various techniques used for labelling probes and detecting hybrids have been reviewed by Matthews and Kricka (1988).

Applications for Nucleic Acid Probes

Nucleic acid probes have broad applications. They can be used not only for the detection and identification of bacteria but also for the diagnosis of infectious disease, the detection of pathogenic and spoilage bacteria in food products, and for studying microbial ecosystems. Nucleic acid probes are highly specific, and their genomic targets are not affected by changing environmental or physiological conditions.

The technique is faster than conventional identification methods and can be applied in the identification of organisms grown in pure or mixed cultures. Nucleic acid probes are especially useful for the identification of slow-growing and fastidious organisms. They can also be used for the direct detection of bacteria in their natural habitat. Even bacteria that have not yet been cultivated can be detected, either through the use of reverse transcriptase sequencing of extracted rRNA (Weller and Ward, 1989) or by applying the polymerase chain reaction (Saiki et al., 1988) with oligonucleotide primers that are complementary to conserved regions of 16 or 23S rRNAs.

A major disadvantage of the nucleic acid probe technique has been its lack of sensitivity. However, this problem can now be solved since there are several ways to increase sensitivity, e.g., amplification of target nucleic acids or signals, using high copy-number target sequences or improved detector groups. Amplification of target nucleic acids with polymerase chain reaction has considerably improved the sensitivity. This technique was used to detect as few as 35 colony-forming units of L. pneumophilia in a water sample (Starnbach et al., 1989).

Physiology and Metabolism

Basic Energy Metabolism

Physiological "properties" of bacteria are expressions of metabolic pathways present in the respective species. As far as types of overall energy metabolism are concerned, the relation to oxygen and light of a given bacterium will decide whether it performs photosynthesis, fermentation, or respiration as the main energy-yielding process.

The ability to utilize light as the sole source of energy clearly separates the phototrophic bacteria (including the cyanobacteria) from all other prokaryotes. Of course, this ability requires the presence of the numerous properties that constitute a functioning photosynthetic apparatus (see The Phototrophic Way of Life in Volume 2). Strict anaerobiosis (see The Anaerobic Way of Life in Volume 2) is also linked to a unique set of properties. In addition, a large number of bacteria have the ability to grow in the absence of oxygen as well as in its presence. These facultatively anaerobic bacteria possess more than one energy-yielding system.

Nutritional and Metabolic Characters

Metabolic properties of a given bacterium are detected and determined by studying the utilization of nutrients, the production of metabolites, or the presence of typical enzymes. In diagnostic bacteriology, especially in the medical and food sectors, such specific biochemical tests are routinely performed for the identification of bacteria.

The fermentation of glucose, lactose, mannitol, or other sugars may be easily detected by the production of gas or by change in color of an indicator dye caused by the production of acids. The qualitative and quantitative analysis of acid end products is an important aid in the identification of bacteria, especially of anaerobic bacteria of the genera *Clostridium*, *Bacteroides*, *Peptococcus*, and *Peptostreptococcus*. Volatile fatty acids may be estimated in the culture suspension, in an ether extract of the culture, or in the bacterial cell directly or after derivative formation, by gas-liquid chromatography or by other techniques, such as isotachophoresis.

In addition to the acid end products, the presence in the medium of other fermentation breakdown products, such as indole and hydrogen sulfide, is characteristic for certain bacteria. Thus, the ability of an organism to produce indole by the enzymatic cleavage of the amino acid tryptophan has long been used as part of the IMViC reactions used to separate *Escherichia coli* from *Enterobacter aerogenes*.

In most treatments on identification in this book, the pattern of utilization of carbon sources plays an important role in the differentiation of species. The ability of certain microorganisms to utilize organic acids such as acetate, citrate, and malonate as sole source of carbon for metabolism has been useful, especially in the differentiation of Gram-negative rods. The alkaline reaction products present in the medium of microorganisms that utilize the carbon of these substances change the color of the pH indicator.

In thiobacilli, the utilization and formation of sulfur compounds serves in identification (see The Colorless Sulfur Bacteria in this Volume). In lactic acid bacteria, the formation of L- versus D-lactic acid is of diagnostic value.

Special Nutritional Requirements

Many bacteria of medical importance do not grow on media generally used for screening and

identification, because they need accessory growth factors. Some of these bacteria, such as members of the rickettsiae and chlamydiae, are highly fastidious in their requirements and grow only inside living cells (obligate intracellular parasites). The determination of nutritional requirements can provide diagnostic characters for some bacteria. Thus, certain species of *Haemophilus*—minute, Gram-negative, rod-shaped bacteria—are characterized by their requirement for NAD (V factor) and for the heme portion of hemoglobin (X factor) for growth on laboratory media, whereas other species of the genus require only one of these factors. These growth requirements are also responsible for a phenomenon called satellitism, which can be a help in the preliminary identification of *Haemophilus* in clinical specimens. In satellitism, members of the genus such as *Haemophilus influenzae*, which are dependent on both factors, develop significant colonies on blood-agar plates only near colonies of *Staphylococcus aureus*, which produce the required substances.

Enzymes

Enzymes used for diagnostic purpose include catalase, coagulase, β-galactosidase, urease, gelatinase, lysine decarboxylase, lecithinase, and phosphatase. As an example, the detection of lysine or ornithine decarboxylase activity is useful in the identification of organisms within the Enterobacteriaceae. The enzyme removes the carboxyl group from the amino acid, thus producing the corresponding amine with liberation of carbon dioxide and a change in color of a pH indicator in the medium.

Some bacteria produce hemolysin, i.e., enzymes that destroy red blood cells. The different types of hemolysin action observed on blood agar plates are an aid in the identification of certain species. For example, some strains produce a greenish discoloration of the medium around the colonies accompanied by the partial lysis of the red blood cells (alpha hemolysis), other strains form a clear zone by the complete lysis of the cells (beta hemolysis), and still others are found to cause no lysis at all.

Based exclusively or predominantly on physiological properties, rapid and widely automated identification procedures have been developed and are being used in clinical and food microbiology (e.g., Gyllenberg, 1976; Goodfellow et al., 1987; d'Amato et al., 1985; Bascomb, 1985).

Ecological Parameters

Since all living beings have one or more ecological niches, they have a set of optimal ecological parameters that can be used as part of their description. The optimal growth temperature and the temperature tolerance of a prokaryote culture are both of great importance for characterization and identification. Psychrophilic, thermophilic, and extremely thermophilic species are easily classified by these parameters. Pathogenic as well as rumen and intestinal bacteria usually have an optimal growth temperature near the body temperature of their hosts.

In a similar way, the optimal pH as well as the tolerated pH range are very important properties for devising media for the enrichment of bacterial strains.

Further ecological parameters, such as redox conditions, salinity (osmolarity), light intensity, and hydrostatic pressure, are of equal importance. There are extremely tolerant and highly adapted bacteria for each of these parameters and often for more than one. Often these forms are not just adapted to but have come to require extreme conditions, e.g., the halobacteria and the thermo-acidophilic bacteria.

Whereas high hydrostatic pressure may not affect autochthonous deep-sea bacteria, it slows down the metabolic rates of bacteria that inhabit surface or terrestrial habitats (Jannasch and Wirsen, 1973).

The relationship to other living beings has led to the evolution of bacterial properties that may be useful for identification. Within the nitrogen-fixing, symbiotic genus *Rhizobium*, the species are differentiated by host specificity. Many other bacteria pathogenic for plants, animals, and humans are host specific. Further details may be found in Bacterial Virulence in this Volume and Plant Pathogenic Bacteria in this Volume.

Susceptibility to Bacteriophages, Bacteriocins, and Other Antimicrobial Agents

The susceptibility of bacteria to bacteriophages depends on the presence of specific receptor structures in the surface of the cell to which the phages can adsorb. On the basis of their interaction with known phages, strains of bacteria can be classified into groups, or phagovars, and, to a lesser extent, the genus or the species can be determined. Only a few phages with specific action are used for diagnostic purposes. Thus, strains of *Vibrio cholerae* can be differentiated into classical and El Tor phagovars by phage susceptibility.

Furthermore, certain phages lyse all strains of *Yersinia pseudotuberculosis*, but not strains of *Y. pestis* and *Y. enterocolitica*.

Since a large proportion of strains of certain bacterial species are lysogenic, they may be characterized by indirect phage typing (lysogenotyp-

ing), i.e., the detection and identification of temperate phages present as prophages in bacteria. However, the method used most is the differentiation of the bacterial strains on the basis of their susceptibility to a standard set of known phages (lysotyping).

At present, phage typing is used by specialized diagnostic laboratories especially for the identification of *Salmonella typhi*, *S. paratyphi*, *S. typhimurium*, and *Staphylococcus aureus*.

Another method for differentiating strains of the same species is bacteriocin typing. Bacteriocins are high-molecular-weight bactericidal proteins which are produced by various species of bacteria and are active only against other strains of the same species or closely related species. Because different strains of a species usually produce bacteriocins with different activity patterns, strains can be subdivided by the characterization of their bacteriocin. Besides bacteriocin production, strains can be characterized by the determination of their sensitivity pattern against a standard set of known bacteriocin-producing strains, bacteriocin-containing lysates or extracts, and purified bacteriocins.

Bacteriocin typing has become a useful method for comparing strains of medical isolates such as *Shigella sonnei*, *Serratia marcescens*, other Enterobactericeae, and *Pseudomonas aeruginosa*.

In addition to phage typing and bacteriocin typing, determination of chemical and serological properties, typing by R factors, electrophoresis patterns of cell proteins, and differences in nutritional requirements for amino acids and other selected substances (auxotyping) are used either separately or together to obtain a more detailed level of differentiation within bacterial species (Bergan and Norris, 1978; Kwapinski, 1977).

Resistance and susceptibility to inhibitors such as fuchsin, bismuth sulfite, desoxycholate, tellurite, bile salts, optochine (a derivate of quinine), and higher concentrations of sodium chloride are also generally stable characters and can serve as diagnostic aids. For example, most strains of pneumococcus (*Streptococcus pneumoniae*) are characteristically susceptible to optochine, whereas most strains of related alpha-hemolytic "viridans" streptococci are resistant. Furthermore, the patterns of susceptibility to antibiotics can be useful in distinguishing between similar species as well as between strains of the same species.

Identification Keys and Diagnostic Tables

The types of characters to be tested during the identification of prokaryotes and eukaryotes are often presented in the form of dichotomous keys and diagnostic tables.

A taxonomic key consists of a steplike series of questions. If these are all of the "yes-no" kind, the key is called a dichotomous one. Dichotomous keys are ideally suited for computerization. There are few successful applications of keys to microbiology, however, as Cowan (1968) pointed out, mainly because the constructor soon runs out of known contrasts.

The first successful modern key for the identification to generic level of bacteria was that developed by Skerman (1967). Skerman also constructed the key to the genera contained in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974).

Keys within clearly defined groups of bacteria are much more common than general bacteriological keys. Such keys are found in *Bergey's Manual of Systematic Bacteriology*, as well as in many chapters of this handbook.

The characters used for the construction of keys must be constant characters; i.e., they must always be present in or absent from a taxon. Such properties become "key" characters. Positive "key" characters are also called "diagnostic" or "marker" characters. Since keys are usually constructed for diagnostic purposes, the characters used are weighted ones, and they form a hierarchy with the most important characters usually being listed first.

Diagnostic tables are tables of characters for the identification of taxa that are based upon the sharing of several (usually unweighted) characters. Such tables contain more information than dichotomous keys and therefore appear to be more complicated. But, in many cases, identification is easier using diagnostic tables. When several characters are variable, the diagnostic table is much more successful as a determinative aid than a key in which (if all characters of the table are included) there are too many routes leading to the same identification (Cowan, 1968).

Minimal Requirements for a Description of a New Species

Sneath (1977) has pointed out that a species can be visualized as a cluster of a very large number of strains in a character space. As an approximation, this space may be treated as roughly spherical, and it can therefore be described using sampling theory, by its center, while its radius defines an envelope that includes about 100% of the strains. When a reference description of a species is set up based on a small number of strains, one is in effect making an estimate of the true position of the center and of the true

Table 2. Minimum data required for the description of a new species.

	Required data	Desired/required data, if applicable
Cell morphology	Cell shape[a]	Color
	Cell size (diameter, length)	Flagellation type[a]
	Motility	
	Visible internal or external structures[a]	Spores[a], appendages[a], capsules[a], sheaths[a]
	Formation of typical cellular aggregate[a]	
	Occurrence of cell differentiation[a]	Life cycle[a], heterocysts[a], hormogonia[a]
	Ultrastructure (general)[b]	Ultrastructure of flagella, envelope, cell wall[b]
Colonial morphology	Appearance of cell suspensions	Color of suspension (absorption spectra)
	Appearance of colonies	Color of colonies
		Motility of colonies
		Formation of fruiting bodies[a]
		Formation of mycelia[a]
Staining behavior	Gram stain	Acid-fast stain, spore stain, flagellum stain
Cell constituent	DNA base ratio	Nucleic acid homology; rRNA sequences
	Reserve materials	Cellular pigments
		Cell wall and membrane constituents
		Typical enzymes
Physiology	Temperature range and optimum	Salinity or osmolarity requirements
	pH range and optimum	Vitamin requirements
	Modes of energy metabolism (phototrophy, chemotrophy, lithotrophy, organotrophy)	Typical metabolic products formed (acids, osmolytes, pigments, antibiotics, toxins, antigens)
	Relation to oxygen	
	List of electron acceptors	Tolerances and susceptibilities
	List of carbon sources	
	List of nitrogen sources	
	List of sulfur sources	
Ecology	Natural habitat(s)	Pathogenicity, host range
		Antigen formation
		Serology
		Phage susceptibility
		Symbiosis

[a]To be demonstrated by light microscopy.
[b]To be demonstrated by electron microscopy.

radius. A sample of only one strain cannot tell anything about the radius of the cluster and is only a poor guide for defining the position of the cluster's center. Sneath postulated that 25 strains are needed for a rather accurate definition of the center and the radius of a cluster and suggested that the minimum number is about 10 strains.

If this number were to have been internationally agreed upon for descriptions of new species (taxa), many if not most of the new prokaryote species described since the times of M. W. Beijerinck and S. N. Winogradsky could not have been described as such. In the majority of cases, new species and genera have been and still are described on the basis of only a few strains, or even only one.

Usually, a newly isolated strain is not isolated accidentally or at random. It has either been recognized microscopically by its morphology and then been sought by use of various enrichment and isolation procedures, or a special selective nutrient medium or enrichment procedure is designed first, and the predominant organism is then isolated.

If a prokaryote strain is considered as a new species, authors should at first explain why they consider this as a new species; i.e., they should carry out a thorough comparison between the "new" and the known species (and genera) of the bacterial group with which the new organism shows most similarities. If, as is usually the case, the new organism can be allotted to one of the major groups of prokaryotes, the description of a new species (and/or genus) should contain at least the information presented in Table 2. The requirements demanded and recommended by the International Code of Nomenclature of Bacteria when a new species is described were discussed in "Nomenclature."

Literature Cited

Adanson, M. 1763. Familles des plantes. Vincent, Paris.

Albritton, W. L., X. P. Chen, V. Khanna. 1988. Comparison of whole-cell electrophoretic profiles of *Haemophilus influenzae*: implementation of a microcomputer mainframe linked system and description of a new similarity coefficient. Can. J. Microbiol. 34:1129–1134.

Allen, R. D., P. Baumann. 1971. Structure and arrangement of flagella in species of the genus *Beneckea* and *Photobacterium fischeri*. Journal of Bacteriology 107:295–302.

Amann, R. I., L. Krumholz, D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bact. 172:762–770.

Anderton, W. J., S. G. Wilkinson. 1985. Structural studies of mannitol teichoic acid from the cell wall of bacterium NCTC 9742. Biochem. J. 226:587–599.

Archibald, A. R. 1974. The structure, biosynthesis and function of teichoic acid. Adv. Microb. Physiol. 11:53–95.

Austin, B., R. R. Colwell. 1977. Evaluation of some coefficients for use in numerical taxonomy of microorganisms. International Journal of Systematic Bacteriology 27: 204–210.

Barksdale, L., K. S. Kim. 1977. *Mycobacterium*. Bacteriological Reviews 41:217–372.

Bascomb, S. 1985. Growth-independent rapid automated enzyme identification system. 367–376. K. O. Habermehl (ed.) Rapid methods and automation in microbiology and immunology. New York, Springer Verlag.

Beers, R. J., W. R. Lockhard. 1962. Experimental methods in computer taxonomy. Journal of General Microbiology 28:633–640.

Bergan, T. Norris., I. R. (ed.). 1978. Methods of microbiology, vols. 10 and 11. London, Academic Press.

Beveridge, T. J. 1981. Ultrastructure, chemistry and function of bacterial wall. Int. Rev. Cytol. 72:229–317.

Böck, A. 1985. Analysis of ribosomal proteins by two-dimensional gel elctrophoresis. 109–122. G. Gottschalk (ed.) Methods in microbiology, vol. 18. Academic Press. London.

Buchanan, R. E., N. E. Gibbons (ed.). 1974. Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins. Baltimore.

Buchanan, R. E., J. G. Holt, E. F. Lessel, Jr. (ed.). 1966. Index Bergeyana. Williams & Wilkins. Baltimore.

Busse, J., G. Auling. 1988. Polyamine pattern as a chemotaxonomic marker within the Proteobacteria. Syst. Appl. Microbiol. 11:1–8.

Cohn, F. 1872. Untersuchungen über Bacterien. Beiträge zur Biologie der Pflanzen 1(2):127–224.

Cohn, F. 1875. Untersuchungen über Bacterien II. Beiträge zur Biologie der Pflanzen 1(3):141–208.

Collins, M. D., D. Jones. 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. Microbiol. Rev. 45:316–354.

Colwell, R. R. 1970. Polyphasic taxonomy of bacteria. 421–436. H. Iizuka and T. Hasegawa (ed.) Culture collections of microorganisms. University of Tokyo Press. Tokyo.

Cowan, S. T. 1968. A dictionary of microbial taxonomic usage. Edinburgh, Oliver & Boyd.

Cowan, S. T. 1975. Manual for the identification of medical bacteria. Cambridge University Press. Cambridge.

Cowan, S. T. 1978. Dictionary of microbial taxonomy. Hill, L. R. (ed.) Cambridge University Press. Cambridge.

D'Amato, R. F., J. C. McLaughlin, M. J. Ferraro. 1985. Rapid manual and mechanized automated methods for the detection and identification of bacteria and yeasts. 52–65. E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy, (ed.) Manual of clinical microbiology, 4th ed. American Society for Microbiology. Washington, DC.

Darland, G., T. D. Brock, W. Samsonoff, S. F. Conti. 1970. A thermophilic mycoplasma isolated from a coal refuse pile. Science 170:1416–1418.

Dattagupta, N., P. M. M. Roe, E. D. Huguenel, E. Carlson, A. Lygu, J. A. Shapiro, J. P. Albarella. 1989. Rapid identification of microorganisms by nucleic acid hybridization after labeling the test sample. Anal. Biochem. 177:85–89.

DeLong, E. F., G. S. Wickham, N. R. Pace. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363.

Dobson, G., D. E. Minnikin, S. M. Minnikin, J. H. Parlett, M. Goodfellow, M. Ridell, M. Magnusson. 1985. Systematic analysis of complex mycobacterial lipids. 237–265. M. Goodfellow and D. E. Minnikin (ed.) Chemical methods in bacterial systematics. Academic Press. London.

Edwards, J. G. 1978. Computer-assisted identification of unknown bacteria. 280–292. A. N. Sharpe, and D. S. Clark. (ed.) Mechanizing microbiology. Charles C. Thomas. Springfield, IL.

Erola, E., O.-P. Lehtonen. 1988. Optimal data processing procedure for automatic bacterial identification by gas-liquid chromatography of cellular fatty acids. J. Clin. Microbiol. 26:1745–1753.

Endl, J., P. H. Seidl, F. Fiedler, K. H. Schleifer. 1983. Chemical composition and structure of cell wall teichoic acids of staphylococci. Arch. Microbiol. 135:215–223.

Endl, J., P. H. Seidl, F. Fiedler, K. H. Schleifer. 1984. Determination of cell wall teichoic acid structures of staphylococci by rapid chemical and serological screening methods. Arch. Microbiol. 137:272–280.

Erdman, V. A., J. Wolters, E. Huysman, R. De Wachter. 1985. Collection of published 5S, 5.8S, and 4.5S ribosomal RNA sequences. Nucleic Acids Research 13:105–153.

Ezaki, T., Y. Hashimoto, E. Yabuuchi. 1989. Fluorometric deoxyribonucleic acid deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39:224–229.

Faller, A. M., F. Götz, K. H. Schleifer. 1980. Cytochrome patterns of staphylococci and micrococci and their taxonomic implications. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. I. Orig. C1:26–39.

Faller, A. M., K. H. Schleifer. 1981. Effect of growth phase and oxygen supply on cytochrome composition and morphology of *Arthrobacter crystallopoietes*. Curr. Microbiol. 6:253–258.

Festl, H., W. Ludwig, K. H. Schleifer. 1986. A DNA hybridization probe for the *Pseudomonas fluorescens* group. Appl. Environ. Microbiol. 57:1190–1194.

Fiedler, F., M. J. Schäffler, E. Stackebrandt. 1981. Biochemical and nucleic acid hybridization studies on *Brevibacterium linens* and related strains. Arch. Microbiol. 129:85–93.

Gibbons, N. E., R. G. E. Murray. 1978. Proposals concerning the higher taxa of bacteria. International Journal of Systematic Bacteriology 28:1–6.

Giovannoni, S. J., E. F. DeLong, G. J. Olsen, N. R. Pace. 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. 170:720–726.

Glauert, A. M., M. J. Thornley. 1969. The topography of the bacterial cell wall. Ann. Rev. Microbiol. 23:159–198.

Goldfine, H., T. A. Langworthy. 1988. A growing interest in bacterial ether lipids. Trends Biochem. Sci. 13:217–221.

Goodfellow, M. 1977. Numerical taxonomy. 579–596. A. I. Laskin, and H. A. Lechevalier. (ed.) CRC handbook of microbiology, 2nd ed., vol. 1. CRC Press. Cleveland.

Goodfellow, M., C. Lonsdale, A. L. James, O. C. McNamara. 1987. Rapid biochemical tests for the characterisation of streptomycetes. FEMS Microbiology Letters 43:39–44.

Goren, M. B., M. Cernich, O. Brokl. 1978. Some observations on mycobacterial acid-fastness. Ann. Rev. Respir. Dis. 118:151–154.

Gyllenberg, H. G. 1965. A model for computer identification of microorganisms. Journal of General Microbiology 39:401–405.

Gyllenberg, H. G. 1976. Application of automation to the identification of streptomycetes. 299–321. T. Asai (ed.) Actinomycetes: the boundary microorganisms. Toppan Co. Tokyo.

Hatt, H. D., E. Zvirbulis. 1967. Status of names of bacterial taxa not evaluated in Index Bergeyana (1966) (Addendum I). I. Names published circa 1950–1967 exclusive of the genus *Salmonella*. International Journal of Systematic Bacteriology 17:171–225.

Hill, L. R. 1974. Theoretical aspects of numerical identification. International Journal of Systematic Bacteriology 24:494–499.

Hofstad, T. 1974. The distribution of heptose and 2-keto-3-deoxyoctonate in Bacteroidaceae. J. Gen. Microbiol. 85:314–320.

Holmes, B., L. R. Hill. 1985. Computers in diagnostic bacteriology, including identification. 265–287. M. Goodfellow, D. Jones and F. G. Priest. (ed.) Computer assisted bacterial systematics. Academic Press. London.

Höpfl, P., K. H. Schleifer, N. Larsen. 1989. 23S ribosomal RNA higher order structure of *Pseudomonas cepacia* and other prokaryotes. Eur. J. Biochem. 185:355–364.

Jackman, P. J. H. 1987. Microbial systematics based on electrophoretic whole-cell protein pattern. 209–225. R. R. Colwell, and R. Grigorova (ed.) Methods in microbiology, vol. 19. Academic Press. London.

Jann, K., O. Westphal. 1975. Microbial polysaccharides. 1–125. M. Sela (ed.) The antigens, vol. 3. Academic Press. London.

Jannasch, H. W., C. O. Wirsen. 1973. Deep-sea microorganisms: In situ response to nutrient enrichment. Science 180:641–643.

Johnson, J. L. 1986. Determination of DNA base composition. 1–31. G. Gottschalk (ed.) Methods in microbiology, vol. 18. Academic Press. London.

Jones, C. W., R. K. Poole. 1985. The analysis of cytochromes. 285–328. G. Gottschalk (ed.) Methods in microbiology, vol. 18. Academic Press. London.

Kandler, O., H. Hippe. 1977. Lack of peptidoglycan in the cell walls of *Methanosarcina barkeri*. Archives of Microbiology 113:57–60.

Kandler, O., H. König. 1978. Chemical composition of the peptidoglycan-free cell wall of methanogenic bacteria. Archives of Microbiology 118:141–152.

Kandler, O., H. König. 1985. Cell envelopes of archaebacteria. 413–457. C. R. Woese and R. S. Wolfe (ed.) The bacteria: a treatise on structure and function, vol. 8, Archaebacteria. Academic Press. London.

Kaneko, T. 1979. Correlative similarity coefficient: New criterion for forming dendrograms. International Journal of Systematic Bacteriology 29:188–193.

Keddie, R. M., I. J. Bousfield. 1980. Cell wall composition in the classification and identification of coryneform bacteria. 167–188. M. Goodfellow and R. G. Board (ed.) Microbiological classification and identification. Academic Press. London.

Kellogg, S. T. 1979. MICRID: A computer-assisted microbial identification system. Applied and Environmental Microbiology 38:559–563.

Kersters, K. 1985. Numerical methods in the classification of bacteria by protein electrophoresis. 337–368. M. Goodfellow, D. Jones, and F. G. Priest (ed.) Computer-assisted bacterial systematics. Academic Press. London.

Kluyver, A. J., C. B. van Niel. 1936. Prospects for a natural system of classification of bacteria. Zentralblatt für Bakteriologie, Parasitenkunde and Infektionskrankheiten, Abt. 2 94:369–403.

Knox, K. W., A. J. Wicken. 1973. Immunochemical properties of teichoic acids. Bacteriol. Rev. 37:215–257.

Kuhn, H. M., U. Meier-Dieter, H. Mayer. 1988. ECA, the enterobacterial common antigen. FEMS Microbiol. Rev. 54:195–222.

Kwapinski, J. B. G. 1977. Immunological and immunochemical classification of microorganisms. 463–517. A. Laskin, and H. A. Lechevalier. (ed.) CRC handbook of microbiology, 2nd ed., vol. 1. CRC Press. Cleveland.

Lapage, S. P., B. Bascomb, W. R. Willcox, M. A. Curtis. 1970. Computer identification of bacteria. 1–22. A. Baillie and R. J. Gilbert (ed.) Automation, mechanization, and data handling in microbiology. Academic Press. London.

Lapage, S. P., B. Bascomb, W. R. Willcox, M. A. Curtis. 1973. Identification of bacteria by computer: general aspects and perspectives. Journal of General Microbiology 77:273–299.

Lapage, S. P., P. H. A. Sneath, E. F. Lessel, V. B. D. Skerman, H. P. R. Seeliger, W. A. Clark (ed.). 1975. International code of nomenclature of bacteria. Washington, D.C. American Society for Microbiology.

Lederer, E., A. Adam, R. Ciorbaru, J.-F. Petit, J. Wietzerbin. 1975. Cell walls of mycobacteria and related organisms; chemistry and immunostimulant properties. Mol. Cell. Biochem. 7:87–104.

Liebl, W., R. Rosenstein, F. Götz, K. H. Schleifer. 1987. Use of staphylococcal nuclease gene as DNA probe for *Staphylococcus aureus*. FEMS Microbiol. Lett. 44:179–184.

Lüderitz, O., C. Galanos, V. Lehmann, H. Mayer, E. Th. Rietschel, J. Weckesser. 1978. Chemical structure and biological activities of lipid A's from various bacterial families. Naturwiss. 65:578–585.

Mandel, M. 1969. New approaches to bacterial taxonomy: perspective and prospects. Ann. Rev. Microbiol. 23:239–274.

Marx, A., M. Peteovici, J. Bittner, M. Antohi, J. Ardeleanu. 1982. Isolation and characterization of a cross-reacting antigen in strains of Bacteroidaceae. Infect. Immun. 36:943–948.

Matthews, J. A., L. J. Kricka. 1988. Analytical strategies for the use of DNA probes. Anal. Biochem. 169:1–25.

Meier-Dieter, U., G. Acker, H. Mayer. 1989. Detection of enterobacterial common antigen on bacterial cell surfaces by colony-immunoblotting: effect of its linkage to lipopolysaccharide. FEMS Microbiol. Lett. 59:215–220.

Miller, L. T. 1982. A single derivatization method for bacterial fatty acid methyl esters including hydroxy acids. J. Clin. Microbiol. 16:584–586.

Minnikin, D. E., M. Goodfellow. 1980. Lipid composition in the classification and identification of acid-fast bacteria.

189–256. M. Goodfellow and R. G. Board (ed.) Microbiological classification and identification. Academic Press. London.

Minnikin, D. E., M. Goodfellow, M. D. Collins. 1978. Lipid composition in the classification and identification of coryneform and related taxa. I. J. Bousfield. and A. G. Callely. (ed.) Coryneform bacteria. Academic Press. London.

Morotomi, M., T. Ohno, M. Mutai. 1988. Rapid and correct identification of intestinal *Bacteroides* spp. with chromosomal DNA probes by whole-cell dot blot hybridization. Appl. Environ. Microbiol. 54:1158–1162.

Moseley, S. L., P. Echeverria, J. Seriwatana, C. Tirapat, W. Chaicumpa, T. Sakuldaipeara, S. Falkow. 1982. Identification of enterotoxigenic *Escherichia coli* using three enterotoxin gene probes. J. Infect. Dis. 145:863–869.

Murray, R. G. E., D. J. Brenner, R. R. Colwell, P. De Vos, M. Goodfellow, P. A. D. Grimont, N. Pfennig, E. Stackebrandt, G. A. Zavarzin. 1990. Report of the ad hoc Committee on Approaches to Taxonomy within the Proteobacteria. Int. J. Syst. Bacteriol. 40:213–215.

Nahaie, M. R., M. Goodfellow, D. E. Minnikin, V. Hajek. 1984. Polar lipid and isoprenoid quinone composition in the classification of *Staphylococcus*. J. Gen. Microbiol. 130:2427–2437.

Naumova, I. 1988. The teichoic acids of actinomycetes. Microbiological Sciences 5:275–279.

Nikaido, H. 1970. Lipopolysaccharide in the taxonomy of Enterobacteriaceae. Int. J. Syst. Bacteriol. 20:383–406.

O'Donnell, A. G., M. R. Nahaie, M. Goodfellow, D. E. Minnikin, V. Hajek. 1985. Numerical analysis of fatty acid profiles in the identification of staphylococci. J. Gen. Microbiol. 131:2023–2033.

Oelze, J. 1985. Analysis of bacteriochlorophylls. 257–284. G. Gottschalk (ed.) Methods in microbiology, vol. 18. Academic Press. London.

Orla-Jensen, S. 1909. Die Hauptlinien des natürlichen Bakteriensystems. Centralblatt für Bakteriologie, Abt. 2 22:305–346.

Ourisson, G., M. Rohmer, K. Poralla. 1987. Prokaryotic hopanoides and other polyterpenoidsterol surrogates. Ann. Rev. Microbiol. 41:301–333.

Pfennig, N., H. G. Trüper. 1971. Higher taxa of the phototrophic bacteria. International Journal of Systematic Bacteriology 21:17–18.

Regensburger, A., W. Ludwig, K. H. Schleifer. 1988. DNA probes with different specificities from a cloned 23S rRNA gene of *Micrococcus luteus*. J. Gen. Microbiol. 134:1197–1204.

Reichelt, J. L., P. Baumann. 1973. Taxonomy of the marine luminous bacteria. Archiv für Mikrobiologie 94:283–330.

Rhodes, M. E. 1965. Flagellation as a criterion for the classification of bacteria. Bacteriological Reviews 29:442–465.

Rose, C. S., S. J. Pirt. 1981. Conversion of glucose to fatty acids, and methane: roles of two mycoplasmal agents. J. Bacteriol. 147:248–254.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.

Sambrook, J., E. F. Fritsch, T. Mariatis. 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. 1. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, USA.

Sawada, S., T. Kawamura, Y. Masuho, K. Tomibe. 1985. A new common polysaccharide antigen of strains of *Pseudomonas aeruginosa* detected with a monoclonal antibody. J. Infect. Dis. 152:1290–1299.

Scherer, P., H. Kneifel. 1983. Distribution of polyamines in methanogenic bacteria. J. Bacteriol. 154:1315–1322.

Schindler, J., J. Duben, O. Lysenko. 1979. Computer-aided numerical identification of Gram-negative fermentative rods on a desk-top computer. Journal of Applied Bacteriology 47:45–51.

Schleifer, K. H., O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36:407–477.

Schleifer, K. H., R. Kilpper-Bälz. 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci. Syst. Appl. Microbiol. 10:1–19.

Schleifer, K. H., W. Ludwig. 1989. Phylogenetic relationships among bacteria. 103–117. Fernholm, B., K. Bremer and H. Jörnvall (ed.) The Hierarchy of life. Elsevier Science Publishers B.V. Amsterdam.

Schleifer, K. H., E. Stackebrandt. 1983. Molecular systematics of prokaryotes. Ann. Rev. Microbiol. 37:143–187.

Schleifer, K. H., W. P. Hammes, O. Kandler. 1976. Effect of endogenous and exogenous factors on the primary structures of bacterial peptidoglycan. Adv. Microb. Physiol. 13:245–292.

Schmidhuber, S., W. Ludwig, K. H. Schleifer. 1988. Construction of a DNA probe for the specific identification of *Streptococcus oralis*. J. Clin. Microbiol. 26:1042–1044.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. 51:873–884.

Sharpe, M. E. 1970. Cell wall and cell membrane antigens in the classification of lactobacilli. Int. J. System. Bacteriol. 20:519–533.

Shaw, N. 1975. Bacterial glycolipids and glycophospholipids. Adv. Microb. Physiol. 12:141–167.

Shively, J. M. 1974. Inclusion bodies of prokaryotes. Annual Review of Microbiology 28:167–187.

Simpson, G. G. 1961. Principles of animal taxonomy. Columbia University Press. New York.

Skerman, V. B. D. 1967. A guide to the identification of the genera of bacteria, 2nd ed. Williams & Wilkins. Baltimore.

Skerman, V. B. D. 1974. A key for the determination of the generic position of the organisms listed in the manual. 1098–1146. R. E. Buchanan, and N. E. Gibbons, (ed.) Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins. Baltimore.

Skerman, V. B. D., V. McGowan, P. H. A. Sneath, (ed). 1980. Approved lists of bacterial names. International Journal of Systematic Bacteriology 30:225–420.

Sleytr, U. B., P. Messner. 1983. Crystalline surface layers on bacteria. Ann. Rev. Microbiol. 37:311–339.

Sneath, P. H. A. 1971. Theoretical aspects of microbiological taxonomy. 581–586. A. Pérez-Miravete, and D. Peláez, (ed.) Recent advances in microbiology. Asociacióon Mexicana de Microbiología. Mexico City.

Sneath, P. H. A. 1972. Computer taxonomy. 29–98. J. R. Norris, and D. W. Ribbons, (ed.) Methods in microbiology, vol. 7A. Academic Press. London.

Sneath, P. H. A. 1977. The maintenance of large numbers of strains of microorganisms, and the implications for culture collections. FEMS Microbiology Letters 1:333–334.

Sneath, P. H. A. 1979a. BASIC program for a significance test for two clusters in Euclidian space as measured by their overlap. Computers & Geosciences 5:143–155.

Sneath, P. H. A. 1979b. BASIC program for identification of an unknown with presence-absence data against an identification matrix of percent positive characters. Computers & Geosciences 5:195–213.

Sneath, P. H. A. 1979c. BASIC program for character separation indices from an identification matrix of percent positive characters. Computers & Geosciences 5:349–357.

Sneath, P. H. A. 1980a. BASIC program for the most diagnostic properties of groups from an identification matrix of percent positive characters. Computers & Geosciences 6:21–26.

Sneath, P. H. A. 1980b. BASIC program for determining the best identification scores possible for the most typical example when compared with an identification matrix of percent positive characters. Computers & Geosciences 6:27–34.

Sneath, P. H. A. 1980c. BASIC program for determining overlap between groups in an identification matrix of percent positive characters. Computers & Geosciences 6:267–278.

Sneath, P. H. A., R. R. Sokal. 1973. Numerical taxonomy, the principles and practice of numerical classification. Freeman. San Francisco.

Sokal, R. R., P. H. A. Sneath. 1963. Numerical taxonomy. Freeman. San Francisco.

Sprott, G. D., R. C. McKellar. 1980. Composition and properties of the cell wall of *Methanospirillum hungatei*. Can. J. Microbiol. 26:115–120.

Stackebrandt, E., R. G. E. Murray, H. G. Trüper. 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives." Internat. J. System. Bacteriol. 38:321–325.

Staehelin, L. A., J. R. Golecki, R. C. Fuller, G. Drews. 1978. Visualization of the supramolecular architecture of chlorosomes (chlorobium type vesicles) in freeze-fractured cells of *Chloroflexus aurantiacus*. Archives of Microbiology 119:269–277.

Stanier, R. Y. 1971. Toward an evolutionary taxonomy of the bacteria. 595–604. A. Pérez-Miravete. and D. Peláez (ed.) Recent advances in microbiology. Asociación Mexicana de Microbiologia. Mexico City.

Stanier, R. Y., J. L. Ingraham, M. L. Wheelis, P. R. Paintes. 1986. The microbial world, 5th ed. Prentice-Hall. Englewood Cliffs, New Jersey.

Stanier, R. Y., C. B. van Niel. 1941. The main outlines of bacterial classification. Journal of Bacteriology. 42:437–466.

Starnbach, M. N., S. Falkow, L. S. Tompkins. 1989. Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridization. J. Clin. Microbiol. 27:1257–1261.

Stetter, K. O., M. Thomm, J. Winter, G. Wildgruber, H. Huber, W. Zillig, D. Janekovic, H. König, P. Palm, S. Wunder. 1981. *Methanothermus fervidus,* sp. nov., a novel extremely thermophilic methanogen isolated from Icelandic hot spring. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. I. Orig. C2:166–178.

Taylor, P. W. 1974. An unusual acid polysaccharide produced by a rough strain of *Escherichia coli*. Biochem. Biophys. Res. Commun. 61:148–154.

Tornabene, T. G. 1985. Lipid analysis and the relationship to chemotaxonomy. 209–234. G. Gottschalk (ed.) Methods in microbiology, vol. 18. Academic Press. London.

Uchida, K., K. Aida. 1977. Acyl type of bacterial cell wall: Its simple identification by colorimetric method. J. Gen. Appl. Microbiol. 23:249–260.

van Niel, C. B. 1946. The classification and natural relationships of bacteria. Cold Spring Harbor Symposia on Quantitative Biology 11:285–301.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, H. G. Trüper. 1987. Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463–464.

Weckesser, J., G. Drews, H. Mayer. 1979. Lipopolysaccharides of photosynthetic prokaryotes. Ann. Rev. Microbiol. 33:215–239.

Weitzman, P. D., D. Jones. 1975. The mode of regulation of bacterial citrate synthase as a taxonomic tool. Journal of General Microbiology 89:187–190.

Weller, R., M. Ward. 1989. Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. Appl. Environ. Microbiol. 55:1818–1822.

Wilkinson, S. G. 1977. Composition and structure of bacterial lipopolysaccharides. 97–175. I. W. Sutherland (ed.) Surface carbohydrates of the prokaryotic cell. Academic Press. London.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Woitzik, D., J. Weckesser, U. J. Jürgens. 1988. Isolation and characterization of cell wall components of the unicellular cyanobacterium *Synechococcus* sp. PCC 6307. J. Gen. Microbiol. 134:619–627.

Zvirbulis, E., H. D. Hatt. 1969a. Status of names of bacterial taxa not evaluated in Index Bergeyana (1966). Addendum II. *Acetobacter* to *Butyrivibrio*. International Journal of Systematic Bacteriology 19:57–115.

Zvirbulis, E., H. D. Hatt. 1969b. Status of names of bacterial taxa not evaluated in Index Bergeyana (1966). Addendum III. *Achromobacter* to *Lactobacterium*. International Journal of Systematic Bacteriology 19:309–370.

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CHAPTER 1.5

Principles of Enrichment, Isolation, Cultivation and Preservation of Prokaryotes

JÖRG OVERMANN

Introduction

Currently, a total of 4700 validly published prokaryotic species are recognized (as of July 2002; DSMZ, 2002; Euzéby, 2001; Fig. 1). In contrast, the number of bacterial species present in just one type of forest soil has been estimated to be 13,000 or even 53,000 species (Torsvik et al., 1994; Sandaa et al., 1999). In light of these more recent findings, the earlier estimate of the fraction of already cultured bacterial species of 20% (Wayne et al., 1987) appears to be much too optimistic. This view is also supported by numerous molecular investigations of 16S rRNA gene sequences in natural bacterial assemblages that indicate a significant fraction of bacteria present in the environment has not yet been recovered by cultivation-based approaches (Fuhrman et al., 1992; Ward et al., 1992; Barns et al., 1994; DeLong et al., 1994; Hiorns et al., 1997; Kuske et al., 1997; Ludwig et al., 1997; Suzuki et al., 1997; Gich et al., 2001; Béjà et al., 2002). The 16S rRNA gene libraries of natural bacterial communities usually do not match sequences of strains isolated from the same or similar samples (Hiorns et al., 1997; Suzuki et al., 1997; Hugenholtz et al., 1998). Even a large cultivation campaign during which 659 bacterial isolates were obtained from grassland soil did not yield any strain with a 16S rRNA sequence corresponding to the dominant strains in the natural community (Felske et al., 1999). In some cases, this discrepancy may simply be the result of the low number of cultivation attempts, since even conventional cultivation trials continue to yield novel phylotypes of bacteria (Pinhassi et al., 1997; Suzuki et al., 1997) and the number of recognized species continuously increases at a rate of about 190 species per year (Fig. 1).

In many instances, key reactions in biogeochemical cycles appear to be mediated not by the frequently isolated prokaryotes but rather by other prokaryotes that are phylogenetically unrelated and mostly not-yet-cultured. Examples include members of the *Nitrosospira*-group which (instead of *Nitrosomonas* spp.) catalyze nitrification in at least some environments (Hastings et al., 1998; Schramm et al., 1999), *Achromatium* spp. and coccoid magnetotactic bacteria participating in the sulfur cycle (Spring et al., 1993), archaea which apparently mediate anaerobic methane consumption (Hinrichs et al., 1999), and novel type II methanotrophic bacteria oxidizing methane at atmospheric concentrations (Holmes et al., 1999; Roslev et al., 1999). Some environments harbor whole groups of entirely unknown bacteria and archaea, which most likely are important for the biogeochemical transformations (Hugenholtz et al., 1998). These include for instance members of the green filamentous bacteria (Coolen et al., 2002) or freshwater actinobacteria (Glöckner et al., 2000). In addition, first culture-independent analyses of large genome fragments retrieved from natural samples have recently uncovered bacteria present in the natural environment that have a previously unrecognized physiology (Béjà et al., 2000). Only in a few instances could numerically important bacterial species apparently be isolated (Ferris et al., 1996; Kalmbach et al., 1997). With the aid of modern molecular methods, some information on the physiological capabilities of "not-yet-cultured prokaryotes" can now be obtained even down to the single-cell level (Ouverney and Fuhrman, 2000).

Though culture-independent techniques have improved, they still provide only limited insights into the physiology of prokaryotes. Therefore, the role of not-yet-cultured prokaryotes in the environment cannot be fully appreciated until these microorganisms are available for detailed physiological and molecular studies. So far, our present methods for the enrichment, isolation and cultivation are largely based on concepts developed first and foremost for the isolation of medically important bacteria. The failure to cultivate many of the ecologically relevant bacteria clearly indicates that there is a need for the development of novel cultivation methods.

Successful enrichment, isolation and cultivation of prokaryotes critically depend on the choice of appropriate growth media and incubation conditions. In the following chapters, the requirements of prokaryotic cells for growth in

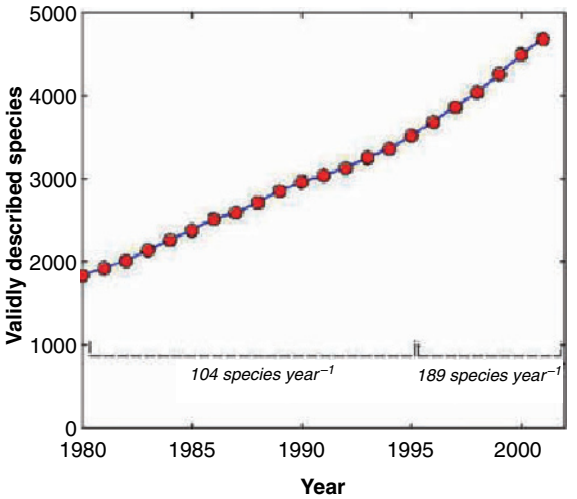

Fig. 1. Increase in validly described species since the appearance of the approved list of bacterial names in 1980. Data were taken from Euzéby (2001) and the (http://www.dsmz.de/bactnom/bactname.htm) Deutsche Sammlung für Mikroorganismen und Zellkulturen and do not include Comb. nov. and *Nomines nova*.

the laboratory and the different existing methods for their cultivation are discussed.

Historical Perspective

Girolamo Fracastoro (1478–1553) first proposed the existence of minute living bodies. He proposed that transfer of this "contagium" was the cause of infectious diseases. Prokaryotes were first made visible by Antonie van Leeuwenhoek (1632–1723), a draper and amateur lens maker living in Delft, The Netherlands. In one of his letters to the Royal Society of London published in 1676, he gave the first account of "kleine diertjes" (little animals), descriptions proving he indeed had seen bacteria. In another letter of September 17, 1683, he provided the first picture of bacteria. These now famous drawings depict cocci, rods, vibrios and spirilla that van Leeuwenhoek had observed in scrapings from his teeth. Although Robert Hooke had described cork "cells" and fossilized foraminiferan shells earlier (Hooke, 1665), he never recorded observations of bacteria, most likely because he only used dry inanimate preparations. The success of van Leeuwenhoek was based on 1) the high quality of the lenses (magnifications up to 280 times) he used and mounted in homemade single-lens microscopes (Dobell, 1932; Porter, 1976), 2) his way of mounting and viewing specimens with a kind of dark-field illumination, and 3) the time he devoted to microscopic investigation.

The work of Louis Pasteur (1822–1895), which marks the beginning of scientific microbiology, follows the scientific discussion over the next 200 years on the issue of spontaneous generation, which assumed mice, frogs, and "lower" forms of life could develop spontaneously in decaying organic matter and mud. Pasteur's experiments with his famous swan-necked flasks ruled out spontaneous generation and at the same time laid the foundations for aseptic manipulation and sterilization. Questioning the belief of eminent chemists of his time, Pasteur provided the first notion of the microbial nature of fermentation in milk (Pasteur, 1862) and of anaerobic life. Other studies revealed that fermentation of sugar to alcohol, wine to vinegar, and the putrefaction of meat all were caused by microorganisms. Each particular kind of fermentation was accompanied by the development of a specific type of microorganism.

Until 1884, the bacterial pleomorphism versus monomorphism controversy was a central issue of microbiology. The description of rust and smut fungi and their morphological changes during development had caused confusion among the early bacteriologists, and it was assumed that, depending solely on growth conditions, one and the same bacterium is able to appear in different forms (pleomorphic), can cause completely different diseases, and form totally different metabolic products. Of outstanding importance for our current thinking in microbiology is the contribution of Robert Koch (1843–1910), the founder of medical bacteriology. In his experimental work, Koch established a series of criteria, the so-called "Koch's postulates," for the identification of causative agents of infectious diseases:

1. The microorganism should be constantly present in animals suffering from the disease, but should not be present in healthy individuals
2. The microorganism must be cultivated in pure culture outside the diseased animal
3. Healthy animals infected with these pure cultures must display the characteristic disease symptoms
4. The microorganism should be reisolated from the experimental animals and shown to be the same as the original

In 1876, Koch isolated the anthrax bacillus from diseased cattle and conclusively demonstrated that the large nonmotile bacilli caused the disease. In 1873, Joseph Lister (1827–1912) had introduced the serial dilution technique to achieve pure cultures. However, this procedure did not yield pure cultures in the hands of every researcher. Pure culture techniques employing solid nutrient media containing gelatin were developed by Anton de Bary (1831–1888) and Oscar Brefeld (1839–1926) for the study of fungi (Bull and Slater, 1982). Although the latter scientists succeeded in cultivating fungi on solid

media, their techniques were not suited for the isolation and growth of bacteria for several reasons: various bacteria were found to liquefy gelatin, on hot days it melted spontaneously, and gelatin-based media could not be incubated at the temperature that most human pathogens needed to grow in a convenient time span. Subsequently, agar-solidified media (around 1881), covered culture dishes, and staining techniques were developed in the laboratory of Robert Koch and, in nearly unaltered form, have remained major tools in bacteriology and medical microbiology until today. After employing agar media, Koch announced his discovery of the tubercle bacillus as the causative agent of tuberculosis in 1882, at the time when this disease had many victims in Europe (Groeschel, 1982). Two years later, he published the discovery of the cholera bacillus.

Subsequently, microorganisms were found to be also of crucial importance for the geochemical cycles. Ferdinand Cohn was the first to realize the role of microorganisms in the transformation of organic matter and inorganic substances on earth (Cohn, 1872). The most significant contributions to the knowledge of the various types of microorganisms responsible for specific chemical transformations in nature (especially for the nitrogen and sulfur cycles) came largely from the laboratories of Sergius Winogradsky (1856–1953) and Martinus Willem Beijerinck (1851–1931). During the 1880s, Winogradsky advanced the concept of chemosynthesis on the basis of his studies of the colorless sulfur bacteria, *Thiothrix* and *Beggiatoa* (Winogradsky, 1949), concluding that these aerobic bacteria obtain their energy for autotrophic growth by oxidizing reduced sulfur compounds (such as sulfides and sulfur) to sulfate.

The work of Beijerinck, with the contributions from Winogradsky, led to the development of the enrichment culture technique. Instead of directly isolating microorganisms from nature by exposing nonselective growth media to some environment and allowing chance to dictate what grew, Beijerinck proposed a different approach. Tailoring culture conditions to favor microbes with a particular metabolic activity usually leads to a rapid enrichment of the desired organism, even if its original numbers are very low in the sample (Van Iterson et al., 1940). One of the early examples of the application of this principle was in Beijerinck's paper on sulfate reduction, which led to the discovery and isolation of *Spirillum* (*Desulfovibrio*) *desulfuricans*, described in a preliminary paper in 1894. Besides root nodule bacteria, *Lactobacillus* species, and others, Beijerinck also isolated the first pure cultures of aerobic nitrogen-fixing bacteria from a culture obtained by Winogradsky. Winogradsky applied enrichment of selective cultures for his research on sulfur bacteria and nitrifying bacteria and also used this technique to identify nitrogen-fixing bacteria in soil. In the years to come after Beijerinck, the success of the batch enrichment culture technique was demonstrated over and over again in the work of Kluyver, Van Niel, Stanier and their students and associates (Veldkamp, 1965; Van Niel, 1967; Pfennig, 1993).

At a time when the discovery of a bewildering variety of microbial forms and activities reached its height, Albert J. Kluyver (1884–1957) and H.J.L. Donker published a seminal paper on the unity in biochemistry (Kluyver and Donker, 1926). This synthetic paper was based on an integration of the ideas of Carl Neuberg, Heinrich Wieland, Otto Warburg, and A. Harden. The concept was that, in addition to a number of hydrolases, cells contained various oxidoreductases catalyzing the transfer of hydrogen from one molecule, the hydrogen (H) donor, to another, the H acceptor, along a gradient of energy. Hydrogen acceptors other than oxygen might also be used (e.g., sulfate). The first report on the view that photosynthesis can be considered as a light-dependent reaction in which different substrates, specific for the different kinds of photosynthetic organisms, serve as H donors for the reduction of carbon dioxide came from Van Niel (1930). Van Niel (1967) described a general formula of photosynthesis. His work was extended greatly by N. Pfennig and his students, who grew many of the anaerobic photosynthetic bacteria in pure culture (Pfennig, 1993).

Early attempts to devise truly anoxic culturing techniques included the use of deep-agar shake tubes gassed with hydrogen, the use of pyrogallol to remove oxygen, sealed tubes, and the isolation of colonies by picking with capillary pipettes. Still, the only anaerobes isolated until 1940 were sporeformers and several nonsporeforming bacteria of clinical importance, while identities of the majority of the anaerobic prokaryotes in sediments, soils, and the gastrointestinal tract remained unknown. This was in part due to the fact that techniques were insufficient to reach the necessary anaerobic conditions, but also to the fact that the media used were not habitat-simulating. Hungate (1950), the first student of Van Niel from the United States, developed an anoxic roll tube technique based on various approaches from the older literature and used habitat-simulating media in the isolation of cellulolytic rumen bacteria (Hungate, 1966; Hungate, 1969; Hungate, 1985). The Hungate technique was very successful and has been widely adapted by microbiologists from various other fields (dentistry, sewage and sediment microbiology).

Many soil bacteria were studied in pure culture after their discovery to examine their basic

metabolism and metabolic capacities. Winogradsky emphasized that the conclusions drawn from laboratory studies on the behavior of bacteria in the natural habitat may be misleading because laboratory strains might only be artefacts, i.e., selected by the chosen nutrient medium, temperature, aeration and agitation. Consequently, he already warned against the use of bacterial strains from culture collections as typical wild-type cells, and together with some of his students, designed methods to study bacteria in situ, within their natural habitats. Winogradsky's concepts on soil microbiology and on the differentiation between autochthonous and allochthonous populations in the soil are the basis of relevant, current research.

The majority of physiological investigations have been limited to pure laboratory cultures. According to the statement of Oscar Brefeld, work with impure cultures yields nothing but nonsense and *Penicillium glaucum* (Brefeld, 1881). However, Koch's methods may also have hindered the understanding of the microbial interactions as they occur in complex natural microbial communities, e.g., in soil (Winogradsky, 1949). In fact, interactions between microorganisms under natural conditions may lead to transformations that are unknown from pure cultures. The shortcomings of pure culture techniques are illustrated best by the interspecies hydrogen transfer described first by M.J. Wolin in 1975, as well as by the inhibition of growth of a *Micrococcus* culture by a chance *Penicillium* contaminant (Fleming, 1929), which led to the discovery of antibiotics. For an in-depth understanding of microbial physiology, microbial transformations therefore have to be studied also in natural mixed microbial communities.

Prokaryotic Growth

Parameters of Prokaryotic Growth

In media of appropriate composition and in the presence of suitable substrates, bacteria multiply. The rate of growth is dependent on substrate concentration, temperature, pH, and osmolarity and can be assessed from the increase in bacterial cell number or in bacterial biomass. This distinction has to be made since under certain conditions growth is not completely balanced, and the increase in biomass is not paralleled by an increase in cell numbers or vice versa. As an example, cells in the early stationary phase can undergo reductive division whereby cell numbers continue to increase while a constant overall biomass of the culture is maintained (Kolter et al., 1993).

Upon inoculation of bacterial cells into fresh growth medium, a period without growth is frequently observed (the so-called "lag phase") during which adaptation of the cells occurs by, e.g., synthesis of cellular enzymes. Afterwards, a phase of exponential growth occurs until cell growth is limited by substrate availability and by accumulation of toxic metabolic products or unfavorable pH (stationary phase). The stationary phase is then followed by a phase of prokaryotic biomass reduction due to lysis of cells. If changes in bacterial cell numbers N are followed during the exponential phase, the specific division rate ε and the generation time g are used for the mathematical description of growth (N_0 denoting the initial cell number, and t the time of incubation):

$$N = N_0 \cdot 2^{\nu t} \tag{1}$$

$$g = \frac{1}{\nu} \tag{2}$$

In a growing culture, the concentration of prokaryotic biomass X increases autocatalytically (a first-order reaction), that is, the instantaneous biomass changes $\mathrm{d}X/\mathrm{d}t$ are proportional to the biomass present (Lengeler et al., 1999). Here the specific growth rate μ and the doubling time t_d are used to describe prokaryotic growth:

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \mu \cdot X \tag{3}$$

and hence

$$X = X_0 \cdot e^{\mu t} \tag{4}$$

The biomass doubling time is then calculated according to

$$t_\mathrm{d} = \frac{\ln 2}{\mu} \tag{5}$$

The two parameters μ and ν are related according to

$$\mu = \nu \cdot \ln 2 \tag{6}$$

The specific growth rate is determined by the substrate concentration which, based on the theoretical consideration of Monod (Monod, 1942; Monod, 1950), can be described by

$$\mu = \mu_\mathrm{max} \cdot \left(\frac{S}{K_s + S} \right) \tag{7}$$

where μ_max is the maximum value of the specific growth rate attained at unlimiting substrate concentrations S and K_s (the saturation constant numerically equal to the substrate concentration at which $\mu = 0.5\ \mu_\mathrm{max}$).

For comparison of growth characteristics between different cultures, the K_s value has been

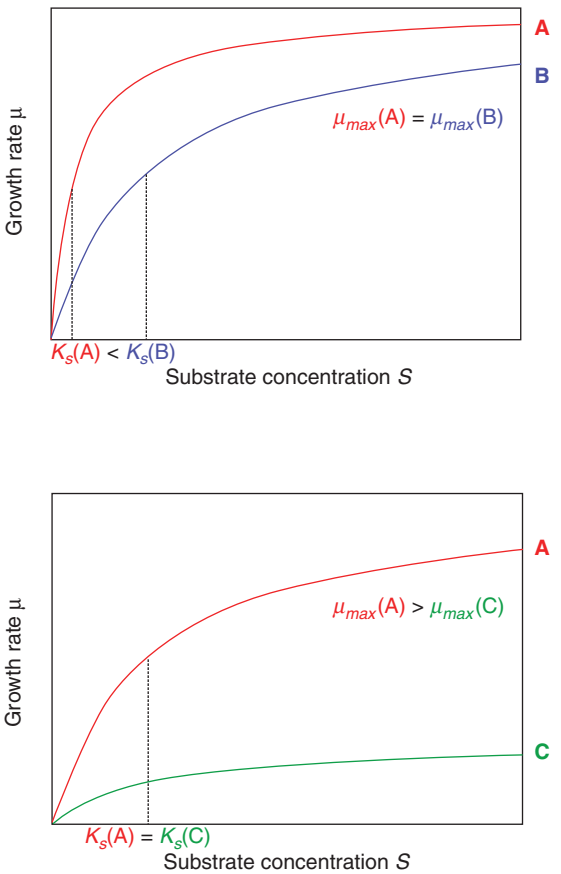

Fig. 2. Significance of the substrate affinity of growth (μ_{max}/K_s, i.e., the initial slope of the μ versus S curve) for three species A, B and C. Comparison of substrate-dependent specific growth rates for two strains A and B with the same maximum growth rate but different K_s values (upper panel), and for two strains A and C with the same K_s value but different maximum growth rates μ_{max} (bottom).

frequently invoked as a measure of affinity for a given substrate. However, prokaryotes with similar K_s values may differ substantially in their affinity (Fig. 2) since the substrate affinity of growth is not solely determined by the K_s value of the prokaryote, but rather by the ratio μ_{max}/K_s (Schut et al., 1997).

Modes of Prokaryotic Growth

One of the most pronounced differences between prokaryotes and eukaryotes is the high metabolic versatility of the former. Among the outstanding metabolic properties of prokaryotes is the use of inorganic electron donors for energy generation, anaerobic growth, and the fixation of molecular nitrogen. In eukaryotes, these properties are either restricted to a few species or completely lacking. Novel ways of energy generation continue to be uncovered (Zengler et al., 1999; Schink and Friedrich, 2000).

Table 1. Classification of microbial metabolism on the basis of energy source, the type of electron donor, and the type of carbon source.

Energy source				
Chemical:	*Chemo-*			
Light:	*Photo-*			
Electron donor				
Inorganic:		*-litho-*		
Organic:		*-organo-*		
Carbon source				
CO_2:			*-auto-*	
Organic:			*-hetero-*	
Both:			*-mixo-*	
				-troph

According to the concept of unity in biochemistry, redox reactions are central to life. Only a few exceptions exist to the rule that biological energy is produced in redox processes (Thauer et al., 1977). Such catabolic nonredox processes comprise the arginine fermentation in *Enterococcus faecalis*, xanthine fermentation in *Clostridium cylindrosporum*, and the pyruvate fermentation to acetate and formate in *Proteus rettgeri*. In these cases, substrates are metabolized by lytic rather than redox reactions, and energy is conserved by substrate level phosphorylation. Also, photophosphorylation in *Halobacterium halobium* proceeds without the participation of an electron chain.

In the case of chemotrophic prokaryotes, the energy driving cellular processes is derived from an exergonic chemical reaction (Table 1), during which an exogenously supplied substrate reduces an external electron acceptor (e.g., during respiration with oxygen, nitrate or sulfate). Alternatively, a metabolic product of the substrate reduces an electron acceptor formed intracellularly from the substrate (fermentation).

The standard free energy change of a specific type of microbial metabolism can be assessed from the difference between the standard redox potential of the electron donors (E_0'[don]) and that of the electron acceptors (E_0'[acc]; tabulated in Thauer et al., 1977), according to

$$\Delta G_0' = -n \cdot F \cdot \Delta E_0' = -n \cdot F \cdot \left[E_0'(\text{acc}) - E_0'(\text{don}) \right] \quad (8)$$

where n is the number of electrons transferred and F is the Faraday constant (96.48 $kJ \cdot V^{-1}$).

The standard free energy change of a reaction can also be calculated from the free energy of formation (G_f^0) of the products and reactants (Thauer et al., 1977) and, in case of energy-yielding reactions involving H^+, the number of protons formed (m) and the free energy of

formation of a proton at pH 7 and 25°C ($\Delta G_f'[H^+]$):

$$\Delta G_0' = \sum G_f^0(\text{products}) - \sum G_f^0(\text{reactants}) + m \cdot \Delta G_f'(H^+)$$
$$= \sum G_f^0(\text{products}) - \sum G_f^0(\text{reactants}) + m \cdot (-39.83\ \text{kJ}) \quad (9)$$

(Madigan et al., 2000a). It has been suggested that microbes can exploit a reaction for growth, if the Gibbs free energy is ≥20 kJ·(mol substrate)$^{-1}$ and hence sufficient for the translocation of one proton across the cytoplasmic membrane (Schink, 1991; Schink, 1997). However, experiments with syntrophic cultures indicate that even smaller amounts of energy may be utilized and that syntrophic associations with methanogens metabolize near thermodynamic equilibrium at values as little as $\delta G' = -4.5$ kJ·(mol substrate)$^{-1}$ (Scholten and Conrad, 2000; Jackson and McInerney, 2002). In ecological niches where available energy is at a minimum, prokaryotes are hence capable of maximizing energy conservation when thermodynamic constraints begin to limit substrate degradation. It has been suggested that the underlying mechanisms control changes in cellular phosphorylation potential or the electronmotive membrane potential of the syntrophically metabolizing bacteria and that these mechanisms may be triggered by signals from their hydrogen-scavenging partner (Jackson and McInerney, 2002).

In contrast to chemotrophs, phototrophic prokaryotes exploit electromagnetic energy for growth. During photosynthesis, light absorption converts a cellular pigment (the reaction center chlorophyll or bacteriochlorophyll) from a weak to a strong reductant. Electrons are transferred to associated electron carriers and—during cyclic electron transport (cyclic photophosphorylation)—finally returned to the oxidized reaction center pigment. As a principal difference from chemotrophic prokaryotes, phototrophic prokaryotes can convert light energy into chemical energy (a proton gradient) without the need of an external electron donor.

Clearly, organic or inorganic carbon compounds represent the most important nutrients for prokaryotic growth. Traditionally, prokaryotes have been divided into two major groups with respect to their carbon requirement, namely autotrophs and heterotrophs. Autotrophic prokaryotes assimilate carbon dioxide as the principal source of cellular carbon. It has to be noted, however, that some inorganic carbon-fixing enzymes, such as acetyl-CoA carboxylase or propionyl-CoA carboxylase (present in certain autotrophs), as well as anaplerotic enzymes such as phosphoenol pyruvate (PEP) carboxylase or biosynthetic enzymes such as carbamoyl phosphate synthetase fix bicarbonate instead of CO_2 (Neuhard and Kelln, 1996; van der Meer et al., 2001). As a rule, these microbes use light or the oxidation of inorganic compounds for the generation of metabolic energy; hence they grow as photolithoautotrophs or chemolithoautotrophs (Table 1), respectively. Facultative autotrophs can also grow at the expense of organic carbon compounds, whereas mixotrophs can utilize CO_2 and organic carbon compounds simultaneously. One and the same strain can grow chemolithoautotrophically under one set of environmental conditions, but switch to chemoorganoheterotrophic growth under different conditions. Consequently, the metabolic types listed in Table 1 are not mutually exclusive, but may occur in the same prokaryote. In the case of heterotrophic bacteria, organic carbon compounds are used not only for biomass synthesis, but also for energy generation. Approximately half of the organic carbon compounds are assimilated by aerobic microorganisms. Fermenting bacteria assimilate significantly less, typically 10–20%, while the remaining carbon substrate is required as an energy source.

The redox state of carbon in biomass (in $<C_4H_8O_2N>$, on average –0.25) is more reduced than many carbon sources utilized by prokaryotes. This situation is most pronounced in the case of autotrophic growth, but also during growth on organic carbon sources such as formate, glycollate, malate, fumarate, oxaloacetate or citrate. The substrates used in microbial metabolism therefore not only serve to generate energy in the case of chemotrophic species, but also yield reducing power for the cells to carry out a variety of reductive processes during synthesis of new cell biomass. In the classification of microbial metabolism, the (inorganic or organic) nature of the electron donor for these reductive processes is considered (Table 1).

Requirements for Prokaryotic Growth

General Composition of the Prokaryotic Cell

For the understanding of the thermodynamics of bacterial growth and as a basis for the design of appropriate growth media, knowledge of the composition of prokaryotic biomass is essential. Of the more than 100 elements that appear in the periodic table, some 35–40 are considered essential. Six nonmetals (C, O, H, N, S and P), together with four metals (K, Mg, Fe and Ca) comprise an average of 98% of the dry weight of prokaryotes.

A good first approximation of the mean composition of prokaryotic biomass is given by the formula $<C_4H_8O_2N>$, although a more elaborate formula for the composition of bacterial cells, namely $C_4H_{6.4}O_{1.5}NP_{0.09}S_{0.024}$, has been published (Battley, 1995). The total ionic constituents of the cytoplasm account for 1% of its dry weight. Consequently, the so-called "macroelements" (Table 2) are needed in relatively high concentrations in the growth medium. Most bacteria do not require Na, although many marine bacteria, certain phototrophs, and some strict anaerobes require this element. Chloride has been found to be obligatory for the growth of a variety of bacteria (Table 2). It should be noted, however, that certain gliding bacteria, e.g., the marine filamentous sulfate-reducing bacterium *Desulfonema magnum*, strictly require calcium concentrations of ≥4 mM (Widdel et al., 1983). This appears to be related to a higher Ca^{2+} requirement of gliding motility (Castenholz, 1973; Burchard, 1980).

The range of organic carbon compounds utilizable by heterotrophs is vast; virtually any compound synthesized by biological processes, as well as many xenobiotica (compounds synthesized in the laboratory which do not originate in nature), can be degraded by microbes. Different species of heterotrophic prokaryotes utilize considerably different numbers and kinds of carbon substrates. Some, such as the pseudomonads, are versatile and are known to utilize over 100 different carbon compounds as the sole source of carbon and energy. Their substrates include carbohydrates, sugar acids, polyols, fatty acids, primary alcohols, amino acids, and aromatic substances. In contrast to these versatile bacteria, several groups exist that are limited in their ability to decompose organic compounds. In this cat-

Table 2. Macroelements and their physiological functions.

Element	% dry weight	mM[a,b]	Physiological functions
C	48–59[c]		Main constituent of organic cellular material
O	13.1–23.9[c]		Organic material and cytoplasmic water
H	6.4–8[c]		Organic material and cytoplasmic water
N	13.6–14.7[c]		Proteins, nucleic acids, and coenzymes
S	0.9–1.4[c]		Cysteine, methionine in proteins, coenzymes thiamine pyrophosphate, coenzyme A, biotin, and α-lipoic acid
P	2–5.4[d]		Nucleic acids, nucleotides, phospholipids, teichoic acids, and coenzymes
K	0.03–1.4	18–800 *4500*	Predominant monovalent cation in cytoplasm, maintenance of cell osmolarity, cofactor of some enzymes (e.g., pyruvate kinase, peptidyltransferase, L-malate dehydrogenase; Walderhaug et al., 1987)
Mg	0.007–0.05	6–46[e,f]	Predominant intracellular divalent cation, cofactor of many enzymes (e.g., kinases), in phosphate esters, ribosomes, membranes, and cell wall
Ca	0.00006–0.06	0.01–9.5[g]	Present in exoenzymes (α-amylases and proteases) and in cell walls, role in transformation, Ca^{++}-dipicolinate is an important component of endospores
Na	*1.45*	*1400*	Bacterial oxaloacetate, glutaconyl-CoA, and methylmalonyl-CoA decarboxylases; NADH-quinone reductase of halophilic *Vibrio* spp. (Skulachev, 1987), transport, H_4MPT:CoM methyltransferase of methanogenic archaea
Cl	0.05	31–*1600*[f,h]	Essential for active uptake of compatible solutes and flagella formation in halophiles, e.g., *H. halophilus*
Fe	0.003–0.02[i]	1.2–7.7	Present in cytochromes, ferredoxins, and other Fe-S-proteins, cofactor in enzymes (some dehydratases)

Abbreviations: CoA, coenzyme A; NADH, reduced beta-nicotinamide adenine dinucleotide; and H_4MPT:CoM, reduced molybdopterin coenzyme M.

[a]Interconversion between % dry weight and mM was done based on the cytosolic volume of *E. coli* of 0.9 µl·(mg protein)$^{-1}$, equivalent to 0.45 µl·(mg dry mass)$^{-1}$ (Gangola and Rosen, 1987).

[b]Intracellular concentrations in halophilic archaea or bacteria are given in italics.

[c]From Norland et al. (1995) and Battley (1995).

[d]From Damoglou and Dawes (1968) and Norland et al. (1995).

[e]From Schmidt et al. (1971); up to 60 mM in moderately halophilic eubacteria (Shindler et al., 1977).

[f]From Battley (1995).

[g]In *E. coli* depending on extracellular concentrations (Gangola and Rosen, 1987). The intracellular concentration of free Ca^{2+} is 90–700 nM.

[h]In *H. halophilus* (Roeßler and Müller, 1998). Chloride has been shown to be obligatory also for growth of *Paracoccus denitrificans*, *Aeromonas hydrophila*, *Escherichia coli*, *Proteus mirabilis*, *P. vulgaris*, *Vibrio fischeri*, *Bacillus megaterium*, *B. subtilis*, *Staphylococcus aureus*, *Corynebacterium glutamicum*, and *Thermus thermophilus* (Roeßler and Müller, 2002).

[i]Niehaus et al. (1991), calculated from their value of 1.6–6.9 µmol Fe·(g protein)$^{-1}$, and Braun (1997), based on the cellular amount of iron ions of 10^5 cell^{-1} and a cytosolic volume of 0.14 µm^3. Intracellular volume calculated from a protein content of *E. coli* of 156 fg·cell^{-1} (Neidhardt and Umbarger, 1996) and the cytosolic volume given in footnote[a].

egory are the obligate methylotrophs that only use methane, methanol, dimethyl ethers, and a few other compounds. Highly specialized species are restricted to the use of only one type of organic carbon substrate, e.g., *Bacillus fastidiosus* to uric acid. Many chemoorganotrophs still require carbon dioxide in small amounts for anaplerotic reactions, e.g., the synthesis of oxaloacetate by PEP carboxylase, PEP carboxykinase, PEP carboxytransphosphorylase, and by pyruvate carboxylase (Wood-Werkman reactions). Since carbon dioxide is produced during catabolism of organic compounds, it does not normally become a limiting nutritional factor. However, some bacteria, such as *Neisseria* and *Brucella*, require higher concentrations of carbon dioxide (up to 10%) in the atmosphere for good growth in organic media, a need that must be considered in isolating and cultivating such organisms.

Oxygen and hydrogen are derived from water and, in the case of heterotrophic bacteria, from the organic carbon source. Molecular O_2 is required in only few exceptions, where an OH group is introduced by mono- or dioxygenases during the biosynthesis of cell constituents. In cellular material, nitrogen is incorporated in the reduced form as amino groups. Many prokaryotes are capable of assimilatory nitrate reduction and therefore grow with nitrate as nitrogen source. A number of aerobic and anaerobic bacteria are capable of nitrogen fixation and thus grow in media devoid of a combined nitrogen source. Some prokaryotes, such as anoxygenic phototrophs, depend on reduced nitrogen in the form of ammonium salts. Others require amino acids or oligo- or polypeptides as a source of cellular nitrogen.

A large fraction of phosphorus is bound in RNA. As a result, phosphorus demand increases with the specific growth rate of the cells. No reduced phosphorus compounds are stable, and cellular phosphorus is in the oxidation state of phosphate. Hence inorganic phosphate is the usual source of this element for microbial nutrition. Since phosphate is frequently also used as a pH buffer, it is added in excess of the growth requirement. However, high concentrations of inorganic phosphates may be growth inhibitory to at least some aquatic bacteria (Bartscht et al., 1999). Organic phosphorus sources such as glycerophosphate are an alternative supply.

Cellular sulfur is mostly in the reduced state and present in the sulfur-containing amino acids cysteine and methionine. Many bacteria are capable of assimilatory sulfate reduction and thus can utilize sulfate to satisfy the sulfur requirements for growth. Other prokaryotes, such as about half of the strains of anoxygenic phototrophic bacteria or methanogenic archaea, require sulfur in a reduced form as either sulfide or an organic compound with a thiol group, such as cysteine.

Potassium is the principal inorganic cation in the cell and reaches a concentration of about 300 mM in the cytoplasm. Although much of it is bound in the ribosomes, it is also a cofactor of some enzymes, is required for carbohydrate metabolism, and is involved in many transport processes and osmoregulation. Usually, an inorganic potassium salt (K_2SO_4 or KH_2PO_4) is added to the growth medium to satisfy this requirement.

The magnesium requirement of bacteria is principally that of bacterial ribosomes. Magnesium also functions as an enzyme cofactor and is present in cell walls and membranes. It is usually supplied as magnesium sulfate. Iron is required at micromolar concentrations, whereas the trace elements listed in Table 3 should be present in

Table 3. Microelements: their source and physiological functions.

Element	Source	Physiological functions
Mn	Mn^{2+}	Superoxide dismutase, photosystem II, some enzymes (PEP carboxykinase and citrate synthase)
Co	Co^{2+}	Coenzyme B_{12}-containing enzymes (glutamate mutase and methylmalonyl-CoA mutase)
Cu	Cu^{2+}	Cytochrome oxidase, plastocyanin, nitrite reductase, oxygenases, and superoxide dismutases[a]
Mo	MoO_4^{2-}	Nitrate reductase, nitrogenase, xanthine dehydrogenase, and formate dehydrogenase
Zn	Zn^{2+}	Carbonic anhydrase, alcohol dehydrogenase, alkaline phosphatase, aldolase, RNA and DNA polymerase, and proteinases
Ni	Ni^{2+}	Urease, hydrogenase, and cofactor F_{430}
V	$VOSO_4$	V-dependent nitrogenase, and bromoperoxidase
Se	SeO_3^{2-}	Formate dehydrogenase, hydrogenase, and glycine reductase in clostridia
W	WO_4^{2-}	Some formate dehydrogenases
B	H_3BO_3	Formation of heterocysts, akinetes in cyanobacteria; possibly crosslinking of pyranoses in cellular envelope;[b] and AI-2 (cyclic borate diester)[c,d]

Abbreviations: PEP, phosphoenol pyruvate; V-dependent, vanadium-dependent; and AI-2, autoinducer-2.

[a]Certain cyanobacteria such as *Synechococcus* are very sensitive to Cu, which therefore has to be omitted from the respective growth media.

[b]Loomis and Durst (1992).

[c]However, boric acid acts as a bacteriostatic agent under certain circumstances (Lum and Meers, 1989).

[d]Chen et al. (2002).

growth media at concentrations between 0.01 and 1 μM. Trace elements are toxic at higher concentrations. Since divalent and trivalent metal cations tend to form insoluble hydroxides or phosphates at neutral to alkaline pH, they may become unavailable to bacteria in growth media. Concentrated stock solutions of inorganic salts therefore are often kept anoxically at acidic pH, and minimal amounts of complexing agents (ethylenediamine-N,N,N′,N′-tetraacetic acid [EDTA] and nitrilotriacetic acid [NTA]) are incorporated.

In addition, a variety of microelements (Mn, Co, Cu, Mo, Zn, Ni, V and B) is required (Table 3). Certain prokaryotes, in particular strict anaerobes, often also require selenium and tungsten.

Growth Factors

Many bacteria grow with a single carbon compound added to the medium. Additional organic compounds often stimulate the growth of microorganisms when present in small concentrations. These so-called "growth factors" represent building blocks of major cell constituents, and their addition to the medium therefore decreases the need for de novo biosynthesis in the cell, if the compounds can be transported into the cell. Generally, the growth factors recognized are 1) amino acids, 2) purines and pyrimidines, and 3) vitamins. The latter are required only in small amounts (on the order of $mg \cdot liter^{-1}$, or between 0.1 and 1 μM). The biological function of a number of vitamins is listed in Table 4. Synthetic stock solutions of vitamins should be kept dark, cold, anoxic and sterile (by filtration) and at a slightly acidic pH. They should be added to the medium after autoclaving the latter.

However, many prokaryotes obligately depend on the presence of growth factors in the medium because the cells are unable to synthesize all cell constituents from a single carbon source. In more rare cases, unusual compounds such as porphyrins (hemin in the case of *Haemophilus* spp.), short-branched fatty acids (e.g., 2-methyl-*n*-butyric acid in the case of *Methanobacterium ruminantium*) or straight-chain fatty acids, mevalonic acid, cholesterol (*Mycoplasma*), choline, betaine, and polyamines are required. Some vitamins are required only during growth on specific substrates (such as B_{12} by *Escherichia coli*). Essential growth factors may include a few vitamins, such as biotin and *p*-aminobenzoic acid in the case of *Clostridium kluyveri*, or a large variety of compounds (as in the case of lactobacilli, which grow in media supplemented with peptone, Tween 80, acetate and Mn^{2+} salts [MRS-medium]). Many lactic acid bacteria including *Leuconostoc* have a requirement for Mn^{2+} (Boyaval, 1989). However, the addition of growth factors such as yeast extract or casamino acids also clearly inhibits growth of some photolithoautotrophic or chemolithoautotrophic bacteria (Overmann and Pfennig, 1989).

Growth factors may also stimulate microbial growth in an indirect fashion. Recently, evidence has accumulated for a stimulatory role of humic substances and extracellular quinones (e.g., anthraquinone-2,6-disulfonate) in dissimilatory iron reduction (Lovley and Blunt-Harris, 1999a). In addition, organic ligands such as EDTA, NTA, *n*-methyliminodiacetic acid, ethanol diglycine, deferoxamine, or phosphates, if added to growth media, make Fe(III) more available and increase rates of microbial reduction significantly (Lovley et al., 1996b; J. Overmann et al., in preparation).

Table 4. Functions of vitamins and related compounds in prokaryotes.

Vitamin	Physiological functions
p-Aminobenzoic acid	Precursor of tetrahydrofolic acid involved in one-carbon transfer
Biotin	Carbon dioxide fixation and release
Coenzyme M	Methane formation
Folic acid	Tetrahydrofolic acid involved in one-carbon metabolism
Lipoic acid[a]	Transfer of acyl groups, e.g., prosthetic group of pyruvate dehydrogenase complex
Thiamine (B_1)	Thiamine pyrophosphate is prosthetic group of decarboxylases and transketolases
Riboflavin (B_2)	Precursor of FMN and FAD, prosthetic group of flavoproteins, and redox reactions
Pyridoxine (B_6)	Pyridoxalphosphate coenzyme of transaminases and amino acid decarboxylases
Cyanocobalamin (B_{12})	Molecular rearrangement reactions (e.g., glutamate mutase)
Pantothenic acid	Precursor of CoA, prosthetic group of acyl carrier proteins, and participant in metabolism of fatty acids
Nicotinic acid	Precursor of NAD and $NADP^+$, and found in many dehydrogenases
Vitamin K	Precursor of menaquinone, and electron carrier in respiratory chains
Hemin	Precursor of cytochromes, and involved in redox reactions

Abbreviations: FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; CoA, coenzyme A; NAD, nicotinamide adenine dinucleotide; and NADP, nicotinamide adenine dinucleotide phosphate.
[a]Dithiooctanoic acid.
From Gottschalk (1985).

pH

In natural environments, the pH value varies from below 1 in acidic springs to over 11 in soda lakes (Brock, 1978; Grant and Tindall, 1986; Madigan et al., 2000a). Prokaryotes can be isolated from these environments and are capable of growing at such pH values (Brock, 1978; Langworthy, 1978; Horikoshi and Akiba, 1982; Krulwich and Guffanti, 1989). Species with optima for growth below pH 5.5 are usually called acidophiles and can grow even at pH values of 1 (e.g., *Acidithiobacillus* and *Sulfolobus*). Prokaryotes with optima above pH 8.5 are called alkaliphiles and grow up to values of pH 11.5 (e.g., *Sporosarcina pasteurii*). Many enzymes and structural components in these extremophiles cannot function properly at the very high or low pH values found in their external environment, and the intracellular pH is kept at relatively constant values (Krulwich and Guffanti, 1983; Krulwich and Guffanti, 1989; Padan and Schuldinger, 1986; Matin, 1990). In general, the internal pH of acidophiles is regulated to a value of 6.0–7.0, and alkaliphiles maintain an internal pH 1 to 2 units lower than the external value.

The majority of natural environments possess pH values between 5 and 9, and the pH optima for the growth of most prokaryotes (neutrophiles) fall well within this range. Neutrophiles keep the internal pH slightly more alkaline than this value outside. For example, *Escherichia coli* regulates its internal pH at 7.4 to 7.8 during growth over the external pH range of 5.0 to 9.0 (Slonczewski and Foster, 1996).

During the growth of microorganisms in cultures, pronounced pH changes can occur because organic acids (especially during fermentation) and ammonia from nitrogen-containing compounds are produced. When exposed to pH values at the upper or lower end of the permissive range of growth, prokaryotes may exhibit metabolic properties not displayed at normal pH values. A well-known example is the shift towards production of nonacidic end products by fermentative bacteria (Graham and Lund, 1983; Gottwald and Gottschalk, 1985; Ferchichi et al., 1986; Huang et al., 1986; Forsberg, 1987; Hommes et al., 1989). Weak organic acids also influence the cytoplasmic pH in a direct manner since in their undissociated form they are lipophilic and therefore rapidly diffuse through cell membranes, ultimately conducting hydrogen ions along the transmembrane gradient. As a result, the intracellular pH is lowered. At low pH values, the effect of extracellular pH in microbial growth is therefore magnified in the presence of organic acids. Hence, permeant acids such as benzoic, propionic, and sorbic acids are inhibitors of microbial growth (and in fact are used as food preservatives), and their potency increases with decreasing pH (Ingraham and Marr, 1996). However, the sensitivity of bacterial cells to low pH values is less pronounced in the stationary phase (Ingraham and Marr, 1996).

In high nutrient strength complex media, the various acidic and basic functional groups of organic molecules often provide sufficient buffering capacity. In mineral media and low nutrient strength complex media, continuing acidification or alkalinization of the growth medium will rapidly lead to arrest of growth of prokaryotes. Therefore, the pH has to be maintained within the permissive range for growth using appropriate organic or inorganic buffer systems (Table 5). Below pH 3, the actual concentration of hydro-

Table 5. pH buffers used for culturing prokaryotes.

Buffer	pK_a	Buffering range	Concentration	References
HOMOPIPES	4.55 (37°C)	4.0–5.0		Slonczewski and Foster, 1996
MES	5.96 (37°C)	5.5–6.5		Slonczewski and Foster, 1996
$CO_2(H_2CO_3)/NaHCO_3$[a]	6.35 (25°C)	5.4–7.8	30 mM	Widdel and Bak, 1992
PIPES	6.66 (37°C)	6.0–7.0		Slonczewski and Foster, 1996
KH_2PO_4/K_2HPO_4	7.2 (25°C)	5.8–7.8	10 mM	Bast, 2002
MOPS	7.2 (25°C)	6.5–7.7	10 mM	Bartscht et al., 1999
	7.01 (37°C)			Slonczewski and Foster, 1996
HEPES	7.5 (25°C)	6.8–8.2	10 mM	Bartscht et al., 1999
Tris·HCl	8.08 (25°C)	7.2–9.0	10 mM	Bast, 2002
TAPS	8.11 (37°C)	7.5–8.5		Slonczewski and Foster, 1996
CAPSO	9.43 (37°C)	9.0–10.0		Slonczewski and Foster, 1996
CAPS	10.08 (37°C)	9.5–10.5		Slonczewski and Foster, 1996
$NaHCO_3/Na_2CO_3$	10.4 (25°C)	8.5–11.0	50 mM	Horikoshi and Akiba, 1982

Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; HOMOPIPES, homopiperazine-*N*,*N*′-bis-2(ethanesulfonic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-[*N*-morpholino] propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); TAPS, 3-[*N*-Tris(hydroxymethyl)methylamino]propanesulfonic acid; and TRIS·HCl, Tris [hydroxymethyl] aminomethane.

[a]Applicable in closed gas tight vessels only.

nium ions becomes high enough to obviate the need for buffering.

Not all buffers listed can be applied for the isolation and cultivation of all prokaryotes. Phosphate buffers, although widely employed, inhibit the growth of bacteria from low nutrient environments such as freshwater lakes even at comparatively low concentrations of 10 mM (Bartscht et al., 1999). At higher concentrations (≥30 mM), phosphate buffers are inhibitory also to many other microorganisms (Bast, 2001). A second disadvantage of phosphate buffers is their tendency to form precipitates with Ca^{2+}, Mg^{2+} and Fe^{3+} ions already at rather low concentrations (e.g., 7 mM PO_4^{3-} and 0.7 mM Ca^{2+}). In these cases, the Ca^{2+} concentration needs to be decreased to 200 µM, which (as calculated from the cellular Ca^{2+} content; Table 2) still permits a sufficient cell yield of ≥7 grams dry weight·liter^{-1} (for exceptions such as gliding bacteria, see General Composition of the Prokaryotic Cell). The sulfonate buffers listed in Table 5 have been found to be useful in several instances (Slonczewski and Foster, 1996; Bartscht et al., 1999). Substituted amines such as TRIS (tris[hydroxymethyl]aminomethane) and triethanolamine should be avoided as they cross the cytoplasmic membrane in their deprotonated form. In general, the ionic form of a buffering substance is less toxic than its undissociated form, since the charged molecules penetrate the cellular membrane much more slowly. Consequently, cationic buffers (e.g., TRIS) are used at pH values below their p*K*, while anionic buffers are employed at a pH above their p*K* value.

Osmolarity

Microorganisms differ considerably in the amount of water they require in their immediate surroundings (Brown, 1976; Kushner, 1978; Yancey et al., 1982). At higher concentrations of aqueous solutions, forces between solutes and solvent lead to a lower "effective concentration"—the so-called "activity"—of water. Availability of water is measured as the water activity a_w, which is defined by the mole fraction of water N_w and the activity coefficient γ_w according to Schopfer and Brennicke (1999)

$$a_w = \gamma_w \cdot N_w \tag{10}$$

According to Raoult's law, the water activity in a dilute solution is equal to the ratio of the vapor pressure P of the solution relative to the vapor pressure P_0 of pure water at the same temperature

$$a_w = \frac{P}{P_0} = \frac{n_1}{n_1 + n_2} \tag{11}$$

Here, n_1 is the number of moles of the solvent and n_2 the number of moles of ideal solute. Values of water activity range between 0 and 1. The majority of microorganisms known grow well only at values between 0.9 and 1.0 (Nichols et al., 1999), as they are commonly found in aquatic and soil environments. Freshwater media used for routine cultivation usually have a_w values ≥0.99, whereas seawater (3.5% NaCl) has a value of approximately 0.98. Salt lakes in which *Halobacterium* and *Halococcus* thrive are saturated with NaCl, and a_w values are around 0.75. The lowest value reported at which growth of microorganisms still occurs is 0.61 for the xerophytic fungus *Xeromyces bisporus*, which can grow on dry foods.

Since the chemical potential of water μ_w is determined by the chemical potential of the pure solvent μ_w° and the water activity by

$$\mu_w = \mu_w^\circ + RT \ln a_w \tag{12}$$

(where R is the gas constant and T the absolute temperature), higher concentrations of solutes decrease the chemical potential of water. Most prokaryotes maintain an internal activity of water lower than that of the surrounding medium. At the same time, the cytoplasmic membrane is highly permeable to water, and bacteria have a high surface-to-volume ratio. Essentially, the chemical potential of intracellular water must be equal to that outside. As a result of the lower intracellular water activity, a tendency exists for water to enter the cell. The direct consequence is osmosis, defined as diffusion of solvent (water) through a semipermeable membrane in the direction of a decreasing chemical potential of the solvent. During this process, the free enthalpy of the entire system decreases.

Osmotic pressure π is the hydrostatic pressure (in MPa) that must be applied to a solution of lower chemical potential of the solvent to stop net diffusion of solvent through the semipermeable membrane

$$\pi \cdot V^\circ_w = -RT \cdot \ln a_w \tag{13}$$

with V°_w being the partial molal volume of water. In diluted solutions, equation (13) can be simplified (with $N_2 = n_2/(n_1 + n_2)$; $\ln a_w = \ln(1 - N_2) - N_2$; and $n_2 << n_1$):

$$\pi = \frac{R \cdot T}{V^\circ_w} \cdot N_2 \approx \frac{R \cdot T}{V^\circ_w} \cdot \frac{n_2}{n_1} = R \cdot T \cdot c_2 \tag{14}$$

where c_2 denotes the concentration of the solute. Hence, a concentration of 0.1 M of a solute at 25°C results in an osmotic pressure of 2.48 bar or 0.248 MPa. If n different solutes are present, the osmotic pressure amounts to

$$\pi = R \cdot T \cdot \sum\nolimits_i^n c_i \tag{15}$$

The presence of a cell wall permits bacteria to maintain the intracellular osmotic pressure, termed "turgor pressure." Maintenance of this turgor pressure is essential for the growth and division of the prokaryotic cell. It is therefore understandable that bacteria regulate their turgor pressure over a wide range of extracellular values of a_w. Gram-negative bacteria have turgor pressures of 0.3–0.6 MPa, while Gram-positive bacteria such as *Bacillus* sp. maintain a turgor pressure of up to 2 MPa (Reed and Walsby, 1985; Overmann et al., 1991; Ingraham and Marr, 1996; Lengeler et al., 1999).

Two major strategies have been found by which prokaryotes adjust intracellular a_w during changes of extracellular salt concentrations. Aerobic, extremely halophilic Archaea (Halobacteriales) and anaerobic, halophilic bacteria (Haloanaerobiales) accumulate inorganic ions, especially K^+ and Cl^- (Oren, 1986; Oren, 1999; Galinski and Trüper, 1994). As a special adaptation to the resulting high intracellular ion concentrations, these prokaryotes contain salt-adapted or even salt-requiring enzymes that contain a large fraction of polar and acidic amino acids (especially aspartate and glutamate), but only a low fraction of hydrophobic amino acids. Aspartate and glutamate residues bind intracellular water especially well. Consequently, correct folding of these enzymes occurs despite low intracellular water concentrations (Oren, 1999).

At lower external osmolalities (≤1 Osm), turgor pressure in *E. coli* is also mainly regulated by increasing intracellular K^+ concentrations (and its counterion, glutamate) parallel to an increase in external osmolality. The intracellular concentration of Na^+ does not vary directly with external osmolality, and intracellular concentrations of the divalent polyamine putrescine are reduced by excretion of this compound into the medium, thereby decreasing the intracellular concentration from 50 to 5 mM. It has been suggested that putrescine bound to nucleic acids is displaced by high K^+ concentrations (Csonka and Epstein, 1996).

As a second type of osmoregulation, small soluble organic molecules are accumulated upon an increase in extracellular salt concentrations. This type is found widely distributed among bacteria. The solutes accumulated do not strongly interfere with cellular metabolism and have thus been termed "compatible solutes" (Brown and Simpson, 1972; Brown, 1976). In addition, these molecules stabilize cellular proteins and increase their solubility (Cayley et al., 1992; Bolen, 2001). Thus, *E. coli* cells accumulate trehalose (*O*-α-D-glucosyl[1→1]-α-D-glucoside), accompanied by a reduction in the concentration of K^+ and glutamate. It has been suggested that this decrease in ionic strength relieves the inhibition of transcription and translation and that the cytoplasmic membrane is stabilized by interaction of compatible solutes with the polar head groups of the phospholipids (Ingraham and Marr, 1996). Various compatible solutes, zwitterionic derivatives of amino acids, sugars or polyols, have been identified and include proline, glutamate, glutamine, alanine, α-aminobutyrate, glycine betaine (*N*,*N*,*N*-trimethylglycine), ectoine, N^{ε}-acetyl-β-lysine, N^{δ}-acetyl-ornithine, β-dimethylsulfoniopropionate, sucrose and choline (Csonka and Epstein, 1996). Some of these, especially proline, glycine betaine, and choline, are also termed "osmoprotectants," since they can be added to the growth medium where they are taken up by the bacterial cells and alleviate osmotic stress effects through direct interactions with intracellular macromolecules. It has also been proposed that glycine betaine increases the fraction of free water in the cell and it is believed that osmoprotectants stabilize and protect enzymes by being excluded from the protein surface (Cayley et al., 1992). Thus, tolerance of *E. coli* towards high external osmolalities can be increased further by the addition of osmotic protectants such as proline, glycine betaine, choline (which is metabolized to glycine betaine), trimethyl-γ-aminobutyrate, β-alanine betaine, taurine betaine, carnitine, or even MOPS (3-[*N*-morpholino] propanesulfonic acid) to the growth medium (Csonka and Hanson, 1991). The latter is a buffer commonly used in growth media, which has been shown to accumulate in *E. coli* at high osmolality (Cayley et al., 1989). Glycine betaine is the most effective osmotic protectant of *E. coli* and *Salmonella typhimurium*. Accumulation of glycine betaine reduces the concentration of endogenous trehalose as well as ionic solutes.

The osmoregulatory potential varies between microorganisms, which can be divided according to their osmotic tolerance. This classification has been used most often for NaCl tolerance. Nonhalophiles thus are organisms capable of growth at NaCl concentrations of >0.2 M, whereas moderate halophiles and marine species usually grow from 0.2–3.5 M NaCl, and extreme halophiles grow from 1–5.5 M NaCl (Kushner, 1978; Yancey et al., 1982; Epstein, 1986; Imhoff, 1986; Larsen, 1986).

Temperature

Prokaryotes can grow at temperatures between –10 or even lower (Morita, 1975; Baross and Morita, 1978; Mazur, 1980; Carpenter et al., 2000) and +113°C (in the case of the archaeon *Pyrolobus fumarii*; Blochl et al., 1997; Stetter, 2001). The upper limit for microbial growth is suggested to be between 110 and 150°C owing

to constraints on the thermostability of several essential cell components. The half life for ATP under these conditions is <1 second, and it is ~1 millisecond for polynucleotides (Bernhardt et al., 1984; White, 1984; Jaenicke, 1988). At the other extreme, bacterial activity has been shown to proceed at –17°C (Carpenter, 2000), and microbial life has been discovered below 3500 m of ice in the Antarctic subglacial Lake Vostok (Price, 2000). Individual strains can grow over a temperature range of 10 to maximally 60°C (usually 30–35°C; Wiegel and Ljungdahl, 1986; Brock, 1987; Wiegel, 1990). On the basis of their minimum, optimum, and maximum temperatures for growth (the so-called "cardinal temperatures T_{min}, T_{opt}, T_{max}"), organisms are currently divided into four major groups: psychrophiles (T_{opt} ≤20°C), mesophiles (T_{opt} between 20 and 42°C), thermophiles (T_{opt}, 42–70°C) and hyperthermophiles (T_{opt}, >70°C). In addition, the term "psychrotolerant" (previously "psychrotrophic") has been coined to describe those prokaryotes capable of growing at temperatures between 0 and 5°C but reach maximum temperatures of growth exceeding 25°C (Morita, 1975). Different temperature ranges for psychrophilic and psychrotolerant prokaryotes have been used, however (Isaksen and Jørgensen, 1996).

Generally, the optimum temperature for growth is only a few °C lower than T_{max}. Consequently, it is advisable to perform routine cultivation at incubation temperatures below T_{opt}. It is very difficult to determine the minimum growth temperature, as doubling times can become extremely long at the low end of the temperature range.

As a rule of thumb, the rate constants of chemical reactions increase by a factor of about 2 (the so-called "Q_{10} value;" range, 1.5–4) when the temperature is increased by 10°C. This exponential dependence on temperature exists for biochemical reactions (e.g., firefly bioluminescence reaction) and likewise for microbial metabolism (e.g., sulfate reduction; Bak and Pfennig, 1991; Isaksen and Jørgensen, 1996) and microbial growth. Hence, the specific growth rate doubles with a 10°C increase in temperature for many bacteria. Like rate constants of chemical reactions, the temperature dependence of the microbial growth rate between the minimum and optimum temperature can be described by the Arrhenius equation (Arrhenius, 1889):

$$\mu = A \cdot e^{-\frac{E_o}{R \cdot T}} \tag{16}$$

in which μ represents the specific growth rate, E_a the activation energy, R the gas constant (8.31 $J \cdot K^{-1} \cdot mol^{-1}$), T the temperature (in K), and the constant A the collision or frequency factor (in h^{-1}), which in chemical reactions describes the collision frequency and orientation of reacting molecules. If applied to bacterial growth or physiological activity, E_a does not represent activation energy in the chemical sense, but rather a measure of the temperature response of the bacteria. Consequently, E_a has sometimes also been termed "the temperature characteristic" (King and Nedwell, 1984). This equation permits an assessment of the temperature dependence of growth or physiological activity within certain limits. If the logarithm of μ is plotted versus the reciprocal of T, a straight line is obtained with a negative slope of $-E_a/2.303 \cdot R$ (Ingraham, 1962; Harder and Veldkamp, 1971; Ratkowsky et al., 1983):

$$\ln \mu = -\frac{E_a}{R} \cdot \frac{1}{T} + \ln A \tag{17}$$

Typical E_a values for the specific growth rate or the physiological activity of bacteria are in the range of 23–132 $kJ \cdot mol^{-1}$ (Bak et al., 1991; Ingraham and Marr, 1996; Knoblauch et al., 1999b). On the basis of equation (3), the Q_{10}-value can be calculated according to:

$$Q_{10} = \exp\left[\frac{E_a \cdot 10}{R \cdot T(T+10)}\right] \tag{18}$$

At the low, and especially at the high, temperature end, the data of the logarithmic plot of log μ versus T^{-1} deviate from linearity. In the case of psychrophiles, the slope of the Arrhenius plot is linear down to 0°C, whereas for psychrotolerants, it deviates from linearity at about 5°C, and for mesophilic bacteria, it tends to deviate from a straight line at even higher temperatures (Harder and Veldkamp, 1971). At high temperatures, the growth rate decreases sharply due to the thermal inactivation of enzymes and disruption of the membrane structure. It should be mentioned that alternative models for the relationship between temperature and growth rate constant have been developed which fit the experimental data of many bacterial strains more precisely (Ratkowsky et al., 1983).

One important factor that determines minimum and maximum temperatures for growth is the fluidity of the membrane lipids, affected particularly by the ratio of monounsaturated and saturated fatty acids, their length, and by proportion of cyclic fatty acids (Russel, 1984; Herbert, 1986; Russel and Fukanaga, 1990; Jones et al., 2002). For example, if the viscosity of the membrane cannot be maintained within certain limits, it may ultimately become leaky to ions at high temperatures. A second factor is the solute transport capability of the membrane (Baxter and Gibbons, 1962; Rose and Evison, 1965), which is dependent on its fluidity. Thirdly, the forces governing the formation of proper tertiary and

quartenary structure of proteins (Jaenicke, 1988) limit bacterial growth especially in the high temperature range. The first enzyme inactivated (e.g., homoserine transsuccinylase in *Escherichia coli*; Ingraham and Marr, 1996) determines T_{max}. Hence, the latter can be increased when the product of this critical enzyme is supplied exogenously (methionine in *E. coli*). Several lines of evidence suggest that the inability to initiate translation and hence to synthesize protein determines the minimum temperature for growth (Ingraham and Marr, 1996). Finally, an important factor determining maximum and minimum growth temperatures is the temperature sensitivity of regulatory mechanisms, which in case of malfunctioning may cause fatal imbalances in cellular metabolism.

At very high temperatures >80°C, proton circuits can no longer be maintained owing to a high proton permeability of the cytoplasmic membrane. Hyperthermophiles switch to energy coupling via Na^+ since the permeability of the membrane to this ion is less affected by high temperature (Lengeler et al., 1999). Also, the pattern of proteins changes significantly with growth temperatures outside the normal range. Changes that occur at higher temperatures are under the control of the heat shock response. About 20 proteins, many of them molecular chaperones or proteases, exhibit a large (10- to 20-fold) but transient increase in synthetic rate upon temperature upshift (Gross, 1996).

In mesophilic bacteria, a specific group of 14 cold shock proteins (none of which is a heat shock protein) is produced during the period of growth cessation following a shift from 37 to 10°C in *E. coli* (Graumann and Marahiel, 1996). Considerable evidence suggests that the inability to synthesize protein determines the minimum temperature of growth in *E. coli* and that the sensitive step is the initiation of translation (Ingraham and Marr, 1996; Graumann and Marahiel, 1996). Additional limiting factors of growth at low temperature are the fluidity of the cytoplasmic membrane and rate of local melting of DNA by RNA polymerase (Graumann and Marahiel, 1996). In psychrophiles, cell membranes tend to contain more unsaturated fatty acids and short-chain fatty acids than membranes of mesophiles (Bhakoo and Herbert, 1979; Chan et al., 1971). Finally, the affinity for substrate uptake is decreased at low temperatures (Nedwell and Rutter, 1994). Psychrophiles synthesize enzymes with high catalytic activities at low temperatures (Feller et al., 1994b; Trimbur et al., 1994) and produce more enzymes upon a decrease in temperature (Feller et al., 1994a). The lowest optimum temperature for an extracellular enzyme recovered from a bacterial culture was 20°C for a protease. The corresponding bacterial isolate produced the maximum amount of protease at –1°C, thereby counteracting the effects of very low temperatures on the catalytic efficiency of the enzyme (Huston et al., 2000). Chitobiase and leucine-aminopeptidase in situ even have lower T_{opt} values of 15°C (Huston et al., 2000). Enhancement of the catalytic activity of psychrophilic enzymes generally is attributed to an increased flexibility of some of their structural components, which at the same time leads to a reduction of their thermostability. Cold-active enzymes thus exhibit a pronounced heat lability and sensitivity to protein denaturants (Lonhienne et al., 2000). As another aspect of psychrophily, posttranscriptional modification of tRNA is much less pronounced in psychrophiles than in mesophiles. However, significantly higher levels of dihydrouridine occur in psychrophiles as compared to psychrotolerants or mesophiles (Dalluge et al., 1997). This latter finding has been explained by the high conformational flexibility, which is maintained by a high dihydrouridine content of tRNA molecules.

Not only the overall specific growth rate, but also the physiology of a given strain changes upon changes in incubation temperature. Examples are the formation of different fermentation products (Jung et al., 1974), change in yield and maintenance coefficients (Brooke et al., 1989), altered specific extracellular xylanase activity (Suh et al., 1988), changes in the affinity for H_2 and acetate consumption by *Methanosarcina barkeri* (Westermann et al., 1989), changes in the content of unsaturated or branched fatty acids in membrane lipids (Russel, 1984; Kaneda, 1991), and formation of secondary metabolites like pigments, e.g., prodigiosin formed only below 30°C by *Serratia marcescens* (Burkhardt, 1992). Thus, different enzymes in one and the same prokaryotic strain appear to exhibit considerable differences in thermostability.

Over 80% of the biosphere is permanently below 5°C, which is mainly due to a rather constant temperature of about 2°C in two thirds of deep ocean water (Graumann and Marahiel, 1996). Chemostat experiments and isolation attempts suggest that nutrient cycling in the maritime Antarctic may be due to the activities of psychrophilic bacteria (Herbert, 1986). Evidence has been obtained for low rates of bacterial DNA and protein synthesis, which indicates that bacteria clustering with the *Deinococcus-Thermus*-group are metabolically active at ambient subzero temperatures of –12 to –17°C (Carpenter et al., 2000). If some simple procedural precautions are observed, psychrophilic microorganisms can be readily isolated from natural environments. Besides using precooled pipettes, media, diluents, etc., the inoculum should not be exposed to lethal temperatures

above 20°C (room temperature). Isolates obtained belong to the low G+C Gram-positive bacteria, high G+C Gram-positive bacteria, α-, β-, γ- and Δ-subclasses of the Proteobacteria (and include purple nonsulfur bacteria, methanotrophs and sulfate-reducing bacteria), and the *Cytophaga-Flavobacterium* group (Bowman et al., 1997c; Gosink and Staley, 1995; Knoblauch and Jørgensen, 1999a; Knoblauch et al., 1999b; Madigan et al., 2000a). An overview of the diversity of recently described psychrophilic bacteria is presented in Table 6.

Most temperate environments, like deeper parts of the water column of lakes and deeper soil strata, are permanently below 20°C. It therefore appears possible that bacteria in these environments are adapted to these lower temperatures. Indeed, first experiments indicate that the fraction of lake water bacteria growing in artificial liquid media reaches maximum values at 16°C (Bussmann et al., 2001). Hence the preferred temperature used for enrichments of freshwater or marine planktonic bacteria is 15°C. Some psychrotolerant bacteria have been isolated from packed food after storage at 2–4°C for several weeks (Broda et al., 2000; Kato et al., 2000). Like psychrophilic bacteria, psychrotolerants also fall in various phylogenetic groups such as low G+C Gram-positive bacteria (including acetogens; Nozhevnikova et al., 2001), high G+C Gram-positive bacteria, Proteobacteria, but also methanogenic archaea (Nozhevnikova et al., 2001; Table 6). Sulfate-reducing bacteria isolated from cold sediments are psychrotolerants with respect to growth rate and show a T_{opt} of 18–19°C, whereas the maximum growth yield was attained at much lower temperatures of 0 and 12°C (Isaksen and Jørgensen, 1996). However, the sulfate-reducing activity of bacteria in Antarctic sediments had a temperature optimum well above the in situ temperature, exhibiting a mesophilic response (Isaksen and Jørgensen, 1996). Consequently, the temperature response of a given bacterial strain cannot be judged solely on the basis of respiratory activity or growth rate alone.

Hydrostatic Pressure

Besides temperature, osmolarity and pH, hydrostatic pressure has been shown to directly influence the growth of prokaryotes. Hydrostatic pressure is a decisive environmental variable in the deep sea. At nearly 11,000 m, the Challenger Deep is the deepest known oceanic site where pressure values greater than 100 MPa (1000 atm) exist. Since the oceans cover about 71% of the earth's surface at a mean depth of 3700 m, the high-pressure, cold habitat (37 MPa, ≤5°C) represents the largest portion of the biosphere by volume.

As early as 1872, the Challenger expedition definitely revealed the occurrence of living material from depths of at least 8000 m. Twelve years later, the bacteria found in samples from the deep sea were shown to be more pressure tolerant than terrestrial species (Marquis and Matsumura, 1978). These findings have now been rigorously confirmed. Barophilic microorganisms, recently also termed "piezophilic," are defined as those well adapted to growth at high pressure (ZoBell and Johnson, 1949; Jannasch and Taylor, 1984), and hence they exhibit optimum growth rates at elevated pressures. Barophilic prokaryotes are usually found below a depth of 2000 m in the ocean. At high hydrostatic pressures and 2°C, doubling times as short as 7 hours have been observed (Yayanos, 1986). Obligate barophiles grow only at pressures exceeding 0.1 MPa (1 atm). In studies of more than 100 bacterial strains from depths between 2000 and 7000 m, obligate barophily has been detected only in isolates from ≥ 6350 m (Yayanos, 1986). The maximum pressure permitting growth of an obligate barophile was determined as 115 MPa (Deming et al., 1988). Some barophilic bacteria grow at pressures >100 MPa (in one case even 130 MPa, a value reached nowhere in the ocean; Yayanos, 1986). Many abyssal and hadal prokaryotes were shown to be barophilic (Yayanos, 1986). On the contrary, barotolerant microorganisms, which are also abundant at great depth, grow fastest at 0.1 MPa and more slowly as hydrostatic pressure is increased. Barophilic bacteria isolated from the deep sea are also psychrophiles (T_{opt} 8–10°C). However, the maximum temperature of growth (T_{max}) is higher at high pressure (Yayanos, 1986). On the other hand, barophilic bacteria show barotolerant properties if cultured not at 10–15°C, but at 4°C, which corresponds to the actual temperature of their environment, and decreasing substrate concentrations induce a more efficient barophilic response in certain deep-sea psychrophiles (Wirsen and Molyneaux, 1999).

Pressure-retaining devices have been designed which allow the sampling of barophilic prokaryotes without decompression (Jannasch et al., 1976; Yayanos, 1978). Phylogenetically, many barophilic bacteria fall in the γ-subclass of Proteobacteria (DeLong et al., 1997). The major genera of cultivated barophiles include the γ-proteobacterial genera *Shewanella*, *Photobacterium*, *Colwellia*, and *Moritella*. In addition, members of other physiological and phylogenetic groups have been described, e.g., the sulfate-reducing *Desulfovibrio profundus* (Bale et al., 1997). Most characterized barophiles are thus closely related to shallow-water marine bacteria. Most of the prokaryotes isolated are also psychrophiles and some are capable of growing at

Table 6. Some psychrophilic and psychrotolerant bacteria described to date.

Group	Species	Strain	References
Psychrophiles			
Low G+C Gram+	*Bacillus marinus*	DSM 1297^T	Ruger et al., 2000
	Sporosarcina psychrophila	DSM 3^T	Euzéby, 2001
High G+C Gram+	*Arthrobacter psychrolactophilus*	ATCC 700733^T	Loveland-Curtze et al., 1999
	Clostridium vincentii	DSM10228^T	Mountfort et al., 1997
	Cryobacterium psychrophilum	NCIMB 2068^T	Suzuki et al., 1997
	Ditzia psychralcaliphila	NCIMB13777^T	Yumoto et al., 2002
	Frigoribacterium faeni	DSM 10309^T	Kampfer et al., 2000
	Subtercula boreus	DSM 13056^T	Mannisto et al., 2000
	Subtercula frigoramans	DSM 13057^T	Mannisto et al., 2000
CFB	*Flavobacterium frigidarium*	ATCC 700810^T	Humphry et al., 2001
	Gelidibacter algens	ACAM 536^T	Bowman et al., 1997b
	Polaribacter franzmannii	ATCC 700399^T	Gosink et al., 1998
	Polaribacter filamentus	ATCC 700397^T	Gosink et al., 1998
	Polaribacter irgensii	ATCC 700398^T	Gosink et al., 1998
	Psychroflexus torquis	ACAM623^T	Bowman et al., 1998a
	Psychroserpens burtonensis	ACAM 188^T	Bowman et al., 1997b
β-Proteobacteria	*Polaromonas vacuolata*	34-P^T	Irgens et al., 1996
	Rhodoferax antarcticus	ATCC700587^T	Madigan et al., 2000
γ-Proteobacteria	*Acinetobacter calcoaceticus*	LP009	Pratuangdejkul and Dharmsthiti, 2000
	Glaciecola pallidula	ATCC 700757^T	Bowman et al., 1998b
	Glaciecola punicea	ATCC 700756^T	Bowman et al., 1998b
	Methylosphaera hansonii	ACAM 549^T	Bowman et al., 1997a
	Moritella marina	ATCC 15381^T	Urakawa et al., 1998
	Psychrobacter pacificensis	IFO 16270^T	Maruyama et al., 2000
	Psychromonas antarcticus	DSM 10704^T	Mountfort et al., 1998
	Shewanella frigidimarina	ATCC 700753^T	Bozal et al., 2002
	Shewanella gelidimarina	ATCC 700752^T	Bowman et al., 1997c
	Shewanella livingstonensis	LMG 19866^T	Bozal et al., 2002
	Thiocapsa sp.	Ant.Rd	Madigan, 1998
δ-Proteobacteria	*Desulfofaba gelida*	DSM 12344^T	Knoblauch and Jørgensen, 1999
	Desulfofrigus fragile	DSM 12345^T	Knoblauch and Jørgensen, 1999
	Desulfofrigus oceanense	DSM 12341^T	Knoblauch and Jørgensen, 1999
	Desulfotalea arctica	DSM 12342^T	Knoblauch and Jørgensen, 1999
	Desulfotalea psychrophila	DSM 12343^T	Knoblauch and Jørgensen, 1999
Psychrotolerants			
Archaea	*Methanococcoides burtonii*	DSM 6242^T	Franzmann et al., 1992
CFB	*Gelidibacter* sp.	IC158	Nichols et al., 1999
	Psychroflexus gondwanense	ACAM 48^T	Bowman et al., 1998
Low G+C Gram+	*Acetobacterium tundrae*	DSM 9173^T	Simankova et al., 2000
	Carnobacterium funditum	DSM 5970^T	Franzmann et al., 1991
	Clostridium gasigenes	DSM 12272^T	Broda et al., 2000
	Lactobacillus bavaricus	DSM 20269^T	Euzéby, 2001
	Lactobacillus algidus	JCM 10491^T	Kato et al., 2000
High G+C Gram+	*Arthrobacter flavus*	MTCC 3476^T	Reddy et al., 2000
	Arthrobacter globiformis	DSM 20124^T	Euzéby, 2001
	Brevibacterium	NCIMB 13216	Nedwell and Rutter, 1994
	Micrococcus agilis		Siebert and Hirsch, 1988
	Micrococcus roseus		Siebert and Hirsch, 1988
β-Proteobacteria	*Hydrogenophaga pseudoflava*	NCIMB 13215	Nedwell and Rutter, 1994
γ-Proteobacteria	*Pseudomonas alcaliphila*	IAM 14884^T	Yumoto et al., 2001

Abbreviations: CFB, cytophaga/flexibacter/bacteroides; DSM, Deutsche Sammlung von Mikroorganismen; ATCC, American Type Culture Collection; NCIMB, National Collections of Industrial Food and Marine Bacteria; ACAM, Australian Collection of Antarctic Microorganisms; IFO, Institute for Fermentation Culture Collection; LMG, Universiteit Gent, Laboratorium voor Mikrobiologie, Gent, Belgium; JCM, Japanese Collection of Microorganisms; MTCC, Microbial Type Culture Collection at Institute of Microbial Technology, Chandigarh, India; and IAM, Institute of Applied Microbiology, University of Tokyo.

very low nutrient concentrations (Deming and Colwell, 1985; Deming, 1986). The latter observation indicates that barophiles are not confined to nutrient-rich niches, such as fecal pellets and inside higher organisms, but are also found free in the water column and in the sediment. Since at least some barophilic prokaryotes are oligotrophs, high-pressure continuous culture techniques had to be developed which now permit a study of barophilic prokaryotes at pressures of up to 71 MPa and at low and precisely controlled nutrient concentrations (Jannasch et al., 1996; Wirsen and Molyneaux, 1999).

High pressures affect different aspects of cell structure and metabolism, such as membrane structure, transcription, translation, and the quartenary structure of enzymes, and hence their activity (Marquis, 1976; Marquis and Matsumura, 1978; MacDonald, 1984; DeLong and Yayamos, 1986; Morita, 1986; Wirsen et al., 1987; Jaenicke, 1988; Somero, 1992; Welch et al., 1993). On the basis of direction of the change in molecular volume, biochemical reactions can be slowed down or accelerated. In fact, increased hydrostatic pressure can accelerate the fructose bisphosphate reaction in a barophilic organism, while decreasing it in a non-barophilic one (Hochachka et al., 1972). However, most of the biological reactions are slowed down at pressures of 30 MPa or more (Ingraham and Marr, 1996). *Escherichia coli* are moderately barotolerant and withstand a maximum pressure of 56 MPa in complex medium. Five MPa cause a detectable decrease in growth rate. The pressure sensitive steps are polysome formation (Schultz et al., 1976) and translocation. Genetic changes in ribosome structure can increase the barotolerance of *E. coli* (Ingraham and Marr, 1996).

The molecular adaptations required for barophily are largely unknown. Changes in membrane fluidity (which is negatively influenced by high pressures) are known to occur through changes in the ratio of unsaturated over saturated fatty acids in membrane phospholipids (MacDonald, 1984; DeLong and Yaymos, 1986; Wirsen et al., 1987), similarly to responses to temperature changes. The barophilic bacterium *Photobacterium profundum* strain SS9 preferentially synthesizes a 37-kDa protein, designated "OmpH," in response to an elevated pressure of 290 atm (29 MPa). On the basis of amino acid sequence comparison, OmpH is an outer membrane porin and possibly especially adapted to a high pressure (Bartlett et al., 1989), and it may represent a member of a high-pressure regulon (Bartlett and Welch, 1995). Control of the abundance of OmpH is probably regulated at the transcriptional level. Unusual putative regulatory sequences have been found upstream of the *ompH* gene (Bartlett et al., 1993; Bartlett and Welch, 1995). In addition to hydrostatic pressure, OmpH is induced by carbon starvation and subject to catabolite control (Bartlett and Welch, 1995). Additional pressure-regulated operons have been identified, and for instance comprise, a gene involved in the assembly of the cytochrome *bd* complex (Li et al., 1998). Alternative RNA polymerase σ factors (e.g., the *rpoE* gene product) and modifiers are involved in genetic regulation by hydrostatic pressure (Chi and Bartlett, 1995; Nakasone et al., 1998).

Treatment of Growth Media

Types of Culture Media

Conventional bacteriological culture media are provided as either liquid broths or solid media. Liquid media are used in studies of growth and metabolism in which homogenous media conditions are mandatory. Usually, optical density can be followed easily in liquid media and subsamples for the analysis of substrates and metabolic products can be withdrawn. In addition, many bacteria, especially from planktonic samples, do not appear to grow on solid media and have to be isolated in liquid dilution series. To maintain an adequate supply of oxygen, cultures of aerobic prokaryotes need to be shaken vigorously, leaving a large head space (gas phase volume).

Solid media were originally designed for the enumeration and isolation of bacteria, but are now also used routinely for general culture work. On solid media, colony morphology and other properties (such as swarming over the agar surface) can be easily observed. Extracellular enzymes originating from the cells but diffusing into the surrounding non-colonized media can be detected as a result of their action on insoluble substrates (cellulose, starch, and lipid emulsions), which can be maintained evenly distributed in solid as opposed to liquid media. Hydrolysis of starch can be visualized after flooding plates with iodine, since only the intact starch molecules produce the characteristic deep-blue to purple color. Lipids can be stained with Sudan black. Furthermore, the effects of antibiotic substances on colony growth can be tested on solid media. Solidifying agents include agar, Gelrite and silica gel. Agar is used most frequently and is sulfated polygalactans (D-galactose and 3,6-anhydrogalactose linked by $1 \rightarrow 3$ and $1 \rightarrow 4$ bonds) produced by marine red algae of the genera *Gelidium*, *Pterocladia* and *Gracilaria*. This polymer is degraded by only very few bacteria such as some *Cytophaga*, *Pseudomonas* and *Vibrio* spp. Unusual is the large difference between the temperatures for melting (~100°C) and solidification (~40°C).

Consequently, many temperature-sensitive constituents of the media, e.g., vitamins, may be added at temperatures slightly above 40°C. Agar-containing media should not be adjusted to pH values <6.0 before sterilization, because the agar may be hydrolyzed. If lower pH values are required, the adjustment should be done by aseptic addition of acid after heat treatment or, alternatively, solid silica gel media should be used. Agar may contain variable amounts of impurities such as Ca, Mg, and other minerals (Bromke and Hammel, 1987). One means of reducing soluble nonpolymeric contaminants is the repeated washing in distilled water (e.g., five times in double-distilled water, using 300% of the final volume; Widdel and Bak, 1992). Alternatively, agar has been purified by subsequent extraction with acetone and ethanol. For direct plating of oligotrophs from the marine environment and if even the washed agar inhibits growth, a glass filter may be used as a substitute.

Gellan gum (or Gelrite, Phytagel, Sigma-Aldrich) is another alternative for solidification of microbial media. Harris (1985) proposed gellan gum as an agar substitute since agar at high concentrations may be toxic to methanogens. Gellan gum (which is produced by strains of *Sphingomonas* spp.) is an anionic acidic heteropolysaccharide consisting of glucose, glucuronic acid and rhamnose. Gellan gum overcomes some of the toxic effects that agar has on some groups of microorganisms (Ferris et al., 1996; Liesack et al., 1997) and starts to solidify upon addition of Ca^{++}; the concentration of divalent cations influences gel strength and solification temperature (Bast, 2001).

Silica gel media have been developed for use when solid media free of any organic contamination are needed, low pH is desired, or agar-degrading microorganisms are to be cultivated (Funk and Krulwich, 1964; Bast, 2001).

Media may affect the growth of microorganisms nonselectively or selectively. Selective media favor the growth of only some bacteria by the inclusion of a particular substrate as a carbon/energy source, by the presence or absence of specific nutrients (e.g., nitrogen or vitamins), or by the presence of compounds with differential toxicity. Inhibitory compounds include: 1) antibiotics with rifampicin (2.4), kanamycin (5.0), erythromycin (50), penicillin (50), tetracycline (100) and fosfomycin (100 [maximum concentrations in $mg \cdot liter^{-1}$]) acting against bacteria, and cycloheximide (100), tunicamycin (0.25), colchicine (20) or cordycepin (25) inhibiting eukarya; 2) dyes (such as crystal violet, methylene blue, or brilliant green) inhibit many Gram-positive bacteria; 3) high concentrations of glycine and LiCl, which permit the growth of many staphylococci but not that of physiologically similar other bacteria; 4) bile salts, which permit the growth of enteric bacteria but not that of many other bacteria; and 5) bromoethane sulfonate (BES) for inhibition of methanogenic archaea, or sodium molybdate for the inhibition of sulfate-reducing bacteria.

Selective media are especially used for the isolation, detection and recognition of pathogenic bacteria from mixed cultures, but are equally important for the isolation of selected groups of slow-growing bacteria from environmental samples. In a strict sense, however, all media are at least slightly selective.

Differentiating media are designed to distinguish one type of microorganism from another in a mixed culture. A differentiating medium contains a special ingredient that changes during growth of a certain type of bacterium. They are designed to differentiate between morphologically or physiologically similar microorganisms (e.g., hemolytic reaction on blood agar, urease, production of acetoin, and reduction of tellurite). Differentiating media can be selective or nonselective.

Assay media are used for the quantification of organic substances such as vitamins, amino acids, and growth factors in bioassays, in which the growth response of a certain organism requiring the factor is directly proportional to the concentration of the factor under investigation.

Media Preparation and Sterilization

Heat-labile supplements such as serum, vitamins, or growth factors (e.g., freshly prepared yeast extract or fermented rumen fluid) are added to the basal medium after sterilization to avoid deterioration. This is also a recommended practice for compounds that might react with other medium ingredients during autoclaving. Glucose and other sugars, when autoclaved with salts such as phosphate, may form inhibitory sugar phosphates. The carbonyl groups of reducing sugars react with free amino groups of primary amines and may result in the formation of toxic Maillard reaction (or "browning" reaction) products such as furfurals or furaldehyde. Oxygen or oxidation products accelerate Maillard reactions. Reducing agents such as cysteine and sulfide will be oxidized by other medium ingredients during autoclaving and form toxic radicals (Carlsson et al., 1979; Cypionka et al., 1985) and thus have to be added separately afterwards. The formation of mineral precipitates (e.g., struvite or $MgNH_4PO_4$; Schink et al., 2002) can often be avoided by separate sterilization of solutions of the calcium, magnesium, and/or iron salts, which are added to the cooled medium. Addition of chelating agents such as EDTA helps to prevent the precipitation in some cases.

In many instances the microelements are present in adequate amounts as contaminants of the mineral salts used in media or as contaminants of glassware and water. In several cases, however, microelements need to be added separately to the growth media. Dispensing of media is usually carried out after cooling to below 50°C to avoid formation of condensation.

Treatments can be distinguished on the basis of their effect on total and viable cell numbers with exposure time. Bacteriostatic substances inhibit growth of bacterial cells, while total cell numbers and the viable cell count (determined, e.g., by plating of cells after diluting out the inhibitory substance) remain constant. Bacteriocidal compounds lead to a decrease, hence an irreversible damage, of viable cells, while total cell numbers remain constant. Finally, bacteriolytic compounds lead to a decrease in viable as well as total cell numbers owing to prokaryotic cell lysis.

Sterilization—the complete inactivation or removal of microorganisms—is achieved by applying heat or irradiation (physical methods) or by treatment with toxic compounds and gases (chemical methods). Gases and liquids can also be sterilized by filtration through filters with extremely small pores (preferably ≤0.1 μm). Disinfection is a procedure that results in the inactivation of only a fraction of the microbial population from an object or from a culture and is most often used to inactivate pathogens. When a pure culture is exposed to a lethal agent, the kinetics of death are exponential, i.e., when the logarithm of the number of survivors (usually determined by counting colony-forming units on a suitable medium) is plotted against time, a straight line is obtained whose downward slope is called the "death rate." The time course of killing is described by the *D*-value, which gives the decimal reduction time, i.e., the time it takes for a 10-fold reduction in the microbial population at a particular temperature. The *Z*-value is the number of degrees that the temperature must be raised to reduce the *D*-value 10-fold, hence

$$Z = \frac{T_1 - T_2}{\log D_1 - \log D_2} \qquad (19)$$

The actual number of survivors is then determined by the initial size of the population and the death rate. Differences exist among microbial species in their resistance to heat, radiation, and other treatments. Hence, sterilization procedures need to be designed to kill the most resistant forms of life, namely the endospores of Gram-positive bacteria. Of the latter, *Moorella thermoacetica* forms unusually heat-resistant endospores which reach decimal reduction times of up to 111 min at 121°C (Byrer et al., 2000). Consequently, purified suspensions of bacterial endospores are used as indicators for the effectiveness of the sterilization process. Routine sterilization procedures are designed to provide a wide margin so that the chance of having even a single survivor is less than one in a million.

Direct heat, dry heat, and moist heat are the three most common methods of sterilization. For sterilization by direct heat, objects are exposed to an open flame, and the adhering microorganisms are quickly burned. Small equipment such as inoculating needles and loops, forceps, open ends of culture tubes, and Pasteur pipettes are routinely flame-sterilized. In addition, combustion is the method of choice for destruction of disposables and contaminated wound dressings in hospitals. When employing a bunsen burner for sterilization, it has to be kept in mind that air supply must be regulated such that the flame generated is completely blue (not yellow-orange) and that the point where the highest temperature is reached is found atop of the inner (dark-blue) cone within the flame. A yellow color of the flame indicates the presence of sooty particles, hence incomplete combustion and lower temperatures. Dry heat is used to sterilize empty glassware and other heat-resistant objects such as laboratory instruments, surgical tools, glass syringes, needles, mineral oils, and dry powders of heat-stable substances and involves baking at 170°C for 2 hours in a hot-air oven. The objects should be protected from subsequent contamination by wrapping them in aluminum foil prior to sterilization.

Obviously, dry sterilization cannot be used to sterilize liquids, which would boil at temperatures above 100°C at atmospheric pressure. Also, the method is not suited for the sterilization of heat-sensitive materials such as cotton wool, plastics and rubber. In addition, heat conduction is less rapid in moist air and dried bacterial cells and spores intrinsically have a higher heat resistance than wet cells and wet spores, which makes sterilization by dry heat a more time-consuming process. Therefore, moist heat is the most effective and most commonly used method for sterilization. Autoclaving denotes a heat treatment with a water-saturated atmosphere under pressure. In principle, the autoclave represents a type of pressure cooker in which a pressure of 1 atm and a temperature of 121°C are reached simply by heating or by inflow of preformed pressurized steam. Only if the autoclave chamber is completely filled with steam can a temperature of 121°C be reached. This temperature, provided a sufficient exposure time is chosen, is sufficient to kill even bacterial endospores. Therefore the temperature has to be monitored when checking the reliability of the sterilization procedure. As a

rule of thumb, 50 minutes of autoclaving are sufficient to sterilize one liter of liquid even when contaminated with endospore-forming bacteria (but compare *Moorella thermoacetica*). After sterilization, the steam is allowed to escape slowly to prevent boiling of liquids, which would otherwise occur upon a sudden drop in pressure.

Pasteurization is a mild treatment with moist heat employed to control spoilage of food products and to extend their shelf life without significant decay of heat-labile constituents such as vitamins. One treatment is the low-temperature-long-time (LTLT) procedure in which, e.g., milk is heated for 30 min at 63°C. In the more frequently used high-temperature-short-time (HTST) or flash procedure, heating lasts for 20 sec at a temperature of 72°C followed by rapid cooling to minimize undesirable changes in taste and nutrient content. Pasteurization reduces the number of viable cells by between 97 and 99% and is intended to eliminate pathogens. It was introduced by L. Pasteur to control the spoilage of wine. However, even vegetative cells of certain non-sporeforming bacterial species, such as *Microbacterium lacticum*, *Enterococcus* spp., and *Coxiella burnetii*, are capable of surviving pasteurization and lead to subsequent food spoilage.

Filtration is the method of choice for the sterilization of heat-sensitive liquids and gases, which are passed through filter material with pores small enough to retain the microorganisms. Membrane filters consisting of mixed cellulose esters, polycarbonate, polytetrafluoroethylene bonded to polyethylene, or polypropylene are most frequently used for sterilization of liquids. Pore size of membrane filters is precisely determined during manufacture and different pore sizes between 0.05 and 12 μm are currently available. Passage time is inversely related to pore-size diameter and permeability can be affected by the chemical or electrostatic properties of the filtrate. In some instances (such as natural water samples containing larger concentrations of dissolved organic matter present in colloidal form), filters will be rapidly clogged. The application of prefilters with larger pore sizes will reduce clogging of the membrane. Certain prokaryotes, such as *Flexibacter* (Little et al., 1987), mycoplasmas, or spirochetes, may pass membrane filters. For safe sterilization, 0.1 μm pore-size membrane filters should be employed since the cells of some prokaryotes are known to have diameters of around 0.2 μm. In most cases, viruses cannot be removed by filtration, however. Depth filters clog less rapidly and are therefore frequently used for the clarification of liquids. Depth filters consist of a matrix of randomly oriented fibers bound together in a tortuous maze of flow channels. However, depth filters differ considerably from membrane filters since organisms are trapped within the matrix and gradually go through and hence eventually contaminate the filtrate. This is not the case for dry (sterilized) cotton plugs which therefore can be employed for the sterilization of gases. Also, high-efficiency particulate air filters (HEPA) have become available commercially for the filtration of large volumes of air, e.g., to supply clean rooms or laminar-flow cabinets. Also, glass pipettes plugged with cotton wool or plastic pipet tips equipped with sterile filters should be used in the laboratory, especially where dealing with medically important bacteria.

Chemical sterilization is the method of choice for sterilizing solid objects that cannot be treated without damage by physical methods, such as certain disposable plastic ware, plastic tubing, surgical supplies (sponges), optical equipment, and samples of plant material (if sterile seeds are required; Shockey and Dehority, 1989). While polypropylene plasticware can be autoclaved without problems, polyethylene polymers, polystyrole, and some polysulfonates or polyfluoroethylene materials are more temperature sensitive. Microorganisms are killed by exposing them to toxic chemicals, mostly propylene oxide, β-propiolactone, or the most widely used ethylene oxide (EtO). The latter is an alkylating agent that reacts with hydroxyl-, sulfhydryl, and amino groups in proteins and nucleic acids. Ethylene oxide volatilizes above 10.8°C and sterilization involves an exposure of materials for at least 4 hours to EtO gas in a closed chamber, after which they must be thoroughly flushed for 8–12 hours with sterile inert gas or air. EtO is explosive, flammable, causes skin burns, and is highly toxic. Therefore, nonflammable mixtures of EtO and freon or carbon dioxide with the same microbicidal activity as EtO alone are in use.

For decontamination of laboratory surfaces, clear phenolics and hypochlorites (3%) are most commonly used, but alcohols (ethanol at a final concentration of 70%) or mixtures of alcohols and formaldehyde and iodophores are most effective against spores.

Ionizing radiation is employed in large laboratories for the sterilization of heat-sensitive solid objects such as powdered pharmaceuticals, disposable plastics, or clothing. Because it has the power of penetrating solids, radiation is also used to retard or eliminate spoilage of foods (Murray, 1989). Gamma radiation emitted by a radioactive cobalt source is most commonly applied. A high dose of 2.5 Mrad is sufficient to kill microorganisms, spores, and viruses, but chemical changes in media are possible. Gamma rays interact with water molecules to produce ions (OH^-) and free radicals ($OH^{\cdot}$) that can significantly alter and destroy many different biomolecules in the cell.

Handling of Glassware and Equipment

An important element of the handling of glassware is its proper cleaning. New glassware needs special treatment for the removal of free alkali. Detergents alone often are not sufficient to remove adsorbed compounds that may later inhibit growth especially of fastidious bacteria from natural bacterioplankton. Soaking in 0.2 N HCl often alleviates these problems. Detergents such as Mucasol® have been found to aid in proper cleaning of glassware. Glassware should be rinsed thoroughly with bidistilled water after treatment with detergents, since even traces of the latter are inhibitory to certain bacteria such as certain cyanobacteria or marine oligotrophs (Gottschal et al., 1991).

In extreme cases, in which prokaryotes from oligotrophic environments are to be isolated, it is necessary to rigorously clean water samplers, storage containers, and culture vessels with acid followed by repeated washings with ultrapure deionized or double quartz-distilled water (Waterbury, 1991). Soaking in appropriate detergent for 1 week and subsequent rinsing with especially clean water, followed by 1 week of soaking in 0.5 N HCl and repeated washing with clean water has been recommended for the culturing of cyanobacteria (Waterbury, 1991). At least in some cases, such as cyanobacteria of the genus *Trichodesmium*, special precautions against traces of organic or inorganic contaminants are mandatory (Orcutt et al., 2002). Nonglass systems consisting of Teflon® vessels are used in which natural seawater is sterilized by gentle Tyndalization in a microwave. Furthermore, Millipore Q water is employed for dilution and cultures grown in Nalgene polycarbonate flasks.

Because of the potential danger involved in working with infectious microorganisms, irrespective of their known pathogenic nature, the requirements for sterile working conditions and safe handling of contaminated glassware and equipment should be rigid. All used glassware and other materials should be autoclaved first before unloading in the wash-up room.

Sterile rooms and sterile cabinets supplied with gas, electricity, sterile air, and ultraviolet (UV) irradiation are useful, because they considerably reduce the possibility of air contamination. For the sterilization of inoculation rooms and other work areas, UV irradiation (wavelength, 260 nm) is often applied. Laminar flow (clean air) cabinets are designed to provide a work area that is protected from the environment and are useful for preventing airborne contamination when handling sterile media, such as during aseptically dispensing sterile fluids and culture media. Filtered air is passed in a vertical or horizontal unidirectional (laminar) flow through the cabinet. This air is made sterile by filtering through high-efficiency air filters that can remove particles down to 0.3 μm. In one type of cabinet, the flow of filtered air is directed toward the front. In a second type of cabinet, the flow occurs vertically downward, forming a curtain of sterile air and is subsequently in part recirculated. For decontamination of laminar flow hoods, 3% hypochloric acid can be used. Laminar flow hoods are certified for handling microorganisms of different hazard classes. For handling bacteria from natural samples and with unknown properties, safety class II cabinets should be used.

Removal and Exclusion of O_2, Cultivation of Anaerobes

The fundamental methodology for the cultivation of anaerobic prokaryotes is the Hungate technique (Stewart and Bryant, 1988; Widdel and Bak, 1992). Modifications of the original Hungate technique include the use of butyl rubber stoppers (Hungate et al., 1966) and of serum bottles closed with crimp-closure aluminum seals holding butyl rubber stoppers (Miller and Wolin, 1974), the syringe technique (Macy et al., 1972), and the use of pressurized tubes and vessels for the culture of methanogens (Balch and Wolfe, 1976). Usually, anoxic media contain a bicarbonate-CO_2 buffer system (Table 5), mineral salts, and a reducing agent. Depending on the requirements of the prokaryotes to be cultivated, clarified rumen fluid or low concentrations of yeast extract are added. Resazurin can be used as a redox indicator, provided it does not inhibit growth of the prokaryotes as in the case of many phototrophic sulfur bacteria.

Reductants frequently employed are sulfide or cysteine. A sulfur-free reducing agent is titanium (III) citrate. Owing to complex formation between titanium and citrate, $Ti(OH)_3$-precipitate formation is minimized (Zehnder and Wuhrman, 1976). The solution can be prepared by adding 5 ml of a 15% (w/v) titanium (III) chloride solution to 50 ml of a 0.2 M sodium citrate solution, followed by neutralization with saturated sodium carbonate. Moench and Zeikus (1983) reported an easy method for preparing this reductant with the use of nitriloacetic acid (NTA) instead of citrate as the complexing agent. However, NTA inhibits growth of certain prokaryotes. Furthermore, titanium (III) citrate may be inhibitory to anaerobic prokaryotes at low growth rates (Wachenheim and Hespell, 1984). In cases where organic reducing agents are to be avoided and free sulfide is toxic to the prokaryotes, amorphous ferrous sulfide has been employed as a reducing agent. This reductant can

be easily prepared in the laboratory and reacts much more rapidly with O_2 than does either soluble sulfide or cysteine (Brock and O'Dea, 1977). After preparation, media are dispensed anoxically as described by Widdel and Bak (1992).

Three-electrode poised-potential amperometric culture systems have been developed which consist of a platinum counter electrode, a platinum working electrode, and a AgCl-Ag reference electrode connected to a potentiostat (Emde et al., 1989; Emde and Schink, 1990; Ohmura et al., 2002). These systems can be used to grow anaerobic prokaryotes at a carefully controlled constant redox potential despite continuing redox reactions and flow of electrons to the working electrode. During growth experiments, the redox potential of the growth medium and the electron flow between working and counter electrodes can be recorded.

Even fastidious anaerobic prokaryotes such as methanogenic archaea can now be cultivated on solid agar media, if anaerobic chambers are employed (Leedle and Hespell, 1980). This adaptation of the technique for anaerobes permits the application of replica-plating techniques to ecological and genetic studies of bacterial populations from anaerobic habitats. Sufficient CO_2 (10–20%) should be present in the gas phase to maintain the pH of the medium if a bicarbonate buffer system is used.

Culture Systems

Batch Culture The typical sequence of lag phase, exponential phase, stationary phase (see Prokaryotic Growth) is observed when prokaryotes are grown in batch culture. In this most frequently employed culture system, cells grow suspended in a medium containing sufficient carbon and energy sources and other required nutrients to allow growth at maximum rate for a limited period of time. Since fresh supply of essential components and removal of metabolic waste products do not occur after inoculation, batch cultures are closed systems, and cells grow in a continuously changing environment, which shifts from a nonlimiting supply of nutrients to conditions of starvation. Because of its relative simplicity and ease of operation, the batch culture is nevertheless most widely used as a routine procedure for propagation of bacteria both in research and industry. Various degrees of control can be obtained by regulating important parameters such as pH, temperature, and oxygen tension.

Fed-Batch Culture A further elaboration of the batch cultivation technique was developed especially for certain industrial fermentations (Yoshida et al., 1973; Pirt, 1974; Pirt, 1975). In fed-batch cultures, a continuous supply of fresh medium is fed to a batch culture as soon as the substrate concentrations drop to low (sometimes growth-limiting) levels. As a result, prokaryotic cells continue to grow. Since no medium is allowed to flow out, the culture volume and cell biomass increase. At the same time, the ratio of biomass per amount of nutrients entering the vessel increases such that nutrient limitation becomes more severe over time and the microbial growth rate decreases continuously. Ultimately, fed-batch culture systems provide the means to largely extend the transition between the exponential and stationary growth phases. For some industrial fermentations, these very conditions have proven vital for optimal production of metabolites, e.g., citric acids, penicillin, some enzymes, and alcohols (Yoshida et al., 1973; Esener et al., 1981; Cleland and Enfors, 1983; Gond et al., 1986; Dawson et al., 1988; Kole et al., 1988). Especially for industrial fermentations, the possibility of turning a fed-batch culture into a "repeated fed batch-culture" by withdrawing part of the culture volume at regular time intervals allows the productive growth phase in principle to be extended indefinitely. The major difference with truly continuous culture systems remains that the volume is not kept constant, thus introducing permanent transience in growth rate, which is possibly essential for certain metabolic activities.

Recycling Fermenter, Dialysis Culture, Retentostat, and Recyclostat To overcome the problem of permanently changing volumes and concentrations of metabolic end products, the recycling fermenter was introduced. In this type of fermenter, either complete or partial recycling of the biomass is accomplished, but the culture liquid is continuously replenished with fresh medium and removed at the same rate. This is achieved by a cross-flow type of external membrane that retains prokaryotic cells while allowing spent medium to leave the fermenter (Müller and Babel, 1996; Ahn et al., 2001). Typically, crossflow dialysis membranes with large total membrane areas together with peristaltic pumps are employed for efficient and rapid filtration without clogging of the membrane (Pörtner and Märkl, 1998). This type of fermenter has proven an especially valuable tool to obtain cultures of high cell density since inhibitory metabolic products such as short-chain fatty acids or alcohols are removed. Another important use is to study growth rate dependence of microbial metabolism at very long generation times (i.e., very low specific growth rates; Chesbro et al., 1979; van Verseveld et al., 1984; Chesbro, 1988; Bulthuis et al., 1989; Müller and Babel, 1996). Under natural

conditions, the transition from exponential to stationary phase is expected to occur much more gradually than in conventional batch cultures (Mason and Egli, 1993). Hence physiological properties, such as maintenance energy requirements and resuscitation, of cells at extreme substrate limitation can be studied best in retentostats (Mason and Egli, 1993; Tappe et al., 1996; Tappe et al., 1999). A very simple means of prolonging the transition time between the exponential and stationary phase is through biphasic culture. In this type of culture, a bottom gel layer containing 4% agar is overlain by liquid medium. This arrangement results in a rapid exponential growth based on the nutrients in the liquid, and a subsequent prolonged deceleration phase of growth when cells utilize substrates slowly diffusing out of the agar layer (Chesbro, 1988).

CHEMOSTAT The main difference between continuous culture and other batch-type cultures is that it is a typical open system in which fresh nutrient medium is added at a constant rate to a well-mixed culture while the volume is kept constant through an overflow device (Fig. 3). In general, this culture system is designed to provide a culture growing permanently in an exponential fashion at a constant sub-maximum growth rate. The growth rate is dictated by the rate at which the limiting nutrient is fed to the culture. In this type of arrangement, the continuous culture is termed "a chemostat." In continuous-culture approaches with a chemostat, the continuously changing conditions characteristic of a batch culture are eliminated (Veldkamp and Kuenen, 1973) and relatively large populations of prokaryotic cells with constant physiological state can be maintained in the presence of low concentrations of a limiting nutrient, which resemble those under natural conditions. It has to be acknowledged, however, that in certain natural systems, nutrient supply and cell removal are not closely linked; these environments therefore do not exhibit steady-state conditions (Wirsen and Molyneaux, 1999). Chemostats offer a tremendously powerful tool for studying the physiology and ecology of prokaryotes. Its characteristics render the chemostat one of the most widely used culturing devices for studying microbial metabolism under carefully controlled environmental conditions in both pure and mixed cultures (Tempest, 1970a; Tempest, 1970b; Veldkamp, 1977; Tempest and Neyssel, 1978; Matin, 1981; Kuenen and Harder, 1982b; Gottschal, 1986; Gottschal, 1990; Gottschal and Dijkhuisen, 1988; Overmann and Pfennig, 1992; van den Ende, 1996; van den Ende, 1997).

In a chemostat, growth of prokaryotes is determined by the dilution rate D, defined as the rate of nutrient supply F (dimension: volume per time) divided by the volume of the culture vessel V, and by the concentration of the limiting nutrient in the reservoir medium S_R (Monod, 1950; Novick and Szilard, 1950; Herbert et al., 1956; Tempest, 1970a; Pirt, 1975; Calcott, 1981a; Gottschal, 1990). The combined effect of growth and dilution by the inflowing medium will eventually result in a steady state in which no further change in biomass concentration X occurs:

$$\frac{dX}{dt} = (\mu - D) \cdot X = 0 \qquad (20)$$

At this point, the specific growth rate exactly balances the dilution rate, and therefore a chosen rate of culture dilution fixes the specific growth rate of the culture at a value below μ_{max}. When the specific growth rate is described by the Monod equation (Monod, 1942)

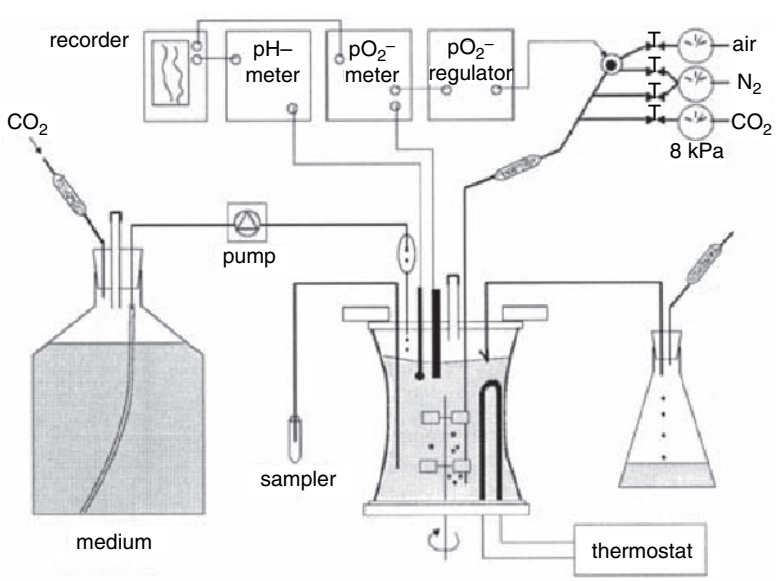

Fig. 3. Example for a chemostat arrangement designed for the continuous cultivation of anaerobic or microaerophilic bacteria. The partial pressure of oxygen can be set to any value between 0 and 21%. A simple regulation of pH is possible by manually controlling the CO_2 flow into the chemostat culture with a high precision needle valve and using a CO_2/bicarbonate buffering system. From Overmann and Pfennig (1992).

$$\mu = \mu_{max} \cdot \frac{S}{K_s + S} \quad (21)$$

in which K_s is the half-saturation constant for growth, the actual substrate concentration S in the culture is fixed at a low, rate-limiting value. In the steady state, the substrate concentration and biomass density are

$$S = K_s \cdot \frac{D}{\mu_{max} - D} \quad (22)$$

$$X = Y \cdot (S_R - S) \quad (23)$$

if the biomass yield Y (the amount of biomass formed per substrate consumed) remains constant over the range of dilution rates employed.

For the optimal production of some microbial metabolites, a continuous culture system is required in which the cells pass through various stages of growth (Ricica and Dobersky, 1981; Thompson et al., 1983; Parkes and Senior, 1988). In these coupled systems, two or more chemostats are arranged serially, by connecting the outlet of the first chemostat to the inlet of the second. Conditions vary between the different chemostat vessels, e.g., with respect to dilution rate, O_2 supply, temperature, or substrate supply. Culturing different prokaryotes in coupled chemostats for instance permits the study of sequential mineralization of recalcitrant organic compounds. Two-stage cascade chemostats are also employed to investigate predator-prey relationships, in which bacteria grown in the first stage are transferred to a second one to serve as food for bacterivorous protozoa (Jost et al., 1973; Swift et al., 1982; Sambanis and Fredrickson, 1987; Simek et al., 1997; Pernthaler et al., 2001).

Even more complex, but rarely used, systems are the bidirectionally linked multistage chemostats, which permit the mutual exchange of bacteria and/or nutrients. In a "gradostat" (Lovitt and Wimpenny, 1981), a series of chemostats (linked by tubing) is fed from both sides with media of different, sometimes complementary, composition. The inoculated bacteria will be exposed to different physicochemical conditions in the different chemostat vessels. In "bidirectional compound diffusion-linked chemostats," the various chemostat vessels are not linked by tubing but through membranes, permitting diffusion of solutes but not bacteria (Keith and Herbert, 1985). Multistage chemostats are especially suited to study prokaryotes under conditions prevailing in the natural habitat, since heterogeneous conditions of environmental parameters and interactions between different species can be reproduced with these systems.

For growth studies at extremely low nutrient concentrations, the chemostat is not a suitable tool since extremely low dilution rates ($<< 0.5\ h^{-1}$) give rise to inhomogeneities due to mixing problems and low steady state biomass concentrations. As a more realistic approach, a retentostat can be employed which offers the advantage of studying physiological properties of cells at extreme substrate limitation (Mason and Egli, 1993; Tappe et al., 1999).

Redox-Controlled Sulfidostat As a special type of continuous culture, the redox-controlled sulfidostat permits the cultivation of phototrophic sulfur-oxidizing bacteria under constant concentrations of hydrogen sulfide (Sánchez et al., 1996). In this type of culture, a constant concentration of sulfide is maintained despite light intensity variations that affect photosynthetic rate and hence sulfide oxidation. A redox controller modulates the rate at which the medium is pumped into the culture and therefore governs the dilution rate. In a similar manner, the system can adjust to new rates of sulfide oxidation caused by changes in light intensity.

Auxostat An essential property of chemostat cultures is that their rate of growth is fixed by the rate at which fresh medium is fed to the culture. Although this rate can be varied over a considerable range of values, steady state cannot be obtained near μ_{max}, and washout of the cells occurs at the critical dilution rate (Pirt, 1975). Furthermore, unbalanced growth (and in some cases washout) may occur when inhibitory metabolites accumulate or potentially toxic substrates are used. An alternative for obtaining controlled growth at an appreciable and constant cell density in such cases is by switching over to some type of internal control of the rate of medium supply. Such a control must be based on a growth-dependent parameter. The first continuous culture with internal control was named "turbidostat" because the feedback control was based on measurements of culture turbidity (Myers and Clark, 1944; Bryson and Szybalski, 1952). However, a continuous and accurate measurement of turbidity represents a major problem in these systems because of wall growth, inhomogeneity of the culture, etc. Therefore, other parameters directly dependent on the culture density are measured with electrodes in more recent systems. Parameters include CO_2, O_2, pH, redox potential, fermentation products, and sulfide (Watson, 1969; Martin and Hempfling, 1976a; Oltmann et al., 1978; Kjaergaard and Jørgensen, 1979; Schauer et al., 1982; Cypionka, 1986; de la Broise and Durand, 1989). These more recent designs have proven reliable and their use is most rewarding in studying growth of microbes in the presence of inhibitory concentrations of substrates or products, and possibly also in selecting mutant strains exhibiting the highest growth rates under such conditions.

Possible Reasons for "Non-culturability"

The titer of colony-forming units obtained from a given sample in almost all cases is significantly lower than the actual titer of prokaryotic cells in the sample as determined by culture-independent microscopic techniques. This observation has been termed the "great plate count anomaly" and is attributed to several factors. One simple explanation would be that only a fraction of the prokaryotic cells are culturable under a certain set of conditions such that no single medium will allow growth of all types of prokaryotes. Indeed, the efficiency of plate counting could be significantly increased when 25 different media were employed instead of a single medium (Balestra and Misaghi, 1997). However, in most cases the numerically dominant species of prokaryotes from natural samples are not recovered. Certain bacteria and archaea, among them the mesophilic crenarchaeota which constitute a fraction of up to 34% of the prokaryotic plankton in subpolar or polar latitudes (DeLong et al., 1994), the clone T78-group of the green gliding bacteria (Coolen et al., 2002), or most of the acidobacteria detected by molecular methods in soils (Barns et al., 1999) have never been isolated in any of the media tested.

It therefore appears reasonable to suggest that 1) cells of not-yet-cultured prokaryotes in natural samples are in a specific physiological state which prevents them from growing in conventional cultivation media and/or 2) the physiology of not-yet-cultured species of prokaryotes is fundamentally different from that of known prokaryotes such that cultivation methods applied do not meet the requirements for growth.

Physiological State of Prokaryotic Cells

Starvation Response The gene product of *rpoS*, namely the transcription factor σ^S, is involved in cellular responses to a diverse number of stresses (Loewen et al., 1998). Induction of *rpoS* results in an increased survival of cells under unfavorable conditions (Munro et al., 1995). Accordingly, and dependent on the growth state of the cells, RpoS (σ^S) can positively influence the culturability of *E. coli* and *Salmonella typhimurium* in oligotrophic seawater (Munro et al., 1995). However, σ^S is also involved in the transition to stationary phase, and cAMP (in a complex with the cAMP receptor protein [CRP-cAMP]) acts as a negative regulator of the transcription of *rpoS* (Loewen et al., 1998). Addition of extracellular cAMP has the same effect (Lange and Hengge-Aronis, 1991). Scavenging transporters (such as LamB), which are regulated by cAMP or endoinduction, are turned on at higher substrate concentrations than RpoS-dependent functions (Notley and Ferenci, 1996). Therefore it appears feasible that, by addition of extracellular cAMP, cells could be maintained more easily in a nutrient scavenging state and hindered from entering the protective stationary phase response, which potentially could facilitate cultivation in the absence of other stress factors.

Presence of Dead Cells, Prevention, and Reversal of Cellular Damage The presence of non-growing cells has been shown in laboratory cultures of *Enterobacter aerogenes*, which at maximum doubling times of 100 h contained up to 50% of cells not capable of growing on agar plates (Tempest et al., 1967). Since culture-independent methods such as microautoradiography or the direct viable count technique have revealed that up to 50%, and in some cases even 90%, of the prokaryotic cells may be metabolically active (Kogure et al., 1979; Fry, 1990; Karner and Fuhrman, 1997), dead cells may actually not be present at high numbers in the natural environment. However, elongating cells may be capable of completing only a limited number of division cycles, which prevents detection of growth by standard methods (Binnerup et al., 1993). This limitation of division cycles can be caused by injury (e.g., to the cell envelope, proteins, or DNA; Barer and Harwood, 1999), caused for instance by oxidative stress. Hence, dormant cells of *Micrococcus luteus* are permeant to certain fluorescent stains but restore the cytoplasmic membrane barrier upon resuscitation (Kaprelyants et al., 1996). Other factors that may limit multiplication are limiting nutrients or an (as yet unexplained) upper limit of cell density as described for *Sphingomonas* sp. strain RB2256 (Schut et al., 1997). Injury of DNA may activate the SOS response which includes expression of SulA, a protein that interacts with the tubulin-like protein FtsZ, thereby preventing septation and thus resulting in a filamentous growth of cells (Bi and Lutkenhaus, 1993; Walker, 1996). Detoxifying enzymes like catalase and superoxide dismutase are involved in the prevention of oxidative stress. Heat shock proteins, peptide methionine sulfoxide reductase, and glutathione reductase are involved in the reversal of damage within the cell (Dukan and Nyström, 1998; Barer and Harwood, 1999).

Dormancy Dormancy is defined as a reversible state of low metabolic activity in which viability is maintained. This physiological state has been studied extensively for *Micrococcus luteus*. Incubation of stationary-phase cultures at room

temperatures for several months results in large numbers of dormant cells. In the case of *Micrococcus luteus*, dormant cells show reduced activity, as exemplified by uptake of the membrane energization-sensitive dye rhodamine-123 (Kaprelyants and Kell, 1993). However, such temporarily non-culturable cells can be resuscitated in the presence of supernatants from growing *M. luteus* cultures (Kaprelyants et al., 1994; Kaprelyants et al., 1996). The agent responsible for resuscitation of dormant cells (Rpf, the resuscitation promoting factor) was identified as a 17-kDa protein exported by *M. luteus* (Mukamolova et al., 1998). In *E. coli*, the protein SdiA regulates the *ftsQAZ* cluster of essential cell division genes and the P_2 promotor (Wang et al., 1991) and is a member of the LuxR subfamily of transcriptional activators. The expression of SdiA is regulated in turn by a factor released by growing cells into the medium (García-Lara et al., 1996). The factor was identified as a *n*-acyl homoserine lactone (Sitnikov et al., 1996). Cell-cell signalling between cells of the same clone therefore may be important in regulating cell division.

Substrate-Accelerated Death If a growth limiting substrate (e.g., glycerol, glucose, ribose, phosphate or ammonia) is added in concentrations of ≥1–10 μM to cells previously starved for the same substrate (Postgate and Hunter, 1963; Postgate and Hunter, 1964; Calcott and Postgate, 1972a), growth of the cells is inhibited. This phenomenon has been called "substrate-accelerated death." Cells challenged with this substrate exhibit low levels of cAMP, and in the presence of extracellular cAMP, substrate-accelerated death disappears (Calcott et al., 1972b). Also, the addition of Mg^{2+} protects cells against substrate-accelerated death. Substrate-accelerated death has been reported to occur in *Klebsiella*, *Escherichia*, *Streptococcus*, *Azotobacter*, *Arthrobacter* and *Mycobacteria* (Calcott and Calvert, 1981b). The significance of substrate-accelerated death on recovery of bacteria from environmental samples remains unknown. However, it has recently been demonstrated that addition of cAMP at low concentrations (10 μM) in certain cases can significantly increase the culturability for natural samples (Bruns et al., 2002).

The Viable But-Non-Culturable State In addition, it has been proposed that certain bacteria may acquire a state termed "viable but-non-culturable" (VBNC; Xu et al., 1982; Roszak and Colwell, 1987). In the proposed VBNC state, bacterial cells, especially of pathogenic bacteria like *Vibrio cholerae* or *Campylobacter jejuni* (Bovill and Mackey, 1997), are believed to become temporarily non-culturable until they are exposed to an environment that stimulates their resuscitation. In *Vibrio vulnificus*, exposure of cells to temperatures around 4°C results in a decline of culturability without cell lysis (Oliver, 1995). Temperature-upshift results in a resuscitation of these non-culturable cells (Whitesides and Oliver, 1997). Those cells that retain the capacity for resuscitation appear to maintain physiological activity. Replacement of the term VBNC by "temporarily non-culturable" or "not immediately culturable" has been proposed since it is unclear whether VBNC is the result of a specific programmed differentiation or an adaptive process (Barer and Harwood, 1999). The view that the VBNC state is a single phenomenon and a valid operational concept has recently been challenged (Barer and Harwood, 1999).

Lysogenic Phages Lysogenic phages may be induced upon plating onto agar media by SOS response-related induction (Barer and Harwood, 1999), leading to a rapid decrease of culturability.

Low Nutrient Concentrations, "Oligotrophic Bacteria"

Media supplemented with comparatively low concentrations of complex carbon sources like yeast extract and peptone (i.e., 0.25 g·liter^{-1} each) have been established for the isolation of fastidious bacteria such as planctomycetes (Schlesner, 1986; Staley et al., 1992). Using concentrations of ≤5 mg of organic carbon·liter^{-1}, which are supplied as casamino acids, or employing straight filtered autoclaved seawater can increase the apparent cultivation success (determined as the ratio of most probable numbers in relation to total cell counts) up to 20–60% (Button et al., 1993; Eguchi et al., 2001). In addition, the qualitative composition of carbon sources determines the cultivation success of naturally occurring prokaryotes. Complex media have been shown to yield higher numbers and a higher diversity of isolates than similar media containing only one defined carbon source at the same concentration: single substrates yielded almost exclusively strains of the γ-subclass of Proteobacteria (Uphoff et al., 2001). For soil pseudomonads, nutrient-poor media containing only ~15 mg C·liter^{-1} have been shown to yield higher numbers of culturable cells than conventional organic-rich media (Aagot et al., 2001). Strongly diluted nutrient broth resulted in significantly increased numbers of Acidobacteria, Actinobacteria, α-Proteobacteria, Low G+C-Gram-positive bacteria, and Verrucomicrobia from soil samples (Janssen et al., 2002). This combined evidence suggests that the majority of heterotrophic prokaryotic cells in many natural

samples require lower concentrations of organic carbon for growth. The cultivation success of freshwater planktonic bacteria could also be increased in media with reduced nitrogen and phosphorus concentrations. In the latter case, nutrient levels were decreased to match the maximal ambient levels in the planktonic environment (50 μM nitrogen and 1.5 μM phosphorus; Eilers et al., 2001). In general, viable cell counts of freshwater or marine planktonic bacteria reached on agar-solidified media are orders of magnitude smaller than those obtained as most probable numbers in liquid extinction dilution series (Eguchi et al., 2001; Jaspers et al., 2001).

Ultramicrobacteria are defined by a cell volume of <0.1 μm^3 (Eguchi et al., 2001) and prevail in oligotrophic marine waters (Schut et al., 1997). In this respect, an important question is whether ultramicrocells represent a distinct class of prokaryotes that maintain their small biovolume independently of their growth status, or whether they are capable of becoming larger. In many bacterial species, nutrient deprivation results in pronounced changes of cell morphology and size (Torella and Morita, 1981; Morita, 1982; Amy and Morita, 1983; Kjelleberg et al., 1993). Upon starvation, many non-growing marine isolates produce ultramicrocells by reductive division and these can have volumes as little as 1% that of rapidly growing cells. The resulting daughter cells retain measurable metabolic activity, at least in an initial period following their formation (Amy and Morita, 1983). It appears likely that such ultramicrocells contribute significantly to observed populations of prokaryotes in ocean waters. However, in cells of other species, like for instance *Sphingomonas alaskensis* within the α-Proteobacteria (Eguchi et al., 2001; Vancanneyt et al., 2001) or the Verrucomicrobiales, a small cell volume (0.03–0.06 μm^3) is a stable characteristic and independent of substrate concentrations (Schut et al., 1993; Janssen et al., 1997).

Oligotrophic bacteria are defined as those that on first cultivation develop on media containing 1–15 mg C·$liter^{-1}$ (Kuznetsov et al., 1979). Consequently, such bacteria cannot be cultivated directly on nutrient-rich agar (Vancanneyt et al., 2001). Actually, the range of carbon concentrations reported for the pelagic and deep ocean is 30–200 μM, corresponding to 0.36–2.4 mg C·$liter^{-1}$ (Jannasch et al., 1996). Therefore, bacto yeast extract (Difco) is added from autoclaved stock solutions to a final concentration of 10–1 mg·$liter^{-1}$ (corresponding to 3.3–0.33 mg C·$liter^{-1}$ or 270–27 μM carbon; Jannasch et al., 1996) in media designed to recover marine pelagic prokaryotes. It has been demonstrated, however, that prolonged incubation over 1 year at 5–8°C or storage and monthly reculturing at 15°C yields cultures which eventually can multiply on high-nutrient laboratory media (Eguchi et al., 2001; Vancanneyt et al., 2001). Oligotrophic bacteria may generally represent novel types of bacteria with unknown physiological properties. In contrast to all other (mostly copiotrophic) bacteria investigated to date, the marine ultramicrobacterium *Sphingomonas alaskensis* RB2256 exhibits a high level of inherent stress resistance towards oxidative stress (hydrogen peroxide [H_2O_2]), but no starvation-induced stress against H_2O_2 (Ostrowski et al., 2001). Stress resistance is negatively correlated to growth rate, but only marginal changes in catalase activity were observed; hence, other factors must be critical to the stress resistance of this strain.

Growth on Multiple Substrates

In spite of the fact that bacteria in natural environments grow in the presence of a large diversity of compounds, most laboratory studies have focused on growth with single substrates. When bacteria are grown in batch culture with more than one growth substrate, sequential utilization of these substrates is often observed. In some cases, this results in a typical diauxic growth pattern (Monod, 1942; Lengeler et al., 1999) in which the substrate that is used first represses the synthesis of enzymes required for the utilization of the other substrates. Only after (almost) complete utilization of the first substrate is growth on the second one induced. It is noteworthy that sugars may cause catabolite repression of inducible enzyme systems even if the sugars are not utilized themselves (Pastan and Perlman, 1969). In enteric bacteria, cAMP is part of the pleiotropic *crp* activation system which regulates most peripheral catabolic operons and carbohydrate transport systems, and it typically mediates carbon catabolite repression (Lengeler et al., 1999). Sugars transported by the phosphotransferase system (PTS) decrease intracellular cAMP levels which in turn prevents the expression of genes necessary for the uptake of non-PTS sugars. After depletion of glucose, intracellular cAMP concentrations rise from 0.3 up to 3 μM, and other sugars can be taken up and metabolized after a lag period required for the induction of the necessary genes. This regulatory pattern leads to a biphasic growth curve. The intermediate lag phase can be abolished by addition of extracellular cAMP when added at millimolar concentrations (Epstein et al., 1975), whereas the growth rate itself does not change (Okada et al., 1981). The involvement of cAMP in regulation of catabolic enzymes has also been demonstrated for a wide range of nonenteric bacteria, including other γ-Proteobacteria, the α-Proteobacteria, β-Proteobacteria, and the cyanobacteria (Botsford et al., 1992).

However, this type of clearcut diauxic pattern with a lag period between consumption of the first and the second substrate is by no means very common. More often, a gradual transition between the use of two (or more) substrates is observed, and in some cases, no enzyme repression is evident at all (Harder and Dijkhuizen, 1982; Gottschal, 1986). Moreover, it is questionable whether distinct preferences for one substrate would be functionally valuable in nutrient-poor natural environments with many different substrates available at the same time. Under nutrient limitation, one would rather expect organisms to develop physiological strategies enabling them to make use of several nutrients simultaneously. Growth in chemostats under limitation of mixtures of different substrates has provided ample evidence that a multitude of substrates, serving similar physiological functions, can be growth limiting at the same time (Harder and Dijkhuizen, 1982; Egli et al., 1983; Gottschal, 1986; Gottschal and Dijkhuizen, 1988). Low K_s values have been found in marine isolates (Schut et al., 1995). However, the reported V_{max} values are generally so low that specific affinities and oligotrophic capacities are insufficient to allow growth on single substrates. In a marine coryneform bacterium, the presence of amino acids can enhance the uptake of glucose and lowers the threshold concentations for growth (Law and Button, 1977). On the other hand, growth on alanine of *Sphingomonas alaskensis* strain RB2256 occurred with a lower affinity in the presence of glucose (Schut et al., 1995).

Metabolically versatile bacteria exist that are specialized in using several different substrates at the same time if the latter are present at growth-limiting concentrations (Laanbroek et al., 1979; Dykhuizen and Davies, 1980; Gottschal and Kuenen, 1980; Beudeker et al., 1982; Legan and Owens, 1988). Since these prokaryotes usually display lower maximum growth rates relative to more specialized species, metabolically versatile bacteria will in most cases be missed when batch-type enrichment techniques are used, even if mixtures of several substrates are present. Instead, chemostat cultures need to be employed for enrichment.

Effect of Inhomogeneities

Inhomogeneities can have a profound effect on the physiology of prokaryotes. Many environments such as soils, sediments and marine snow are highly heterogeneous, and therefore microbial substrates and bacterial productivity are distributed in microscale patches of variable concentration and size (Azam, 1998; Ploug, 1999). Under such conditions, steep gradients, e.g., of molecular oxygen, may result in a close proximity of aerobic and anaerobic species and transformations (Revsbech and Jørgensen, 1986).

Attachment of prokaryotic cells has been shown to be of significance under some conditions. Some compounds are optimally metabolized only if oxygen-dependent and strictly anoxic steps are coupled by diffusion of metabolic products and thus proceed in close proximity. This principle has been demonstrated in an elegant study of the degradation of DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane), using calcium alginate beads for immobilization of bacteria (Beunink and Rehm, 1988). In this system, the attached cells of *Alcaligenes* species and *Enterobacter cloacae* performed reductive dechlorination of DDT inside the beads, and the partly dechlorinated products were metabolized oxidatively. Since highly chlorinated compounds are dehalogenated more readily under anoxic conditions, this same principle may hold for many other halogenated xenobiotics as well.

Besides affinity for a given substrate and cell yield, attachment to solid surface is an additional determinant of the outcome of competition between prokaryotes with similar physiology. Attachment to solid surfaces can thus change the metabolic interrelations between competing bacteria. When growing in suspension, *Pseudomonas putida* R1 and *Acinetobacter* C6 compete for the substrate benzyl alcohol, with *Acinetobacter* largely outcompeting *P. putida*. However, both strains formed stable and structured biofilms when glass as solid substrate was available (Christensen et al., 2002). Under the latter conditions, *P. putida* is capable of utilizing benzoate which *Acinetobacter* uses only inefficiently and therefore excretes to a large extend.

In the natural environment, nonpolar organic carbon substrates occur in the adsorbed state. In the adsorbed state, these substrates are often not directly available and rates of desorption control the rate of degradation. However, some prokaryotes can degrade adsorbed substrates more rapidly than can be accounted for by the rates of desorption into the aqueous phase (Harms and Zehnder, 1995). Prokaryotes thus differ in their capability of using adsorbed substrates (Guerin and Boyd, 1997; Stringfellow and Aitken, 1994; Crocker et al., 1995). Consequently, enrichment strategies, in which especially nonpolar substrates are offered in the adsorbed state, may provide a more relevant low-bioavailability environment, and hence lead to the enrichment and isolation of novel types of prokaryotes (Tang et al., 1998; Grosser et al., 2000).

Interactions with Other Microorganisms

Most likely, one considerable problem in current enrichment and cultivation techniques is that

microbial interactions cannot be reproduced adequately (see Alternative and Novel Concepts for Cultivation). In nature, prokaryotes reach cell densities of about $10^6 \cdot ml^{-1}$ in most aquatic environments, $10^9 \cdot cm^{-3}$ in sediments, and $10^{11} \cdot cm^{-3}$ in soils. If a homogenous distribution of the cells is assumed, the average cell-to-cell distance at these increasing densities would amount to 112, 10 and 1 μm, respectively. Over such small distances, transport of small molecules by molecular diffusion proceeds at a rapid rate and takes between microseconds and a few seconds (Overmann, 2002a). Owing to the laws of three-dimensional diffusion, the flux of metabolites experienced in the vicinity of a prokaryotic cell by another one decreases to as little as 0.01% when the cell-to-cell distance increases to 10 μm (Overmann and Schubert, 2002b). This simple calculation indicates that 1) interactions between prokaryotes may influence their growth under natural conditions and most likely need to be considered in cultivation attempts of some not-yet-cultured bacteria, and 2) a strong selective pressure must exist for interacting prokaryotic cells to maintain close spatial proximity.

At high cell densities, even monospecific associations of some bacteria exhibit physiological traits that differ from those of dilute cultures. In the case of quorum sensing, the excretion of autoinducer molecules signals high cell density and triggers light production, expression of virulence factors, and swarming (Fuqua and Greenberg, 1998) or prevents cell aggregation (Puskas et al., 1997). Similarly, myxobacteria exhibit complex social interactions: when deprived of nutrients they enter into a complex developmental cycle that results in the formation of a multicellular fruiting body that contains myxospores (Reichenbach, 1984). Other cases of monospecific associations have been described occasionally (Lins and Farina, 1999). However, it has also been found that different types of bacteria can form associations. These comprise highly structured associations of defined composition, so-called "consortia" (Schink, 1991; Overmann, 2002a). In addition, less structured assemblages, like microcolonies, net-like structures, biofilms, or aggregates of up to 18 different prokaryotic genera, have commonly been observed in natural samples (Weise and Rheinheimer, 1978; Paerl, 1982; Alldredge and Youngbluth, 1985; van Gemerden et al., 1989; Dubinina et al., 1993; Kolenbrander and London, 1993; Seitz et al., 1993; Overmann et al., 1996; Jacobi et al., 1997; Moissl et al., 2002). Least structured and hence difficult to detect are 10–100 μm-large patches of free-living bacterial cells, but evidence has accumulated for the existence of such inhomogeneities in the pelagic habitat (Krembs et al., 1998). Under certain natural conditions, the growth of prokaryotes is therefore not only influenced by the various physicochemical factors, but also by interactions with other microorganisms. Cells may need to communicate with each other for growth.

So far, the compounds known to be exchanged between prokaryotes include signal compounds (as in the case of quorum sensing), growth factors, and compounds directly involved in energy metabolism (mostly electron donors/electron acceptors such as hydrogen or inorganic sulfur compounds in syntrophic interactions). Besides enabling prokaryotes to perform novel syntrophic reactions, interactions can also lead to altered kinetics of microbial transformations. For example, the high affinity oxidation of methane observed in soil could be reproduced in a coculture of a non-novel methanotroph with a *Variovorax* strain (Dunfield et al., 1999). This finding indicates that the physiology and ecology of prokaryotes can only be completely appreciated if interactions with other prokaryotes are considered as well.

Overall, eight types of interspecies interrelationships can be distinguished based on the effect of each of the two populations (Atlas and Bartha, 1993; Table 7), ranging from mutualistic to antagonistic interactions. It has to be kept in mind, however, that the different categories listed in Table 7 represent only concepts, which in many cases fail to account for all facets of microbial interactions.

Neutralism This describes the situation of a complete lack of interaction between two populations. This situation is more likely to occur

Table 7. Types of interspecies interactions.

Name of interaction	Effect of interaction
Neutralism	Neither population affects the other
Competition	Populations inhibit each other when resources are in limiting supply
Amensalism	Population 1 is negatively affected by 2, but 2 is not affected by 1
Parasitism	Population 1 consumes population 2, but usually not in a destructive manner
Predation	Population 1 consumes population 2 in a destructive manner
Commensalism	Population 1 benefits from population 2 without affecting it in a negative sense
Synergism (protocooperation)	Both populations benefit from the interaction, which is not obligatory
Mutualism (symbiosis)	Both populations benefit from the interaction, which is obligatory

between populations with very different metabolic capabilities. However, neutralism is defined in a negative way and therefore is most difficult to verify experimentally. Most likely interactions are absent at very low population densities and if prokaryotes form physiologically largely inactive resting stages (Atlas and Bartha, 1993). However, it has been demonstrated that even endospores can affect the surrounding environment by the presence of extracellular enzymes (i.e., Mn^{2+} oxidase; Francis and Tebo, 2002).

Competition Since energy and nutrient sources are often present in limiting concentrations, competition for growth-limiting resources is one of the major types of interactions between cells of one population or between different prokaryotes, and it results in a reduction of growth rate. Eventually, it may lead to the exclusion of the slower-growing species, a process also termed "competitive exclusion" (Gause, 1934; Hardin, 1960). Since the introduction of continuous culture techniques which allowed cultivation under conditions of permanent nutrient limitation, the competition in mixed cultures has been studied under a great variety of environmental conditions (Powell, 1958; Veldkamp and Jannasch, 1972; Fredrickson, 1977; Veldkamp, 1977; Fredrickson and Stephanopoulos, 1981; Kuenen and Gottschal, 1982a; Kuenen and Harder, 1982b; Gottschal and Dijkuizen, 1988; Visscher et al., 1992b). These studies were mostly concerned with simple and pure competition for single growth-limiting nutrient in the absence of other interactions, and the results supported the competitive exclusion principle. The outcome of the competition between two or more species is merely dependent on the shape of the μ versus S relationship of the competitors (Fig. 2). Those prokaryotes which reach a higher specific growth rate at a given substrate concentration always outcompete the slower-growing species. In some instances, the μ versus S curves cross and as a result the outcome of the competition depends on the dilution rate chosen. Apparently, certain species are much better adapted to growth at very low substrate concentrations (and exhibit a relatively low μ_{max} value), whereas others are more specialized in growth at high rates in the presence of high substrate concentrations. Examples have been reported for aerobic and anaerobic heterotrophs, chemolithotrophic species, and phototrophic organisms (Harder and Veldkamp, 1971; Jannasch and Mateles, 1974; Fredrickson, 1977; Harder et al., 1977; Mur et al., 1977; Veldkamp, 1977; Matin and Veldkamp, 1978; Kristjansson et al., 1982; Lovley et al., 1982; Laanbroek et al., 1983; Laanbroek et al., 1984; King, 1984; Kuenen and Robertson, 1984; Robinson and Tiedje, 1984; Veldkamp et al., 1984; Legan et al., 1987; Legan and Owens, 1988). Competition between microorganisms exists not only for organic carbon but also for nutrients like phosphate (Rhee, 1972; Currie and Kalff, 1984).

Amensalism The production of lactic acid by lactic acid bacteria or sulfuric acid by *Thiobacillus thiooxidans*, which inhibit other non-acidophilic microorganisms, are examples of amensalistic relationships. Similarly, the inhibition of *Salmonella enterica* by acetate and propionate produced by *Clostridium lactatifermentans* at low pH (5.8) has been demonstrated (van der Wielen et al., 2002). At these low pH values, considerable acetate and propionate present as undissociated acids penetrate the cytoplasmic membrane and hence decrease the membrane potential (see pH).

Predation The best documented example for predatory prokaryotes are bacteria of the genus *Bdellovibrio* which attack Gram-negative bacteria by attaching to their prey, penetrating the cell wall, and subsequently multiplying within the periplasmic space. Multiplication of *Bdellovibrio* sp. occurs at the expense of cellular components of the host cell and leads to the formation of 4–20 daughter cells by segmentation within 2–3 hours after infection (Stolp and Starr, 1963; Stolp and Starr, 1965; Varon and Shilo, 1980; Shilo, 1984). The cell content of the host is partially degraded and utilized. Although growth and survival of wild-type *Bdellovibrio* spp. is strictly dependent on the availability of appropriate prey cells, this parasite differs fundamentally from viruses in that it does not depend directly on the metabolic machinery of the host cell. *Bdellovibrio* species and similar bacteria appear to be widespread, having been isolated from many different aquatic and terrestrial ecosystems (Varon and Shilo, 1980; Burnham and Conti, 1984). Additional predatory bacteria that require cell contact have been described. *Vampirococcus* sp. is a nonmotile, Gram-negative anaerobic bacterium which occurs as an epibiont of phototrophic *Chromatium* spp. to which it adheres by means of specific attachment structures without penetrating the outer cell layers. Concomitant to growth and division of the epibiont, the host cell cytoplasm is degraded, leaving behind an almost empty cell envelope (Guerrero et al., 1986). A second type of predatory bacterium, *Daptobacter* sp., penetrates the cell envelope of cells of various genera of the Chromatiaceae and degrades the cytoplasm of its prey. The Gram-negative *Daptobacter* is a facultative predator, facultatively anaerobic and motile (Guerrero et al., 1986). Other bacteria can lyse prokaryotes without direct contact. An isolate of *Stenotrophomonas maltophilia* was

found to lyse cells of *Chlorobium limicola* and several heterotrophic bacteria (Nogales et al., 1997). Finally, myxobacteria can cause lysis of susceptible strains at some distance, apparently with the aid of exoenzymes. The myxobacteria derive their nutrition from material released by the lysed cells.

Commensalism In the non-obligatory commensal relationship, one population benefits, e.g., from growth factors excreted by a second population, while the latter remains unaffected. This type of relationship thus is unidirectional in character. Commensalism within one and the same population is of significance if, for instance, the growth substrates are insoluble (such as lignin or cellulose). These substrates are made available by extracellular enzymes, and while the exoenzymes themselves are kept at the cell surface (i.e., in the periplasmic space in the case of Gram-negative bacteria or by attachment to the cytoplasmic membrane in the case of Gram-positives), the resulting substrates often are rapidly lost by diffusion from the vicinity of single cells. In contrast, soluble products can be utilized at high efficiency if cell densities are high. As an example, *Mxyococcus xanthus* does not grow on insoluble casein at cell densities lower than 10^3 per ml, whereas growth rates increased with cell densities above this value (Rosenberg et al., 1977). This effect of cell density is not observed on prehydrolyzed casein.

Not in all cases does the limitation of two populations of prokaryotes by one substrate lead to competitive exclusion, but it can result in stable mixed cultures. The underlying reasons include the occurrence of additional interactions, especially commensalism and mutualism, the formation of self-inhibitory products, the presence of predators, selective adhesion, fluctuations in physical parameters (pH, temperature, light, and oxic/anoxic conditions), or an alternating supply of differing growth-limiting substrates (Bungay and Bungay, 1968; Megee et al., 1972; Jost et al., 1973; Meers, 1973; van Gemerden, 1974; Meyer et al., 1975; Lee et al., 1976; Fredrickson, 1977; de Freitas and Fredrickson, 1978; Slater and Bull, 1978; Gottschal et al., 1979; Miura et al., 1980; Bull and Slater, 1982; Kuenen and Robertson, 1984; Kuenen et al., 1985). For example, purple sulfur bacteria and colorless sulfur bacteria, which are expected to compete for H_2S as electron-donating substrate, are found to thrive in high population densities in the same layer of microbial mats (Visscher et al., 1992a). This has been explained by the removal of oxygen by colorless sulfur oxidizers and the formation of incompletely oxidized inorganic sulfur intermediates, which then serve as alternative electron donor for the anoxygenic phototroph (Visscher et al., 1992b; van den Ende et al., 1996). Also, competition can be alleviated and stable cocultures obtained if conditions for competition do not exist over an entire 24-h period but only for a shorter time interval (van Gemerden, 1974). This fact for instance can be exploited for the enrichment of more fastidious purple sulfur bacteria (like the large-celled *Chromatium* spp.) by using light-dark cycles instead of continuous light.

As an example of a commensalistic relationship based on crossfeeding between nonrelated bacteria, *Propionibacterium shermanii* can grow in mixed continuous culture at the expense of lactate produced by *Lactobacillus plantarum* from glucose, the growth-limiting nutrient in the chemostat (Lee et al., 1976). Lactate was also the mediator in a commensal relationship between *Streptococcus mutans* and *Veillonella alcalescens*. Both strains are commonly found in dental plaque and were shown to coexist in mixed continuous cultures supplemented with glucose as the limiting nutrient (Mikx and van der Hoeven, 1975). In this mixed culture, the lactate produced by *S. mutans* was metabolized to acetate, propionate, and ethanol, which, owing to lower dissociation constants, might reduce the demineralization of tooth enamel. Also, consumption of lactate by a second organism (*Pseudomonas stutzeri*) under anaerobic conditions and in the presence of nitrate in a lactose-limited mixed chemostat culture results in a marked stimulation of the growth yield of *Lactococcus cremoris* (Otto et al., 1980). The latter is capable of an electrogenic lactate export during which two H^+ are translocated across the cytoplasmic membrane together with one lactate molecule, thereby generating a membrane potential. Since the driving force of this transport is the transmembrane lactate gradient, the stimulatory interaction is based on the very low external lactate concentration maintained by *Pseudomonas*. This enables *S. cremoris* to gain more energy from the efflux of lactate than at higher external concentrations (Michels et al., 1979). Commensalistic interactions are also established in mixed cultures where either stimulatory compounds are formed or inhibitory compounds are being removed. As an example of the removal of inhibitory compounds, methane-consuming pseudomonads grew in a mixed culture with a *Hyphomicrobium* species, the latter removing small inhibitory amounts of methanol formed during methane oxidation (Wilkinson et al., 1974).

Synergism Synergism (protocooperation) occurs between *Lactobacillus arabinosus* and *Enterococcus faecalis* in a minimal medium (Nurmikko, 1956) in which each organism is unable to grow on its own. The synergism is

based on the fact that *S. faecalis* requires folic acid, which is produced by *Lactobacillus*, whereas the latter requires phenylalanine, which is produced by *Streptococcus*. Reciprocal stimulation also can occur in yoghurt, where *Lactobacillus bulgaricus* produces amino acids that stimulate the growth of *Streptococcus thermophilus*. The latter produces small quantities of formic acid, which in turn stimulates *L. bulgaricus* (Driessen, 1981). Chemolithotrophic and phototrophic organisms growing with CO_2 as carbon source are known to excrete substantial quantities (>50%) of the carbon fixed as carbohydrates, peptides, amino acids, lipids or vitamins (Clark and Schmidt, 1966; Czeczuga, 1968; Fogg, 1971; Cohen et al., 1979; Jones, 1982; Coveney, 1982; Wolter, 1982; Soendergaard et al., 1985; Bateson and Ward, 1988). In these interactions, the benefit for the chemo- or photolithoautotrophic partner is often less evident and may comprise the supply of vitamins (Jones, 1982), the reduction of oxygen concentration around heterocysts (Bunt, 1961; Paerl, 1978; Paerl, 1982)—which has been disputed on physical grounds (Overmann, 2002a)—and the formation of CO_2 (Lange, 1971). A reciprocal relationship was also suggested in a mesophilic estuarine microbial mat system in which the cyanobacterium *Microcholeus chtonoplastes* excreted organic matter that stimulated growth of *Thiocapsa roseopersicina*, which in turn prevented accumulation of excess amounts of hydrogen sulfide (de Wit and van Gemerden, 1988).

Many different types of interspecies interactions occur in laminated microbial ecosystems, or so-called "microbial mats," which are found on seashores, estuarine areas, salt marshes, and along the effluents from geothermal springs. Metabolic interactions include 1) the excretion of photosynthates and cell lysis of cyanobacteria as the primary colonizers, 2) respiration of the organic substances by heterotrophic bacteria, thereby generating anoxic conditions, 3) fermentation and reduction of sulfate by anaerobic bacteria, and 4) oxidation of the reduced sulfur compounds by anoxygenic phototrophs and chemolithotrophs (Stal et al., 1985). In addition, a multitude of interactions, most of them beneficial, occur between the different groups of prokaryotes (Overmann and van Gemerden, 2000). In microbial mats, sulfate-reducing bacteria can reach their highest abundance at the surface, rather than at the deeper, permanently anoxic sediment layers. Accordingly, the sulfate-reducing bacterium *Desulfovibrio desulfuricans* has been found to be capable of syntrophic growth with the colorless sulfur-oxidizing bacterium *Thiobacillus thioparus* under oxygen limitation and with lactate as the electron-donating substrate (van den Ende et al., 1997). In this association, *Thiobacillus* removed molecular oxygen inhibiting *Desulfovibrio* and provided the latter with soluble polysulfide compounds as electron acceptor. *Desulfovibrio* in turn oxidized lactate to acetate, forming sulfide concomitantly, which served again as electron-donating substrate of *Thiobacillus*.

Syntrophic Interactions Under certain conditions, synergism between different groups of anaerobic prokaryotes is essential for the degradation of organic matter. In contrast to aerobic decomposition, anaerobic mineralization involves the participation of different groups of prokaryotes with only a limited physiological flexibility. Consequently, a tight cooperation of mixed populations exists. In this multistep process, polymeric organic matter (such as cellulose, proteins, and lipids) is first hydrolyzed to oligomers and monomers that are subsequently fermented (Hungate, 1960; Bryant, 1976; Laanbroek and Veldkamp, 1982; Wolin, 1982; Nedwell, 1984). The first step in anaerobic degradation of sugars, most amino acids, and other readily fermentable substrates is the formation of H_2, CO_2, formate, alcohols, acetate and other short-chain fatty acids, sulfide, and ammonia. In the presence of oxidized sulfur compounds, further mineralization may proceed directly through activity of sulfate-reducing bacteria, which, as a group, can directly oxidize the (long-chain) fatty acids, amino acids, alcohols, aromatic compounds, and hydrogen (Widdel, 1988). In the absence of sulfate, CO_2 will serve as the major electron acceptor resulting in the formation of methane. Methanogenic bacteria, however, have a very narrow range of substrates (mainly H_2, acetate, formate, methanol, and several methylamines; Oremland, 1988). Hence, an additional group of prokaryotes, the "acetogenic" bacteria, are involved in the formation of suitable methane precursors. In the absence of sulfate, the subsequent fermentative degradation of some of the products like propionate, butyrate, or benzoate to hydrogen, CO_2, and acetate is an endergonic process under standard conditions, however. A syntrophic relation, the so-called "interspecies hydrogen transfer," is established between hydrogen-producing bacteria (also called "obligate proton-reducing bacteria") on one hand and hydrogen-consuming prokaryotes such as methanogens on the other.

Anaerobic chemotrophic syntrophic cocultures are the only associations of prokaryotes that so far have been investigated in sufficient detail to understand the physiological basis of the interaction (Schink, 1991). Interspecies hydrogen transfer in these cocultures is decreased to values below 10 Pa (Zehnder and Stumm, 1988),

which renders the oxidation of short-chain fatty acids exergonic. Thus, *Syntrophomonas wolfei* in coculture can metabolize even-numbered fatty acids such as butyrate, caproate, and caprylate to H_2 and acetate; odd-numbered fatty acids such as valerate and heptanoate are metabolized to propionate, acetate, and hydrogen (Dwyer et al., 1988; McInerney et al., 1979; McInerney et al., 1981). *Syntrophobacter wolinii* was shown to degrade propionate to acetate, CO_2, and H_2 in coculture with an H_2-consuming *Desulfovibrio* species (Boone and Bryant, 1980). Although acetate can be cleaved to CH_4 and CO_2 by several methanogenic species, a thermophilic acetate-oxidizing organism was shown to convert acetate to CO_2 and H_2 only in coculture with *Methanobacterium thermautotrophicum* (Zinder and Koch, 1984). Benzoate and several other aromatic compounds were also shown to be degraded by cocultures of a proton-reducing species and methanogens, sulfate-reducing bacteria, or both (Mountfort and Bryant, 1985; Dolfing and Tiedje, 1986; Mountfort and Asher, 1986; Szewzyk and Schink, 1989). Although hydrogen represents the best-documented agent for transfer of reducing equivalents, formate serves the same purpose in species lacking hydrogenases (Thauer et al., 1975; Thiele and Zeikus, 1988a; Thiele et al., 1988b). Syntrophic associations can involve even three different physiological types of prokaryotes, e.g., two different sulfate-reducing bacteria and one methanogenic archaeon (Thiele and Zeikus, 1988a).

In other fermentations, proton reduction and subsequent hydrogen transfer is not a strict requirement since the reaction is exergonic under standard conditions, but interspecies hydrogen transfer shifts the fermentation pattern toward the production of more oxidized products plus hydrogen in contrast to the reduced fermentation products formed in pure culture. For example, *Ruminococcus albus* ferments glucose to acetate, H_2, and CO_2 (instead of to ethanol) if cocultured with *Wolinella succinogenes*, which uses hydrogen as electron donor in the reduction of fumarate to succinate (Ianotti et al., 1973). Hydrogen transfer results in an additional energy gain for *R. albus* as more acetyl-CoA is converted to acetate in an ATP-yielding route. Shifts in fermentation products were also demonstrated for cocultures of *Clostridium thermocellum* and *Methanobacterium thermautotrophicum* (Weimer and Zeikus, 1977).

A second type of syntrophic interaction has been found in cocultures of green sulfur bacteria with sulfur- or sulfate-reducing bacteria (Wolfe and Pfennig, 1977; Biebl and Pfennig, 1978; Warthmann et al., 1992). Under conditions of limitation by inorganic sulfur compounds as they prevail in freshwater ecosystems, the sulfide-producing bacterium relies on the activity of the sulfide-oxidizing phototroph for the availability of the electron acceptor, while the activity of the green sulfur bacteria is controlled by the activity of the sulfide-producing organism. In this way, a closed sulfur cycle is established through which each sulfur atom cycles many times (Pfennig, 1980).

Symbiosis Mutual relationships between different prokaryotes render them capable of occupying niches that could not be occupied by either organism alone. "Symbiosis" in its orginal sense is a close association between different species of organisms (de Bary, 1879). Currently, symbiosis is often defined as an obligatory and highly specific mutual relationship that benefits both partners (Atlas and Bartha, 1993; Table 7). Most symbioses of prokaryotes known to date are with eukaryotes. Besides the endosymbiosis of chloroplasts (cyanobacteria) or α- mitochondria (Proteobacteria; Margulis, 1981), the root nodule symbiosis of rhizobia with legumes is another well-studied example. In the latter association, the bacteria provide combined nitrogen and the plant host dicarboxylic acids. In their symbiosis with anaerobic protozoa, prokaryotes appear to act as hydrogen sinks in interspecies hydrogen transfer (Hackstein et al., 1999). In ectosymbiotic association with marine nematodes, especially adapted γ-Proteobacteria oxidize sulfide with concomitant CO_2-fixation (Ott et al., 1991), as do the endosymbionts of the same group in gutless marine oligochetes (Dubilier et al., 1995). Cellulolytic nitrogen-fixing bacteria in shipworms (Bivalvia: Terebrenidae) provide exoenzymes necessary for the digestion of cellulose or keratene (Waterbury et al., 1983). Bacterial symbionts of insects synthesize nutrients such as vitamins (Aksoy, 1995) or recycle amino acids (Douglas, 1998). Intracellular bacterial symbionts of protozoa can provide toxins, which in turn are of selective value for the host in competition with protozoal competitors (Görtz and Brigge, 1998). In other cases, the cytotoxicity of the protozoal host is increased by endosymbiotic bacteria (Fritsche et al., 1998). Most of the prokaryotic symbionts of higher organisms could not be cultured to date and thus have only been identified by 16S rDNA analyses.

In contrast to the hundreds of cases of symbiotic relationships between prokaryotes and eukaryotes, only very few examples of symbioses between different prokaryotes have been described to date (Overmann and Schubert, 2002b). However, evidence from very different environments, which range from the termite hindgut and dental plaque to the chemocline of stratified lakes and deep-sea sediments, indicates

that many more symbiotic associations exist among prokaryotes (Overmann, 2002a; Overmann and Schubert, 2002b). As indicated by microscopic studies, morphologically conspicuous associations of prokaryotes exist in the form of "consortia," in which two or more prokaryotes maintain a permanent cell-to-cell-contact (Hirsch, 1984; Schink, 1991; Trüper and Pfennig, 1971), and most likely represent the extreme case of a mutual interdependence of different prokaryotes. To date, some 16 different types of such consortia have been described (Huber et al., 2002; Overmann, 2002a; Overmann and Schubert, 2002b) and are comprised of very different phylogenetic groups, e.g., filamentous cyanobacteria associated with heterotrophic bacteria, associations between giant sulfur-oxidizing γ-Proteobacteria (*Thioploca*) and sulfate-reducing *Desulfonema*, or the aggregates of sulfate-reducing bacteria with methanogenic archaea, which were detected in methane-hydrate-rich marine sediments. An additional novel type of association has been described (*Nanoarchaeum equitans*; Huber et al., 2002). Recently, advancements have been made towards a more detailed understanding of the structure and function of phototrophic consortia consisting of green sulfur bacterial epibionts surrounding a colorless motile central α-Proteobacterium (Fröstl and Overmann, 1998; Fröstl and Overmann, 2000; Overmann et al., 1998; Tuschak et al., 1999; Overmann and Schubert, 2002b).

Enrichment Techniques

When the desired prokaryote is present in very small numbers, the enrichment-culture technique is used to enable this particular type of microorganism to grow faster than all others in the sample. During selective enrichment, a natural sample harboring many different microorganisms is kept under conditions which favor the growth of a particular physiological type of prokaryote or a group of prokaryotes, thus allowing them to grow faster than accompanying physiologically different types of microorganisms (Schlegel and Jannasch, 1967; Aaronson, 1970; Norris and Ribbons, 1970a; Norris and Ribbons, 1970b; Veldkamp, 1977; Gerhardt et al., 1981; Poindexter and Leadbetter, 1986; Austin, 1988). Usually, no inhibitory ingredient is added to the medium, but the enrichment medium is designed to favor growth of the desired organism. The technique was essentially developed by microbiologists (e.g., Beijerinck and Winogradsky) early in the twentieth century. The enrichment technique is applied if 1) a prokaryote with particular metabolic properties is known to exist or 2) it is to be determined whether prokaryotes exist which grow under specific conditions, e.g., high/low temperature, high osmolarity, extreme pH values, or with xenobiotic carbon substrates.

By far the most common method of enrichments is the batch culture technique, resulting in the selection of those prokaryotes that attain the maximum specific growth rate under the conditions chosen. Since at the same time, bacteria are growing for a substantial period of time at high substrate concentrations, batch culture enrichments frequently select for prokaryotes with high maximum growth rates and low substrate specificity (the so-called "zymogenous organisms" [Winogradsky, 1949], also referred to as "*r*-strategists" [Andrews, 1984; Andrews and Harris, 1986]). It has been argued for many years that prokaryotes obtained in this way are not representative of those which dominate microbial transformations in the natural environment (Jannasch, 1967a; Jannasch, 1967b; Veldkamp and Jannasch, 1972; Veldkamp, 1977; Kuenen and Harder, 1982b; Gottschal, 1986; Poindexter and Leadbetter, 1986; Gottschal and Dijkhuizen, 1988). Nevertheless, nonlimiting growth conditions possibly also occur under natural conditions at least as transient phenomena, for instance after the burial of organic matter (leaves and fecal pellets), during algal blooms, tidal flooding, or upon sudden changes in physicochemical conditions. Similarly, in gastrointestinal tracts, feeding may provide for conditions most suitable for a rapid response of opportunistic microbial species. The batch culture technique can be employed for the enrichment and isolation of *K*-strategists, if the latter outnumber the accompanying *R*-strategists in the original sample and are capable of (albeit slow) growth in the medium used. In these cases, serial dilution series are established, such that *R*-strategists are absent at higher dilutions and cannot overgrow the target prokaryotes (Ferris et al., 1996; Sekiguchi et al., 2001).

The second, less frequently employed technique is the use of a chemostat with one or more growth-limiting nutrients in the inflowing medium. Using this approach, organisms specialized in growing at very low substrate concentrations and usually exhibiting relatively low μ_{max} values and fairly high substrate specificities (so-called "autochthonous" prokaryotes or *K*-strategists) can be obtained and studied (Andrews, 1984; Jannasch, 1967a; Jannasch, 1967b; Schlegel and Jannasch, 1967; Harder et al., 1982; Kuenen and Harder, 1982b; Poindexter and Leadbetter, 1986; Gottschal and Dijkhuizen, 1988). Another advantage of continuous cultures systems over batch cultures is that physical and chemical parameters can be maintained constant over prolonged periods. Alternatively, these conditions may be changed in a controlled fashion,

such as during light/dark cycles or oxic/anoxic transitions.

Isolation of Prokaryotes

The purpose of isolation is the generation of a single clone, i.e., a population consisting of bacteria all derived from a single cell. The strains obtained in this manner are an essential, but not sufficient (see Interactions with Other Microorganisms), prerequisite for thorough, in-depth and unambiguous studies of the ultrastructure, physiology, molecular biology, genetics and autecology of prokaryotes. When free from other (contaminating) microorganisms, a culture is called "axenic." Not all individuals in this population need to be genetically identical as mutation and selection of mutants can take place, but this heterogeneity is accomodated in the pure culture concept.

Since pure cultures almost never occur in nature, their preparation involves the physical separation of single cells from others, inoculation into sterile medium, and incubation under conditions allowing axenic growth. In general, samples are diluted by means of different methods and concomitantly placed on a nonselective medium. Either direct samples or aliquots from appropriate enrichments may be used for isolation of strains. Separation of single prokaryotic cells can be accomplished by streaking on solid media such as agar plates, separation in liquid media as occurs during preparation of pour plates, in liquid dilution series, or with the aid of micromanipulators. Each colony on solid media or culture developing in the highest positive dilutions potentially has grown from a single cell.

For inoculation with diluted sample material, a small amount of liquid (≤100 µl for agar plates) containing not more than about 200 cells is brought on the surface of an agar plate and spread evenly by the use of a Drigalski spatula. Samples with higher cell densities, for instance material from cell colonies, are streaked on the surface with a sterile loop after dipping the latter in the sample. One of the most efficient ways of streaking is the "13-streak method" (Cypionka, 1999), which involves intermittent sterilization of the loop and provides the highest chance to obtain single, well-isolated colonies from samples of widely different cell densities. Physically separated cells grow out into distinct populations visible on the solidified medium as small heaps or spots, called "colonies," varying in shape, color, and size. When streaking an alleged pure culture, all colonies should be identical in appearance, color, and texture, but in crowded parts of the plate, the size of the colonies will be smaller owing to nutrient depletion. Consequently, size difference as a criterion can only be applied to plates with well-separated (>5 mm) colonies. Motile and swarming prokaryotes cannot easily be isolated on agar plates unless the plates are thoroughly dried and chemicals (mostly detergents like Pril® at a concentration of 0.2% w/v, as used in Pril-Mannitol-Agar [Merck]; Pietzsch, 1969; Reusse and Meyer, 1972) added to disintegrate bacterial flagella and thus stop motility. Some bacteria cannot be cultivated or isolated on solid agar media and require sloppy agar or a liquid medium.

An alternate method is the inoculation of agar plates in the liquid stage, shortly before solidification (the so-called "pour-plate" technique). A known volume of a bacterial suspension is mixed with molten agar (kept at 42°C to just prevent solidification) and then poured into a sterile Petri dish where the mixture is allowed to solidify. With some experience, mixing can also be performed in the Petri dish itself. Upon incubation, cells will grow into colonies that mostly develop below the surface. The latter colonies often show a lenticular shape. Gas-forming colonies may produce cracks in the surrounding agar. The pour-plate technique is suited for the isolation of organisms requiring oxygen at pressures lower than atmospheric (microaerobic bacteria). It is not easy, however, to isolate or count subsurface colonies or to assess colony morphology as a criterion for culture purity. Also, some prokaryotes may be killed by exposure to the higher temperatures of the molten agar.

Anaerobic bacteria most frequently are isolated in deep-agar dilution series in pre-reduced media (see Cultivation of Anaerobes in this Chapter). The first tube is inoculated and mixed and an aliquot (usually a tenth of the volume) is transferred to the following tube. In this manner, a serial dilution series is obtained, which is subsequently solidified by cooling in a 20°C water bath, rapidly gassed with N_2 or a mixture of N_2/CO_2, and sealed with gas-tight butyl rubber stoppers.

Liquid dilution series are preferentially employed for more sensitive bacteria that do not readily grow on agar-solidified media or for inocula with cell titers too high for direct plating on solid media. An additional advantage of this technique is that physical separation of bacterial cells in serial dilutions may promote growth of those bacteria which are otherwise inhibited by antibacterial substances (e.g., bacteriolytic enzymes) produced by the accompanying bacteria (Talamoto et al., 1994). Heterogeneous liquid samples are first homogenized by shaking or agitation in some sort of mechanical device, e.g., a tube containing sterile glass beads, or in a mixer. A nontoxic detergent and complexing agents, e.g., Tween 80 (final concentration, 0.05% v/v) and sodium pyrophosphate (10 mM in

10 mM *n*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] [HEPES] buffer), respectively, may be added for detachment of cells adhering to solids. Subsequently, serial dilutions (mostly in 1 : 10 dilution steps) are prepared in sterile medium. Directly after inoculation, each tube has to be mixed carefully, and a fresh glass pipette or pipette tip must be employed for the following dilution step. By inoculating tubes with cell suspensions so dilute that some tubes receive just one single cell or no cell at all, pure cultures may ultimately be obtained and most probable numbers (MPN) of culturable bacteria can be calculated. This method is based on the assumption that bacteria are normally distributed in the liquid media. Numbers of viable cells from natural samples obtained in this way frequently surpass those obtained on agar media (Bartscht et al., 1999; Jaspers et al., 2001). However, the opposite was observed for soil samples, which yielded highest numbers of cultivated bacteria on improved solid media (Janssen et al., 2002).

One drawback of MPN dilution series when employed for the isolation of bacterial strains is that only few cultures can be obtained from the highest positive dilutions (i.e., based on the number of parallel dilution series, usually 3–8). However, provided the average viability for cells in a given sample is known, the number of pure cultures produced can be increased by orders of magnitude without increasing the total number of cultures inoculated. To this end, one appropriate size of inoculum is chosen which statistically contains 0.5 viable cells for parallel inoculation of media. A very efficient way of automated inoculation is the MICRODROP® technique, in which microdroplets (volume of ~100 pl) of a cell suspension are generated by a piezoelectric actuator and positioned directly on agar plates (Schober et al., 1993) or into each well of microtiter plates filled with liquid media. If the procedure is automated, large numbers of inoculation can be performed very rapidly (e.g., 100 inoculations in less than a minute). If each droplet statistically contains 0.5 viable bacterial cells, the number of positive positions on the agar plate or of wells in a microtiter plate follows a Poisson distribution and the most probable number of culturable bacteria can be calculated from fraction p of positive tubes (i.e., the number of positive tubes divided by the total number of tubes n) according to Button et al. (1993)

$$MPN = -\ln(1-p) \tag{24a}$$

The standard deviation is

$$\text{S.D.} = \sqrt{\frac{p}{[n\cdot(1-p)]}} \tag{24b}$$

A much more tedious isolation procedure involves the use of micromanipulator devices (Skerman, 1968), which nowadays include computer-assisted electronic controls, and is used to physically separate single cells or morphologically defined symbiotic consortia under an inverted microcope (Fröhlich and König, 1999; Fröstl and Overmann, 2000). The isolated cells can then be transferred into sterile media. This latter method is especially valuable if morphologically conspicuous prokaryotes, which are easily overgrown by other microorganisms, are to be isolated. This procedure has been used to isolate cyanobacteria, myxobacteria, budding bacteria, and manganese-oxidizing bacteria and phototrophic consortia (Fröhlich and König, 1999; Fröstl and Overmann, 2000; Sly and Arunpairojana, 1987).

Alternative and Novel Concepts for Cultivation

The usually low cultivation success and the molecular detection of entire not-yet-cultured groups of prokaryotes (see Introduction in this Chapter) strongly indicate that specially adapted or novel techniques have to be developed for the isolation of not-yet-cultured prokaryotes from natural samples. Most importantly, novel or alternative cultivation techniques should account for those factors most likely responsible for the low culturability.

Ionic Composition of the Growth Media

Fully synthetic media have been developed which correspond to the ionic strength of freshwater (Bartscht et al., 1999) or seawater (Schut et al., 1993; Sass et al., 2001). For the cultivation of soil prokaryotes, ionic concentrations have been adapted to those typically found in soil solution (Angle et al., 1991; Grosser et al., 2000; for Winogradsky's salt solution, compare Aagot et al., 2001). Synthetic seawater medium has been used in several investigations of the culturability of marine bacteria (Schut et al., 1993; Eguchi et al., 2001; Sass et al., 2001). The various recipes differ slightly with respect to trace element content, salt composition, and the type of buffer used, and they represent an improvement over other types of media employed previously. Reduction or elimination of phosphate and copper concentrations, respectively, has proven very successful for the isolation of "unculturable" cyanobacteria (Waterbury, 1991). Recently, artificial freshwater media have been developed which facilitate the cultivation of planktonic bacteria from limnic sources (Bartscht et al., 1999; Jaspers et al., 2001).

Exploring Novel Types of Metabolism

Novel types of microbial reactions may be postulated on the basis of measurements of concentration changes in potential microbial substrates and products in the environment (Zengler et al., 1999; Schink and Friedrich, 2000). Any reaction can theoretically be assessed for its potential to drive prokaryotic growth by calculating the free energy change. A negative value of the free energy change indicates that a postulated reaction can actually promote microbial growth and can then be verified by trying to enrich for the respective prokaryote.

Cultivation-Independent Physiological Testing of Target Bacteria

The enrichment and isolation of numerically abundant prokaryotes or specific groups of interest can be followed by a phylogenetic screening of enrichments, e.g., by fluorescent in situ hybridization (FISH; Kane et al., 1993; Spring et al., 2000). FISH has recently been used in a combined approach to identify potential growth substrates of target prokaryotes for subsequent enrichment and isolation experiments. In this new approach, the uptake of radiolabeled substrates by target bacteria can be followed if microautoradiography is combined with FISH using oligonucleotide probes designed for selected prokaryotes (Lee et al., 1999; Cottrell and Kirchman, 2000; Gray et al., 2000; Nielsen et al., 2000; Ouverney and Fuhrman, 2000). Pitfalls of this novel technique are 1) that only one growth substrate can be tested at a time and 2) that the added substrate may be rapidly degraded by other (nontarget) microorganisms and that target prokaryotes are actually the only organisms present capable of assimilating the (unidentified) metabolites.

Stable isotope probing is a parallel approach based on labeling with ^{13}C-labeled substrates of genomic DNA of selected prokaryotes. Active prokaryotes can subsequently be identified by 16S rDNA sequencing of the heavy DNA (Radajewski et al., 2000). A limitation of the latter technique is that 1) only those substrates can be tested which are utilized by very few types of prokaryotes (e.g., C_1-compounds), 2) a large excess of labeled substrate and long incubation times are necessary to maximize ^{13}C-uptake and obtain sufficiently heavier genomic DNA—possibly resulting in pronounced shifts in microbial community structure, and 3) unknown intermediates may be rapidly generated by other bacteria thus preventing a correct identification of potential growth substrates.

Thirdly, prokaryotes stimulated by defined substrates can be identified on the basis of the incorporation of bromodeoxyuridine (BrdU) into genomic DNA by metabolically active cells, subsequent immunocapture of BrdU-labeled DNA, and sequencing of the corresponding 16S rDNA sequences (Urbach et al., 1999). An important limitation of the latter approach is that not all prokaryotes assimilate BrdU, even when metabolically active.

As a fourth approach to identify potential growth substrates and optimize growth conditions for yet-to-be-cultured prokaryotes, specific oligonucleotide probes can be developed which are directed against the internal transcribed spacer region of the rRNA (*rrn*) operon and used for FISH to detect selectively, physiologically active cells (Cangelosi and Brabant, 1997; Licht et al., 1999; Oerther et al., 2000; Schmid et al., 2001). All of the above techniques in principle can be employed to perform substrate tests for target prokaryotes under close to in-situ conditions without prior cultivation of the microorganism of interest.

Types and Concentrations of Growth Substrates

Sometimes, specific substrates can selectively promote the growth of numerically abundant bacteria (González et al., 1997). However, target bacteria are frequently slow-growing *K*-strategists and hence easily overgrown by the more rare but rapidly dividing large-celled *R*-strategist bacteria during enrichment in the presence of higher substrate concentrations. Consequently, one novel approach for the isolation of bacteria from natural samples is the utilization of low substrate concentrations of carbon substrates added to synthetic media of appropriate ionic strength (see Ionic Composition of Growth Media). In addition, reduction of the concentrations of N and P compounds (which at certain times may represent the growth-limiting natural substrates) has been found to significantly increase colony counts (Eilers et al., 2001). An alternative approach is to employ sterile filtered seawater directly (Button et al., 1993), which already contains naturally occurring organic carbon substrates as potential substrates for the growth of prokaryotes. Media for the cultivation of soil bacteria can be supplemented with cold soil extract (Olsen and Bakken, 1987) as a carbon and energy source or as a source of supplies. In some instances, application of low nutrient media has yielded comparably high cultivation success (up to 60%; Button et al., 1993). Application of complex mixtures of organic carbon substrates from natural sources can also increase growth of selected groups of anaerobic bacteria. For example, anaerobically prepared sterilized sludge or sediment slurries resulted in higher

MPN of sulfate-reducing bacteria as compared to conventional media (Vester and Ingvorsen, 1998).

As an alternative, typical *K*-strategists can be preconcentrated and potential *R*-strategists selectively excluded by a prefiltration step during which the inoculum is filtered through sterile 0.8-μm pore size membrane filters. This treatment can significantly increase the enrichment efficiency of certain target bacteria. As an example, members of the *Holophaga*/*Acidobacterium* lineage could be successfully enriched from freshwater lake sediments using prefiltration (Spring et al., 2000).

Especially adapted cultivation techniques have been developed to meet the exact physiological requirements of fastidious bacteria. Gradient plates or gradient tubes represent a simple means to cultivate prokaryotes dependent on two substrates, each present at a defined and very narrow concentration range. Examples include chemolithotrophic sulfur-oxidizing bacteria, which are naturally occurring in highly stratified environments such as the chemocline of stratified lakes or sediments. In such cases, artificial agar-stabilized oxygen-sulfide-countergradients have been used very successfully to isolate for the first time *Beggiatoa alba* or magnetotactic cocci (Nelson et al., 1986; Meldrum et al., 1993). In a second type of system, a quartz sand core is employed for gradient stabilization and is sandwiched between a lower anoxic sulfide-containing compartment and an upper oxic compartment (benthic gradient chamber; Pringault et al., 1996). As a great advantage of such gradient systems, the bacteria of interest (by means of their own physiological activity) precisely create the necessary environmental conditions for growth (Jørgensen, 1982).

Recently, the testing of novel classes of substrates has led to the discovery of novel physiological capabilities of known bacterial species, or even to the enrichment and isolation of novel bacteria of a broad phylogenetic diversity (Coates et al., 2002). Many investigations have focussed on the role of humic substances on bacterial growth. Humic substances consist of a skeleton of alkyl or aromatic units which are cross-linked mainly by oxygen and nitrogen groups and which carry carboxylic acid, phenolic and alcoholic hydroxyls, ketone, and quinone groups (Schulten et al., 1991). Contrary to the conservative view of humic substances as being refractory, they in fact can be utilized in different ways by a large number of prokaryotes. Besides the utilization as growth substrates by *Rhodococcus* spp. (Goodfellow, 1992a), and probably also by *Actinoplanes* and related genera (Vobis, 1992), *Microbispora*, *Streptosporangium* (Goodfellow, 1992b), and *Pedomicrobium* spp. (Poindexter, 1992), humic substances represent redox-active compounds that act as electron carriers in abiotic as well as biotic redox reactions (Coates et al., 2002). The redox-active moieties in these reactions are the quinone groups (Lovley et al., 1996; Scott et al., 1998). Humic compounds can act as an electron acceptor for respiratory and fermentative bacteria belonging to diverse bacterial lineages, such as the high G+C Gram-positive bacteria (*Propionibacterium freudenreichii*), γ-Proteobacteria (*Shewanella alga*), δ-Proteobacteria (*Geobacter metallireducens*), or the *Holophaga*/*Acidbacterium* division (e.g., *Geothrix fermentans*, Benz et al., 1998; Coates et al., 1998; Lovley et al., 1999b). Humic compounds as water soluble carriers can shuttle electrons between microorganisms and insoluble terminal electron acceptors (e.g., Fe(III) oxides; Lovley et al., 1996). In addition, humic substances have been shown to be utilized as electron-donating substrates during respiration with nitrate or fumarate, while organic carbon compounds (e.g., acetate) are assimilated heterotrophically (Lovley et al., 1999b; Coates et al., 2002). This latter type of metabolism has been shown for *S. alga*, *G. metallireducens* and *G. fermentans*, but also for the denitrifying bacterium *Paracoccus denitrificans*, the α-Proteobacterium *Agrobacterium* strain PB, the β-Proteobacteria *Dechloromonas agitata*, *Dechloromonas* strain JJ, *Azoarcus* strain HA, the γ-Proteobacteria *Pseudomonas* strain BU, *Pseudomonas* strain NMX, and *Marinobacter* strain SBS, and a γ-Proteobacterium (strain KC; Lovley et al., 1999b; Coates et al., 2002). These microorganisms are capable of exploiting a part of the large pool of humic substances in soils and sediments and gain a competitive advantage over prokaryotes that depend on limited organic compounds (e.g., acetate) as energy source.

Cocultivation

Often, positive interactions between cells of one clone are required for growth of a single cell. This is exemplified by the fact that in the case of fastidious prokaryotes, small inocula can result in an extended lag period or a complete failure of growth. This effect has been attributed to the presence of low-molecular-weight metabolic intermediates excreted by living cells whose introduction into fresh medium depends on the size of the inoculum. This type of isolation difficulties can be remedied by preparing a sterile filtrate of spent enrichment culture medium and including this filtrate as a major constituent of the new medium on which single cells of fastidious microorganisms are to be isolated (Atlas and Bartha, 1993).

As a third point of concern, evidence has accumulated that different prokaryotes may actually depend on phylogenetically nonrelated partner bacteria for a successful enrichment (see Interactions with Other Microorganisms). For example, bacteria of the *Holophaga*/*Acidobacterium* lineage in enrichment cultures as well as natural microbial communities are frequently accompanied by α-Proteobacteria of the *Beijerinckia* group (Felske et al., 1998; Nogales et al., 1999; Radajewski et al., 2000; Spring et al., 2000). Another example is the not-yet-cultured β-Proteobacterium associated with green sulfur bacteria in phototrophic consortia (Fröstl and Overmann, 2000) and to date not enriched or grown in pure culture. Double culture vessels for cocultivation of different bacteria have been described earlier (Jannasch and Mateles, 1974; Wirsen and Jannasch, 1978). Meanwhile, simple incubation devices have been developed which permit the growth of two different prokaryotes in coculture, but separate them by a membrane permeable to metabolites (Fig. 4).

The addition of cAMP, which represents one type of bacterial signal compound at least in some cases, resulted in a significant increase of culturability in natural samples (Bruns et al., 2002). An isolated *Roseovarius* strain exhibited increased cell yield in the presence of cAMP. Similarly, the addition of pyrophosphate to cultures of *E. coli* has been proposed to confer upon the cells a better capacity to use carbon sources, induce biosynthetic processes, and enhance stationary phase survival of *E. coli* cells (Biville et al., 1996).

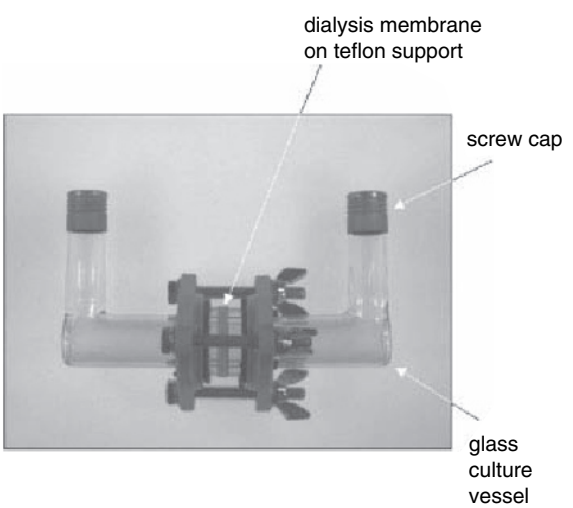

Fig. 4. A simple dialysis coculture setup that permits the growth of two separate prokaryotic cultures while contact via a dialysis membrane is maintained. For growth of aerobic cultures, the screw caps are replaced by cotton plugs and the glass vessels are only half full. Homogeneous cell suspensions can be maintained by adding a small stirring bar to each of the two chambers and placing the set up on a magnetic stirrer.

Chemical Protection

Upon exposure to stress, e.g., heat, osmotic or ethanol shock, bacterial cells may stop growing while their metabolism continues. As a result of this uncoupling, a burst of free radical production occurs which may be lethal to the cells. Exponentially growing bacterial cells are significantly more sensitive to this so-called "suicide response" than cells in the stationary phase (Aldsworth et al., 1999). Entry of *E. coli* into stationary phase growth leads to expression of genes for resistance mechanisms under the control of the alternative sigma factor σ^s, and they include those for resistance against oxidative stress such as *katE* (Loewen and Hengge-Aronis, 1994). At least in some bacteria, cellular damage by oxidative stress can be prevented by the addition of H_2O_2-degrading compounds such as catalase, sodium pyruvate, or α-oxoglutaric acid (Martin et al., 1976b; Brewer et al., 1977; Mossel et al., 1980; Mizunoe et al., 1999). The addition of ascorbic acid to the growth medium or lowering of the oxygen partial pressure has similar effects (Jannasch and Mateles, 1974). The addition of chemical protectants or antioxidants may thus improve the recovery of abundant bacteria from natural samples. Accordingly, addition of activated charcoal as another scavenger of toxic oxygen forms increased the number of colony-forming units in samples from the deep terrestrial subsurface (Stevens, 1995).

Solid Surfaces

Solid surfaces may lead to stimulation of cell division and growth of starved bacteria (Kjelleberg et al., 1982). (See Effect of Inhomogeneities.) So far, however, the capability of attachment has rarely been exploited as a selective factor for the enrichment and isolation of novel types of prokaryotes.

Conservation of Prokaryotic Cultures

For future studies, as reference for standardized assays and tests, for taxonomic purposes, and as valuable stock for biotechnological applications, strains of prokaryotes need to be maintained and preserved over extended periods of time. For long-term maintenance, cultures are routinely stored in a lyophilized or deep-frozen form to prolong their viability and to reduce changes due to the occurrence of mutations.

Maintenance of Working Cultures

Usually, maintenance of working cultures requires the periodic transfer of strains to fresh minimal media. Aerobic strains are streaked on cotton-plugged agar slants or agar plates, whereas stab cultures in agar deeps are produced for facultative anaerobes or microaerophiles and closed by screw caps. Obligate anaerobes are transferred to fresh liquid media. After cultivation, cultures are stored in the refrigerator in the dark at 5–8°C to reduce metabolic activity while maintaining viability. The frequency of transfer should be kept to a minimum, but it has to be determined separately for each organism: some require transfer after just a few days, while some sporeformers can survive in liquid cultures for years. During storage, agar slants and stab cultures must be prevented from drying. To avoid selection of mutants, it is advisable to subculture from the whole plate or slant and not just from one colony.

For different physiological groups of bacteria, specific maintenance requirements exist. Working cultures of rumen bacteria and other fermentative anaerobes can be maintained in slants or sloppy agar (0.5–0.7% [w/v]) prepared from nonselective media. The cultures are incubated at 39°C until growth is just apparent and then stored at 4°C. Such cultures remain viable for a few weeks at least, although the viable count is decreased. Purple sulfur bacteria of the family Chromatiaceae are precultured in sulfide-containing media until the sulfide is depleted and intracellular sulfur has been formed as the intermediate oxidation product in the cells; cultures in this phase of growth have a chalky appearance macroscopically in reflected light. Subsequently, cultures are transferred to the dark at 4°C and can be stored for at least 3 months. Green sulfur bacteria (Chlorobiaceae) are maintained best after the accumulated sulfur has also been depleted. However, cultures of many microorganisms kept at 4°C still show slow growth and turnover of cellular components. Special preservation of cultures is required for extended storage and to prevent genetic and metabolic alterations.

Preservation of Stock Cultures

Spore-Forming Bacteria One method of long-term preservation for spore-forming bacteria is based on the use of sterile soil or sand. A sample of soil or sand is sterilized in a screw-capped bottle by autoclaving for several hours on at least two successive days. Then, 1 ml of the suspension of the organism is added, and the contents dried in a vacuum desiccator with the cap of the tube loosely closed. When the contents are dry, the cap is closed tightly and the bottle stored in the refrigerator. Even easier is the use of sterile filter paper discs or strips soaked with the bacterial suspension. The discs are stored as described for sand above. One may keep several discs in one screw-cap container and remove one disc at a time, taking care not to contaminate the remaining discs.

Lyophilization Freshly grown cells are harvested by centrifugation and placed in an ampoule containing suspending medium and frozen at –60 to –80°C. Rapid freezing is often accomplished in a metal chamber with dry ice (solid CO_2) and ethanol. The water is then removed from the frozen state directly by sublimation in a vacuum. Freeze-dryers consist of a manifold connected to a vacuum pump capable of reducing the pressure to less than 0.01 mmHg. The ampoule is sealed and stored in a cool place. The suspending medium is critical for the rate of survival during the freeze-drying process and for the rate of death of dried bacteria during storage. Among the most commonly used suspending media are serum plus 30% glucose nutrient broths or sterile skimmed milk fortified with 5% sucrose, sterilized in 3-ml amounts.

A percentage of survivors of freeze-thaw stress may lose the ability to form colonies on a minimal salts-glucose medium. In *E. coli*, this effect was not due to a particular nutrient that was absent, but rather to a pertubation of the cell control system since cyclic GMP or ppGpp singly or in combination partly restored the efficiency of plating on a minimal medium of frozen-thawed cells. Mutagenic effects of freeze-thaw stress have been shown to be related to single-stranded breaks in DNA, an effect that might be similar to that of ionizing radiation. The damage can be repaired in nutrient media. In *E. coli*, both the *uvrA*-, *polA*-dependent (excision repair) and the error-prone *rec*-, *polA*-dependent (recombinational repair) DNA repair pathways are required for repair of freeze-thaw-induced DNA damage.

Many bacteria (e.g., purple sulfur bacteria [Chromatiaceae]) cannot be preserved by lyophilization (Pfennig and Trüper, 1989)

Freezing and Ultrafreezing Cultures of rumen bacteria can be kept viable for up to one year by adding 20% (v/v) glycerol as a cryoprotectant and storage at –20°C (Teather, 1982). For long-term storage, cultures are kept at –196°C in liquid nitrogen in the presence of 5% dimethylsulfoxide (Hespell and Bryant, 1981; Pfennig and Trüper, 1989).

Culture Collections

A complete list of worldwide culture collections may be obtained from the following (http://www.dsmz.de/species/abbrev.htm) DSMZ website.

Literature Cited

Aagot, N., O. Nybroe, P. Nielsen, and K. Johnsen. 2001. An altered Pseudomonas diversity is recovered from soil by using nutrient-poor Pseudomonas-selective soil extract media. Appl. Environ. Microbiol. 67:5233–5239.

Aaronson, S. 1970. Experimental Microbial Ecology. Academic Press. New York, NY.

Ahn, W. S., S. J. Park, and S. Y. Lee. 2001. Production of poly (3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant Escherichia coli. Biotechnol. Lett. 23:235–240.

Aksoy, S. 1995. Wigglesworthia gen. nov. and Wigglesworthia glossinida sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of Tsetse flies. Int. J. Syst. Bacteriol. 45:848–851.

Aldsworth, T. G:, R. L. Sharman, and C. E. R. Dodd. 1999. Bacterial suicide through stress. Cell. Molec. Life Sci. 56:378–383.

Alldredge, A. L., and M. J. Youngbluth. 1985. The significance of macroscopic aggregates (marine snow) as sites for heterotrophic bacterial production in the mesopelagic zone of the subtropical Atlantic. Deep-Sea Res. 32:1445–1456.

Amy, P. S., and R. Y. Morita. 1983. Starvation-survival patterns of sixteen freshly isolated open-ocean bacteria. Appl. Environ. Microbiol. 45:1109–1115.

Andrews, J. H. 1984. Relevance of r- and K-theory to the ecology of plant pathogens. *In:* M. J. Klug and C. A. Reddy (Eds.) Current Perspectives in Microbial Ecology. ASM Press. Washington, DC. 1–7.

Andrews, J. H., and R. F. Harris. 1986. r- and K-selection and microbial ecology. *In:* K. C. Marshall (Ed.) Advanced Microbiology and Ecology. Plenum Press. New York, NY. 9:1–7.

Angle, J. S., S. P. McGrath, and R. L. Chaney. 1991. New culture medium containing ionic conentrations of nutreints similar to concentrations found in the soil solution. Appl. Environ. Microbiol. 57:3674–3676.

Arrhenius, S. 1889. Über die Reaktionsgeschwindigkeit bei der Inversion von Rohrzucker durch Säuren. Z. Phys. Chem. 4:226–248.

Atlas, R. M., and R. Bartha. 1993. Microbial Ecology, 3rd ed. Benjamin/Cummings. Redwood City, PA.

Austin, B. 1988. Methods in Aquatic Bacteriology. John Wiley and Sons. Chichester, UK.

Azam, F. 1998. Microbial control of oceanic carbon flux: The plot thickens. Science 280:694–696.

Bak, F., and N. Pfennig. 1991. Microbial sulfate reduction in littoral sediment of Lake Constance. FEMS Microbiol. Ecol. 85:31–42.

Balch, W. E., and R. S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethane sulfonic acid (HS-CoM)-dependent growth of Methanobacterium ruminantium in a pressurized atmosphere. Appl. Environ. Microbiol. 32:781–791.

Bale, S. J., K. Goodman, P. A. Rochelle, J. R. Marchesi, J. C. Fry, A. J. Weightman, and R. J. Parkes. 1997. Desulfovibrio profundus sp. nov., a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea. Int. J. Syst. Bacteriol. 47:515–521.

Balestra, G. M., and I. L. Misaghi. 1997. Increasing the efficiency of the plate counting method for estimating bacterial diversity. J. Microbiol. Meth. 30:111–117.

Barer, M. R., and C. R. Harwood. 1999. Bacterial viability and culturability. Adv. Microb. Physiol. 41:93–137.

Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. 91:1609–1613.

Barns, S. M., S. L. Takala, and C. R. Kuske. 1999. Wide distribution and diversity of members of the bacterial kingdom Acidobacterium in the environment. Appl. Environ. Microbiol. 65:1731–1737.

Baross, J. A., and R. Y. Morita. 1978. Microbial life at low temperatures: Ecological aspects. *In:* D. J. Kushner (Ed.) Microbial Life in Extreme Environments. Academic Press. London, UK. 9–17.

Bartlett, D., M. Wright, A. A. Yayanos, and M. Silverman. 1989. Isolation of a gene regulated by hydrostatic pressure in a deep-sea bacterium. Nature 342:572–574.

Bartlett, D. H., E. Chi, and M. E. Wright. 1993. Sequence of the ompH gene from the deep-sea bacterium Photobacterium SS9. Gene 131:125–128.

Bartlett, D. H., and T. J. Welch. 1995. ompH gene expression is regulated by multiple envirnmental cues in addition to high pressure in the deep-sea bacterium Photobacterium species strain SS9. J. Bacteriol. 177: 1008–1016.

Bartscht, K., H. Cypionka, and J. Overmann. 1999. Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. FEMS Microbiol. Ecol. 28:249–259.

Bast, E. 2001. Mikrobiologische Methoden, 2nd ed. Spektrum Akad. Verlag. Berlin, Germany.

Bateson, M. M., and D. M. Ward. 1988. Photoexcretion and fate of glycolate in a hot spring cyanobacterial mat. Appl. Environ. Microbiol. 54:1738–1743.

Battley, E. H. 1995. An apparent anomaly in the calculation of ash-free dry weights for the determination of cellular yields. Appl. Environ. Microbiol. 61:1655–1657.

Baxter, R. M., and N. E. Gibbons. 1962. Observations on the physiology of psychrophilism in a yeast. Can. J. Microbiol. 8:511–517.

Béjà, O., L. Aravind, E. V. Koonin, M. T. Suzuki, A. Hadd, L. P. Nguyen, S. B. Jovanovich, C. M. Gates, R. A. Feldman, J. L. Spudich, E. N. Spudich, and E. F. DeLong. 2000. Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. Science 289:1902–1906.

Béjà O., M. T. Suzuki, J. F. Heidelberg, W. C. Nelson, C. M. Preston, T. Hamadas, J. A. Eisen, C. M. Fraser, and E. F. DeLong. 2002. Unsuspected diversity among marine aerobic anoxygenic phototrophs. Nature 415: 630–633.

Benz, M., B. Schink, and A. Brune. 1998. Humic acid reduction by Propionibacterium freudenreichii and other fermenting bacteria. Appl. Environ. Microbiol. 64:4507–4512.

Bernhardt, G., H.-D. Lüdemann, R. Jaenicke, H. König, and K. O. Stetter. 1984. Biomolecules are unstable under "black smoker" conditions. Naturwissenschaften 71: 583–586.

Beudeker, R. F., J. C. Gottschal, and J. G. Kuenen. 1982. Reactivity versus flexibility in thiobacilli. Ant. v. Leeuwenhoek 48:39–51.

Beunink, J., and H. J. Rehm. 1988. Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system. Appl. Microbiol. Biotechnol. 29:72–80.

Bhakoo, M., and R. A. Herbert. 1979. The effects of temperature on the fatty acid and phospholipid composition of four obligately psychrophilic Vibrio spp. Arch. Microbiol. 121:121–127.

Bi, E., and J. Lutkenhaus. 1993. Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring. J. Bacteriol. 175:1118–1125.

Biebl, H., and N. Pfennig. 1978. Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Arch. Microbiol. 117:9–16.

Binnerup, S. J., D. F. Jensen, H. Thordal-Christensen, and J. Sorgensen. 1993. Detection of viable, but non-culturable Pseudomonas fluorescens DF57 in soil using a microcolony epifluorescence technique. FEMS Microbiol. Ecol. 12:97–195.

Biville, F., C. Laurent-Winter, and A. Danchin. 1996. In vivo positive effects of exogenous pyrophosphate on Escherichia coli cell growth and stationary phase survival. Res. Microbiol. 147:597–608.

Blochl, E., R. Rachel, S. Burggraf, D. Hafenbradl, H. W. Jannasch, and K. O. Stetter. 1997. Pyrolobus fumarii, gen. nov. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. Extremophiles 1:14–21.

Bolen, D. W. 2001. Protein stabilization by naturally occurring osmolytes. Meth. Molec. Biol. 168:17–36.

Boone, D. R., and M. P. Bryant. 1980. Propionate degrading bacterium Syntrophobacter wolinii sp. nov., gen. nov. from methanogenic ecosystems. Appl. Environ. Microbiol. 33:1162–1169.

Botsford, J. L., and J. G. Harman. 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. 56:100–122.

Bovill, R. A., and B. M. Mackey. 1997. Resuscitation of "non-culturable" cells from aged cultures of Campylobacter jejuni. Microbiology 143:1575–1581.

Bowman, J. P., S. A. McCammon, J. L. Brown, P. D. Nichols, and T. A. McMeekin. 1997a. Psychroserpens burtonensis gen. nov., sp. nov., and Gelidibacter algens, gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. Int. J. Syst. Evol. Microbiol. 47:670–677.

Bowman, J. P., S. A. McCammon, D. S. Nichols, J. H. Skerratt, S. M. Rea, P. E. Nichols, and T. A. McMeekin. 1997b. Shewanella gelidimarina sp. nov. and Shewanella frigidimarina sp. nov., novel Antarctic species with the ability to produce eicosapentaenoic acid (20:5w3) and grow anaerobically by dissimilatory Fe(III) reduction. Int. J. Syst. Bacteriol. 47:1040–1047.

Bowman, J. P., S. A. McCammon, and J. H. Skerratt. 1997c. Methylosphaera hansonii gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. Microbiology 143:1451–1459.

Bowman, J. P., S. A. McCammon, J. L. Brown, and T. A. McMeekin. 1998a. Glaciecola punicea gen. nov., sp. nov., and Glaciecola pallidula gen. nov., sp. nov.: Psychrophilic bacteria from Antarctic sea-ice habitats. Int. J. Syst. Evol. Microbiol. 48:1213–1222.

Bowman, J. P., S. A. McCammon, T. Lewis, J. H. Skerratt, J. L. Brown, D. S. Nichols, and T. A. McMeekin. 1998b. Psychroflexus torquis gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of Flavobacterium gondwanense (Dobson et al. 1993) as Psychroflexus gondwandense gen. nov., comb. nov. Microbiology 144:1601–1609.

Boyaval, P. 1989. Lactic acid bacteria and metal ions. Lait 69:87–113.

Bozal, N., M. J. Montes, E. Tudela, F. Jimenez, and J. Guinea. 2002. Shewanella frigidimarina and Shewanella livingstonesis sp. nov. isolated from Antarctic coastal areas. Int. J. Syst. Evol. Microbiol. 52:195–205.

Braun V. 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. Biol. Chem. 378:779–786.

Brefeld, O. 1881. Botanische Untersuchungen Über Schimmelpilze: Culturmethoden. Leipzig, Germany.

Brewer, D. G., S. E. Martin, and Z. J. Ordal. 1977. Beneficial effects of catalase or pyruvate in a most-probable-number technique for the detection of Staphylococcus aureus. Appl. Environ. Microbiol. 34:797–800.

Brock, T. D., and K. O'Dea. 1977. Amorphous ferrous sulfide as a reducing agent for culture of anaerobes. Appl. Environ. Microbiol. 33:254–256.

Brock, T. D. 1978. Thermophilic Microorganisms and Life at High Temperatures. Springer-Verlag. New York, NY.

Brock, T. D. 1987. Introduction: An overview of the thermophiles. *In:* T. D. Brock (Ed.) Thermophiles: General Molecular and Applied Microbiology. John Wiley and Sons. New York, NY. 1–16.

Broda, D. M., D. J. Saul, P. A. Lawson, R. G. Bell, and D. R. Musgrave. 2000. Clostridium gasigenes sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int. J. Syst. Evol. Microbiol. 50:107–118.

Bromke, B., and J. M. Hammel. 1987. Gelatin as a complete endogenous source of calcium for Serratia marcescens protease activity. J. Microbiol. Meth. 6:253–256.

Brooke, A. G., E. M. Watling, M. M. Attwood, and D. W. Tempest. 1989. Environmental control of metabolic fluxes in thermotolerant methylotrophic Bacillus strains. Arch. Microbiol. 151:268–273.

Brown, A. D., and J. R. Simpson. 1972. Water relations of sugar-tolerant yeasts: The role of intracellular polyols. J. Gen. Microbiol. 72:589–591.

Brown, A. D. 1976. Microbial water stress. Bacteriol. Rev. 40:803–846.

Bruns, A., H. Cypionka, and J. Overmann. 2002. Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. Appl. Environ. Microbiol. 68:3978–3987.

Bryant, M. P. 1976. The microbiology of anaerobic degradation and methanogenesis with special reference to sewage. *In:* H. W. Schlegel and J. Barnea (Eds.) Microbial Energy Conversion. Erick Goltze KG. Göttingen, The Netherlands. 107–1167.

Bryson, V., and W. Szybalski. 1952. Microbial selection. Science 116:45–51.

Bull, A. T., and J. H. Slater. 1982. Microbial interactions and community structure. *In:* A. T. Bull and J. H. Slater (Eds.) Microbial Interactions and Communities. Academic Press. London, UK. 1:13–44.

Bulthuis, B. A., G. M. Koningstein, A. H. Stouthamer, and H. W. van Verseveld. 1989. A comparison between aerobic growth of Bacillus licheniformis in continuous culture and partial-recycling fermenter, with contributions to the discussion on maintenance energy demand. Arch. Microbiol. 152:499–507.

Bungay, H. R., and M. L. Bungay. 1968. Microbial interactions in continuous culture. Adv. Appl. Microbiol. 10: 269–290.

Bunt, J. G. 1961. Nitrogen-fixing blue-green algae in Australian rice soils. Nature 192:479–480.

Burchard, R. P. 1980. Gliding motility of bacteria. BioScience 30:157–162.

Burkhardt, F. 1992. Mikrobiologische Diagnostik. Georg Thieme Verlag. New York, NY.

Burnham, J. C., and S. F. Conti. 1984. Genus Bdellovibrio. *In:* N. R. Krieg (Ed.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:118–124.

Bussmann, I., B. Philipp, and B. Schink. 2001. Factors influencing the cultivability of lake water bacteria. J. Microbiol. Meth. 47:41–50.

Button, D. K., F. Schut, P. Quang, R. Martin, and B. R. Robertson. 1993. Viability and isolation of marine bacteria by dilution culture: Theory, procedures, and initial results. Appl. Environ. Microbiol. 59:881–891.

Byrer, D. E., F. A. Rainey, and J. Wiegel. 2000. Novel strains of Moorella thermoacetica form unusually heat-resistant spores. Arch. Microbiol. 174:334–339.

Calcott, P. H., and J. R. Postgate. 1972a. On substrate-accelerated death in Klebsiella aerogenes. J. Gen. Microbiol. 70:115–122.

Calcott, P. H., W. Montague, and J. R. Postgate. 1972b. The levels of cyclic AMP during substrate accelerated death. J. Gen. Microbiol. 73:197–200.

Calcott, P. H. 1981a. The construction and operation of continuous cultures. *In:* P. H. Calcott (Ed.) Continuous Cultures of Cells. CRC Press. Boca Raton, FL. 1:13–26.

Calcott, P. H., and T. J. Calvert. 1981b. Characterization of 3':5'cyclic AMP phosphdiesterase in Klebsiella aerogenes and its role in substrate accelerated death. J. Gen. Microbiol. 122:313–321.

Cangelosi, G. A., and W. H. Brabant. 1997. Depletion of pre-16S rRNA in starved Escherichia coli cells. J. Bacteriol. 179:4457–4463.

Carlsson, J., G. P. D. Granberg, G. K. Nyberg, and M.-B. K. Edlund. 1979. Bactericidal effect of cysteine exposed to atmospheric oxygen. Appl. Environ. Microbiol. 37:383–390.

Carpenter, E. J., S. Lin, and D. G. Capone. 2000. Bacterial activity in South Pole snow. Appl. Environ. Microbiol. 66:4514–4517.

Castenholz, R. W. 1973. Movements. *In:* N. G. Carr and B. A. Whitton (Eds.) The Biology of Blue-green Algae. Blackwell Scientific Publications. London, UK. 320–339.

Cayley, S., M. T. Record, and B. A. Lewis. 1989. Accumulation of 3-[N-morpholino] propanesulfonate by osmotically stressed Escherichia coli K-12. J. Bacteriol. 171:3597–3602.

Cayley, S., B. A. Lewis, and M. T. Record. 1992. Origins of osmoprotective properties of betaine and proline in Escherichia coli K-12. J. Bacteriol. 174:1586–1595.

Chan, M., R. H. Himes, and J. M. Akagi. 1971. Fatty acid composition of thermophilic, mesophilic, and psychrophilic clostridia. J. Bacteriol. 106:876–881.

Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545–549.

Chesbro, W. R., T. Evans, and R. Eifert. 1979. Very slow growth of Escherichia coli. J. Bacteriol. 139:625–638.

Chesbro, W. 1988. The domains of slow bacterial growth. Can. J. Microbiol. 34:427–435.

Chi, E., D. H. Bartlett. 1995. An rpoE-like locus controls outer membrane protein synthesis and growth at cold temperatures and high pressures in the deep-sea bacterium Photobacterium sp. strain SS9. Molec. Microbiol. 17:713–726.

Christensen, B. B., J. A. J. Haagensen, A. Heydorn, and S. Molin. 2002. Metabolic commensalism and competition in a two-species microbial consortium. Appl. Environ. Microbiol. 68:2495–2502.

Clark, C., and E. L. Schmidt. 1966. Effect of mixed culture on Nitrosomonas europaea simulated by uptake and utilization of pyruvate. J. Bacteriol. 91:367–373.

Cleland, N., and S. O. Enfors. 1983. Control of glucose fed batch cultivations of E.coli by means of an oxygen stabilized enzyme electrode. Eur. J. Appl. Microbiol. Biotechnol. 18:141–147.

Coates, J. D., D. J. Ellis, E. L. Blunt-Harris, C. V. Gaw, E. Roden, and D. R. Lovley. 1998. Recovery of humic-reducing bacteria from a diversity of environments. Appl. Environ. Microbiol. 64:1504–1509.

Coates, J. D., K. A. Cole, R. Chakraborty., S. M. O'Connor, and L. A. Achenbach. 2002. Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. Appl. Environ. Microbiol. 68:2445–2453.

Crocker, F. H., W. F. Guerin, and S. A. Boyd. 1995. Bioavailability of naphthalene sorbed to cationic surfactant-modified smectite clay. Environ. Sci. Technol. 29:2953–2958.

Cohen, Y., I. de Jonge, and J. G. Kuenen. 1979. Excretion of glycolate by Thiobacillus neapolitanus grown in continuous culture. Arch. Microbiol. 122:189–194.

Cohn, F. 1872. Über Bacterien, die kleinsten lebenden Wesen. Carl Habel. Berlin, Germany.

Coolen, M. J. L., H. Cypionka, A. Smock, H. Sass, and J. Overmann. 2002. Ongoing modification of Mediterranean Pleistocene sapropels mediated by prokaryotes. Science 296:2407–2410.

Cottrell, M. T., and D. L. Kirchman. 2000. Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacteria cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. 66:1692–1697.

Coveney, M. F. 1982. Bacterial uptake of photosynthetic carbon from freshwater phytoplankton. Oikos 38:8–20.

Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53:121–147.

Csonka, L. N., and A. Hanson. 1991. Prokaryotic osmoregulation: Genetics and physiology. Ann. Rev. Microbiol. 45:569–606.

Csonka, L. N., and W. Epstein. 1996. Osmoregulation. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella, 2nd ed. ASM Press. Washington, DC. 1210–1223.

Currie, D. J., and J. Kalff. 1984. Can bacteria outcompete phytoplankton for phosphorus? A chemostat test. Microb. Ecol. 10:205–216.

Cypionka, H., F. Widdel, and N. Pfennig. 1985. Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate-free oxygen-sulfide gradients. FEMS Microbiol. Ecol. 31:39–45.

Cypionka, H. 1986. Sulfide-controlled continuous culture of sulfate-reducing bacteria. J. Microbiol. Meth. 5:1–9.

Cypionka, H. 1999. Grundlagen der Mikrobiologie. Springer-Verlag. New York, NY.

Czeczuga, B. 1968. An attempt to determine the primary production of the green sulphur bacteria, Chlorobium limicola Nads, (Chlorobacteriaceae). Hydrobiologia 31:317–333.

Dalluge, J. J., T. Hamamoto, K. Horikoshi, R. Y. Morita, K. O. Stetter, and J. A. McCloskey. 1997. Posttranscriptional modification of tRNA in psychrophilic bacteria. J. Bacteriol. 179:1918–1923.

Damoglou, A. P., and E. A. Dawes. 1968. Studies on the lipid content and phosphate requirement for glucose- and acetate-grown Escherichia coli. Biochem. J. 110:775–781.

Dawson, M. W., I. S. Maddox, I. F. Boag, and J. D. Brooks. 1988. Application of fed-batch culture to citric acid production by Aspergillus niger: The effects of dilution rate and dissolved oxygen tension. Biotechnol. Bioeng. 32:220–226.

De Bary, A. 1879. Die Erscheinung der Symbiose. Naturforschung Versammlung Cassel.

de Freitas, M. J., and A. G. Fredrickson. 1978. Inhibition as a factor in the maintenance of the diversity of microbial ecosystems. J. Gen. Microbiol. 106:307–320.

de la Broise, D., and A. Durand. 1989. Osmotic, biomass, and oxygen effects on the growth rate of Fusarium oxysporum using a dissolved oxygen controlled turbidstat. Biotechnol. Bioeng. 33:699–705.

DeLong, E. F., and A. A. Yayamos. 1986. Biochemical function and ecological significance of novel bacterial lipids in deep-sea prokaryotes. Appl. Environ. Microbiol. 51:730–737.

DeLong, E. F., K. Ying Wu, B. B. Prézelin, and R. V. M. Jovine. 1994. High abundance of Archaea in Antarctic marine picoplankton. Nature 371:695–697.

DeLong, E. F., D. G. Franks, and A. A. Yayanos. 1997. Evolutionary relationships of cultivated psychrophilic and barophilic deep-sea bacteria. Appl. Environ. Microbiol. 63:2105–2108.

Deming, J. W., and R. R. Colwell. 1985. Observations of barophilic microbial activity in samples of sediments and intercepted particulates from the Demerara abyssal plain. Appl. Environ. Microbiol. 50:1002–1006.

Deming, J. W. 1986. Ecological strategies of barophilic bacteria in the deep ocean. Microbiol. Sci. 3:205–207.

Deming, J. W., L. W. Somers, W. L. Straube, D. G. Swartz, and M. T. MacDonell. 1988. Isolation of an obligately barophilic bacterium and description of a new genus, Colwellia gen. nov. Syst. Appl. Microbiol. 10:152–160.

Deutsche Sammlung für Mikroorganismen und Zellkulturen. 2002. Bacterial nomenclature up-to-date. (http://www.dsmz.de/bactnom/bactname.htm).

De Wit, R., and H. van Gemerden. 1988. Interactions between phototrophic bacteria in sediment ecosystems. Hydrobiol. Bull. 22:135–145.

Dobell, C. 1932. Anthony van Leeuwenhoek and His "Little Animals." Harcourt, Brace. New York, NY.

Dolfing, J., and J. M. Tiedje. 1986. Hydrogen cycling in a three-tiered foodweb growing on the methanogenic conversion of 3-chlorobenzoate. FEMS Microbiol. Ecol. 38:293–298.

Douglas, A. E. 1998. Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria Buchnera. Annu. Rev. Entomol. 43:17–37.

Driessen, F. M. 1981. Protocooperation of yoghurt bacteria in continuous cultures. *In:* M. E. Buschell and J. H. Slater (Eds.) Mixed Culture Fermentations. Academic Press. London, UK. 99–120.

Dubilier, N., O. Giere, D. L. Distel, and C. M. Cavanaugh. 1995. Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and in situ hybridization. Appl. Environ. Microbiol. 61:2346–2350.

Dubinina, G. A., N. V. Leshcheva, and M. Y. Grabovich. 1993. The colorless sulfur bacterium Thiodendron is actually a symbiotic association of spirochetes and sulfidogens. Microbiology 62:432–444.

Dukan, S., and T. Nyström. 1998. Bacterial senescence: Stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. Genes Develop. 12:3431–3441.

Dunfield, P. F., W. Liesack, T. Henckel, R. Knowles, and R. Conrad. 1999. High-affinity methane oxidation by a soil enrichment culture containing a type II methanotroph. Appl. Environ. Microbiol. 65:1009–1014.

Dwyer, D. F., E. Weeg-Aerssens, D. R. Shelton, and J. M. Tiedje. 1988. Bioenergetic conditions of butyrate metabolism by a syntrophic, anaerobic bacterium in coculture with hydrogen oxidizing methanogenic and sulfidogenic bacteria. Appl. Environ. Microbiol. 54:1354–1359.

Dykhuizen, D., and M. Davies. 1980. An experimental model: Bacterial specialists and generalists competing in chemostats. Ecology 61:1213–1227.

Egli, T., N. D. Lindley, and J. R. Quayle. 1983. Regulation of enzyme synthesis and variation of residual methanol concentration during carbon limited growth of Kloeckera ap. 2201 on mixtures of methanol and glucose. J. Gen. Microbiol. 129:1269–1281.

Eguchi, M., M. Ostrowski, F. Fegatella, J. Bowman, D. Nichols, T. Nishino, and R. Cavicchioli. 2001. Sphingomonas alaskensis AFO1, an abundant oligotrophic ultramicrobacterium from the North Pacific. Appl. Environ. Microbiol. 67:4945–4954.

Eilers, H., J. Pernthaler, J. Peplies, F. O. Glöckner, G. Gerdts, and R. Amann. 2001. Isolation of novel pelagic bacteria from the German Bight and their seasonal contributions to surface picoplankton. Appl. Environ. Microbiol. 67:5134–5142.

Emde, R., A. Swain, and B. Schink. 1989. Anaerobic oxidation of glycerol by Escherichia coli in an amperometric poised-potential culture system. Appl. Microbiol. Biotechnol. 32:170–175.

Emde R., and B. Schink. 1990. Oxidation of glycerol, lactate, and propionate by Propionibacterium freudenreichii in a poised-potential amperometric culture system. Arch. Microbiol. 153:506–512.

Epstein, W., L. B. Rothman-Denes, J. Hesse. 1975. Adenosine 3′:5′-cyclic monophosphate as mediator of catabolite repression in Escherichia coli. Proc. Natl. Acad. Sci. 72:2300–2304.

Epstein, W. 1986. Osmoregulation by potassium transport in Escherichia coli. FEMS Microbiol. Rev. 39:73–78.

Esener, A. A:, J. A. Roels, and N. W. Kossen. 1981. Fed batch culture: Modelling and application in the study of microbial energetics. Biotechnol. Bioeng. 22:1851–1871.

Euzéby, J. P. 2001. List of bacterial names with standing in nomenclature. (http://www.bacterio.cict.fr).

Evans, C. G., D. Herbert, and D. W. Tempest. 1970. The continuous cultivation of microorganisms. 2. Construction of a chemostat. *In:* J. R. Norris and D. W. Ribbons

(Eds.) Methods in Microbiology. Academic Press. London, UK. 2:277–327.

Feller, G., E. Narinx, J. L. Arpigny, Z. Zekhnini, J. Swings, and C. Gerday. 1994a. Temperature dependence of growth, enzyme secretion and activity of psychrophilic Antarctic bacteria. Appl. Microb. Biotechnol. 41:477–479.

Feller, G., F. Payan, F. Theys, M. Quian, R. Haser, and C. Gerday. 1994b. Stability and structural analysis of α-amylase from the Antarctic psychrophile Alteromonas haloplanctis A23. Eur. J. Biochem. 222:441–447.

Felske, A., A. Wolterink, R. van Lis, and A. D. L. Akkermans. 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl. Environ. Microbiol. 64:871–879.

Felske, A. A. Wolterink, R. van Lis, W. M. de Vos, and A. D. L. Akkermans. 1999. Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. FEMS Microbiol. Ecol. 30:137–145.

Ferchichi, M., D. Hemme, and C. Bouillaune. 1986. Influence of oxygen and pH on methanethiol production from L-methionine by Brevibacterium linens CNRZ 918. Appl. Environ. Microbiol. 51:725–729.

Ferris, M. J., A. L. Ruff-Roberts, E. D. Kopczynski, M. M. Bateson, and D. M. Ward. 1996. Enrichment culture and microscopy conceal diverse thermophilic Synechococcus populations in a single hot spring microbial mat habitat. Appl. Environ. Microbiol. 62:1045–1050.

Fleming, A. 1929. On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of B. influenzae. Brit. J. Exp. Pathol. 10:226–236.

Fogg, G. E. 1971. Extracellular products of algae in freshwater. Arch. Hydrobiol. 5:1–25.

Forsberg, C. W. 1987. Production of 1,3-propanediol from glycerol by Clostridium acetobutylicum and other Clostridium species. Appl. Environ. Microbiol. 53:639–643.

Francis, C. A., and B. M. Tebo. 2002. Enzymatic manganese(II) oxidation by metabolically dormant spores of diverse Bacillus species. Appl. Environ. Microbiol. 68:874–880.

Franzmann, P. D., P. Höpfl, N. Weiss, and B. J. Tindall. 1991. Psychrotrophic, lactic acid-producing bacteria from anoxic waters in Ace Lake, Antarctica: Carnobacterium funditum sp. nov. and Carnobacterium alterfunditum sp. nov. Arch. Microbiol. 156:255–262.

Franzmann, P. D., N. Springer, W. Ludwig, E. Conway de Macario, and M. Rohde. 1992. A methanogenic archaeon from Ace Lake, Antarctica: Methanococcoides burtonii sp. nov. Syst. Appl. Microbiol. 15:573–581.

Fredrickson, A. G. 1977. Behaviour of mixed cultures of microorganisms. Ann. Rev. Microbiol. 31:63–87.

Fredrickson, A. G., and G. Stephanopoulos. 1981. Microbial competition. Science 213:972–979.

Fritsche, T. R., D. Sobek, and R. K. Gautom. 1998. Enhancement of in vitro cytophathogenicity by Acanthamoeba spp. following acquisition of bacterial endosymbionts. FEMS Microbiol. Lett. 166:231–236.

Fröhlich, J., and H. König. 1999. Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. Syst. Appl. Microbiol. 22:249–257.

Fröstl, J. M., and J. Overmann. 1998. Physiology and tactic response of the phototrophic consortium "Chlorochromatium aggregatum." Arch. Microbiol. 169:129–135.

Fröstl, J. M., and J. Overmann. 2000. Phylogenetic affiliation of the bacteria that constitute phototrophic consortia. Arch. Microbiol. 174:50–58.

Fry, J. C. 1990. Direct methods and biomass estimation. *In:* R. Grigorova and J. R. Norris (Eds.) Methods in Microbiology. Academic Press. London, UK. 22:41–85.

Fuhrman, J. A., K. McCallum, and A. A. Davis. 1992. Novel major archaebacterial group from marine plankton. Nature 356:148–149.

Fuhrman, J. A., K. McCallum, and A. A. Davis. 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl. Environ. Microbiol. 59:1294–1302.

Funk, H. B., and T. A. Krulwich. 1964. Preparation of clear silica gels that can be streaked. J. Bacteriol. 88:1200–1201.

Fuqua, C., and E. P. Greenberg. 1998. Self perception in bacteria: Quorum sensing with acylated homoserine lactones. Curr. Opin. Microbiol. 1:50–58.

Galinski, E. A., and H. G. Trüper. 1994. Microbial behaviour in salt-stressed ecosystems. FEMS Microbiol. Rev. 15:95–108.

Gangola, P., and B. P. Rosen. 1987. Maintenance of intracellular calcium in Escherichia coli. J. Biol. Chem. 262:12570–12574.

Garcia-Lara, J., L. H. Shang, and L. I. Rothfield. 1996. An extracellular factor regulates expression of sdiA, a transcriptional activator of cell division genes in Escherichia coli. J. Bacteriol. 178:2742–2748.

Graumann, P., and M. A. Marahiel. 1996. Some like it cold: Response of microorganisms to cold shock. Arch Microbiol. 166:293–300.

Gause, G. F. 1934. The Struggle for Existence. Williams and Wilkins. Baltimore, MD.

Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips. 1981. Manual of Methods for General Bacteriology. American Society for Microbiology. Washington, DC.

Gich, F., J. Garcia-Gil, and J. Overmann. 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. 177:1–10.

Glöckner, F. O., E. Zaichikov, N. Belkova, L. Denissova, J. Pernthaler, A. Perthaler, and R. Amann. 2000. Comparative 16S rRNA analysis of lake bacterioplankton revreals globally distributed phylogenetic clusters including an abundant group of actinoabcteria. Appl. Environ. Microbiol. 66:5053–5065.

Gond, O., J. M. Engasser, C. Matta-El-Amouri, and H. Petitdemange. 1986. The acetone butanol fermentation on glucose and xylose. II: Regulation and kinetics in fed batch cultures. Biotechnol. Bioeng. 28:167–175.

González, J. M., F. Mayer, M. A. Moran, R. E. Hodson, and W. B. Withman. 1997. Sagittula stellata gen. nov., sp. nov., a lignin-transforming bacterium from a coastal environment. Int. J. Syst. Bacteriol. 47:773–780.

Goodfellow, M. 1992a. The family Nocardiaceae. *In:* A. Balows, H. G. Trüper, M. Dworkin, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 1188–1213.

Goodfellow, M. 1992b. The family Streptosporangiaceae. *In:* A Balows, H G. Trüper, M. Dworkin, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 1115–1138.

Görtz, H.-D., and T. Brigge. 1998. Intracellular bacteria in Protozoa. Naturwissenschaften 85:359–368.

Gosink, J. J., and J. T. Staley. 1995. Biodiversity of gas vacuolate bacteria from Antarctic sea ice and water. Appl. Environ. Microbiol. 61:3486–3489.

Gosink, J. J., C. R. Woese, and J. T. Staley. 1998. Polaribacter gen. nov., with three new species, P. irgensii sp. nov., P. franzmannii sp. nov. and P. filamentus so. nov., gas vacuolate polar marine bacteria of the Cytophaga-Flavobacterium-Bacteroides group and reclassification of "Flectobacillus glomeratus" as Polaribacter glomeratus comb. nov. Int. J. Syst. Evol. Microbiol. 48:223–235.

Gottschal, J. C., S. de Vries, and J. G. Kuenen. 1979. Competition between the facultatively chemolithotrophic Thiobacillus A2, an obligately chemolithotrophic Thiobacillus and a heterotrophic Spirillum for inorganic and organic substrates. Arch. Microbiol. 121: 241–249.

Gottschal, J. C., and J. G. Kuenen. 1980. Selective enrichment of facultatively chemolithotrophic thiobacilli and related organisms in continuous culture. FEMS Microbiol. Lett. 7:241–247.

Gottschal, J. C., and J. G. Morris. 1981. The induction of acetone and butanol production in cultures of Clostridium acetobutylicum by elevated concentrations of acetate and butyrate. FEMS Microbiol. Lett. 12:385–389.

Gottschal, J. C. 1986. Mixed substrate utilization by mixed cultures. *In:* E. R. Leadbetter and J. S. Poindexter (Eds.) Bacteria in Nature. Plenum Press. New York, NY. 2:261–292.

Gottschal, J. C., and L. Dijkhuizen. 1988. The place of the continuous culture in ecological research. *In:* J. W. T. Wimpenny (Ed.) Handbook of Laboratory Model Systems for Microbial Ecosystems. CRC Press. Boca Raton, FL. 1:19–79.

Gottschal, J. C. 1990. Different types of continuous culture in ecological studies. *In:* J. R. Norris and R. Grigorova (Eds.) Methods in Microbiology. Academic Press. London, UK. 22:87–124.

Gottschal, J. C., W. Harder, and R. A. Prins. 1991. Principles of enrichment, isolation, cultivation, and preservation. *In:* A. Balows, H. G. Trüper, M. Dworkin, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 149–196.

Gottschalk, G. 1985. Bacterial Metabolism. Springer-Verlag. New York, NY.

Gottwald, M., and G. Gottschalk. 1985. The internal pH of Clostridium acetobutylicum and its effect on the shift from acid to solvent formation. Arch. Microbiol. 143:42–46.

Graham, A. F., and B. M. Lund. 1983. The effect of alkaline pH on growth and metabolic products of a motile, yellow-pigmented Streptococcus sp. J. Gen. Microbiol. 129:2429–2435.

Grant, W. D., and B. J. Tindall. 1986. The alkaline saline environment. *In:* R. A. Herbert and G. A. Codd (Eds.) Microbes in Extreme Environments. Academic Press. London, UK. 25–54.

Gray, N. D., R. Howarth, R. W. Pickup, J. G. Jones, and I. M. Head. 2000. Use of combined microautoradiography and fluorescence in situ hybridization to determine carbon metabolism in mixed natural communities of uncultured bacteria from the genus Achromatium. Appl. Environ. Microbiol. 66:4518–4522.

Groeschel, D. H. M. 1982. The etiology of tuberculosis: A tribute to Robert Koch on the occasion of the centenary of his discovery of the tubercle bacillus. ASM News 48:248–250.

Gross, C. A. 1996. Function and regulation of the heat shock proteins. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella, 2nd ed. ASM Press. Washington, DC. 1382–1399.

Grosser, R. J., M. Friedrich, D. M. Ward, and W. P. Inskeep. 2000. Effect of model sorptive phases on phenanthrene biodegradation: Different enrichment conditions influence bioavailability and selection of phenanthrene-degrading isolates. Appl. Environ. Microbiol. 66:2695–2702.

Guerin, W. F., and S. A. Boyd. 1997. Bioavailability of naphthalene associated with natural and synthetic sorbents. Water Res. 51:1504–1512.

Guerrero, R., C. Pedros-Alió C., I. Esteve., J. Mas, D. Chase, and L. Margulis. 1986. Predatory prokaryotes: Predation and primary consumption evolved in bacteria. Proc. Natl. Acad. Sci. USA 83:2138–2142.

Hackstein, J. H., A. Akhmanova, B. Boxma, H. R. Harhangi, and F. G. Voncken. 1999. Hydrogenosomes: Eukaryotic adaptations to anaerobic environments. Trends. Microbiol. 7:441–447.

Harder, W., and H. Veldkamp. 1971. Competition of marine psychrophilic bacteria at low temperatures. Ant. v. Leeuwenhoek 37:51–63.

Harder, W., J. G. Kuenen, and A. Matin. 1977. Microbial selection in continuous culture. J. Appl. Bacteriol. 43:1–24.

Harder, W., and L. Dijkhuizen. 1982. Strategies of mixed substrate utilization in microorganisms. Phil. Trans. R. Soc. Lond. B 297:459–480.

Hardin, G. 1960. The competitive exclusion principle. Science 131:1292–1297.

Harms, H., and A. J. B. Zehnder. 1995. Bioavailability of sorbed 3-chlorodibenzofuran. Appl. Environ. Microbiol. 61:27–33.

Harris, J. E. 1985. Gelrite as an agar substitute for the cultivation of mesophilic Methanobacter and Methanobrevibacter species. Appl. Environ. Microbiol. 50:1107–1109.

Hastings, R. C., J. R. Saunders, G. H. Hall, R. W. Pickup, and A. J. McCarthy. 1998. Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Appl. Environ. Microbiol. 64:3674–3682.

Herbert, D., R. Elsworth, R. C. Telling. 1956. The continuous culture of bacteria: A theoretical and experimental study. J. Gen. Microbiol. 14:601–622.

Herbert, R. A. 1986. The ecology and physiology of psychrophilic microorganisms. *In:* RA herbert and GA Codd (eds.) Microbes in extreme environments. Academic Press. London, England. 1–23.

Hespell, R. B., and M. P. Bryant. 1981. The genera Butyrivibrio, Succinivibrio, Lachnospira and Selenomonas. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 1479–1494.

Hinrichs, K.-U., J. M. Hayes, S. P. Sylva, P. G. Brewer, and E. F. DeLong. 1999. Methane-consuming archaebacteria in marine sediments. Nature 398:802–805.

Hiorns, W. D., B. A. Methé S. A. Nierzwicki-Bauer, and J. P. Zehr. 1997. Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequence analysis. Appl. Environ. Microbiol. 63:2957–2960.

Hirsch, P. 1984. Microcolony formation and consortia. *In:* K. C. Marshall (Ed.) Microbial Adhesion and Aggregation: Dahlem Konferenzen. Springer-Verlag. New York, NY. 373–393.

Hochachka, P. W., T. W. Moon, and T. Mustafa. 1972. The adaptation of enzymes to pressure in abyssal and midwater fishes. *In:* M. A. Sleigh and A. G. Macdonald (Eds.) The Effects of Pressure on Living Organisms. Academic Press. London, UK. 175–195.

Holmes, A. J., P. Roslev, I. R. McDonald, N. Iversen, K. Henriksen, and J. C. Murrell. 1999. Characterization of methanotrophic bacterial populations in soil showing atmospheric methane uptake. Appl. Environ. Microbiol. 65:3312–3318.

Hommes, R. W. J., P. W. Postma, D. W. Tempest, and O. M. Neijssel. 1989. The influence of the culture pH value on the direct glucose oxidation pathway in Klebsiella pneumoniae NCTC 418. Arch. Microbiol. 151:261–267.

Hooke, R. 1665. Micrographia: Or some physiological descriptions of minute bodies made by magnifying glasses with observations and inqueries thereupon. John Martyn and James Allestry. London, UK.

Horikoshi, K., and T. Akiba. 1982. Alkalophilic Microorganisms. Springer-Verlag. New York, NY.

Huang, L., C. W. Forsberg, and L. N. Gibbins. 1986. Influence of external pH and fermentation products on Clostridium acetobutylicum intracellular pH and cellular distribution of fermentation products. Appl. Environ. Microbiol. 51:1230–1234.

Huber, H., M. J. Hohn, R. Rachel, T. Fuchs, V. C. Wimmer, and K. O. Stetter. 2002. A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. Nature 417:63–67.

Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace. 1998. Novel division level bacterial diversity in a yellowstone hot spring. J. Bacteriol. 180:366–376.

Humphry, D. R., A. George, G. W. Black, and S. P. Cummings. 2001. Flavobacterium frigidarium sp. nov., an aerobic psychrophilic, xylanolytic and laminariolytic bacterium from Antarctica. Int. J. Syst. Evol. Microbiol. 51:1235–1243.

Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14:1–49.

Hungate, R. E. 1960. Microbial ecology of the rumen. Symposium: Selected topics in microbial ecology. Ann. Meeting. Soc. Amer. Bact. Philadelphia 24:353–364.

Hungate, R. E. 1966. The Rumen and its Microbes. Academic Press. New York, NY.

Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes. *In:* J. R. Norris and W. D. Ribbons (Eds.) Methods in Microbiology. Academic Press. London, UK. 3B:117–132.

Hungate, R. E. 1985. Anaerobic biotransformations of organic matter. *In:* E. R. Leadbetter and J. S. Poindexter (Eds.) Bacteria in Nature. Plenum Press. New York, NY. 1:39–96.

Huston, A. L., B. B. Krieger-Brockett, and J. W. Deming. 2000. Remarkably low temperature optima for extracellular enzyme activity form Arctic bacteria and sea ice. Environ. Microbiol. 2:383–388.

Ianotti, E. L., D. Kafkewitz, M. J. Wolin, and M. P. Bryant. 1973. Glucose fermentation products of Ruminococcus albus grown in continuous culture with Vibrio succinogenes. J. Bacteriol. 114:1231–1240.

Imhoff, J. F. 1986. Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol. Rev. 39:57–66.

Ingraham, J. L. 1962. Temperature relationships. *In:* I. C. Gunsalus and R. Y. Stanier (Eds.) The Bacteria. Academic Press. New York, NY. 4:265–296.

Ingraham, J. L., and A. G. Marr. 1996. Effect of temperature, pressure, pH, and osmotic stress on growth. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella typhimurium. ASM Press. Washington, DC. 1570–1578.

Irgens, R. L., J. J. Gosink, and J. T. Staley. 1996. Polaromonas vacuolata gen. nov., sp. nov., a psychrophilic, marine gas vacuolate bacterium from Antarctica. Int. J. Syst. Evol. Microbiol. 46:822–826.

Isaksen, M. F., and B. B. Jørgensen. 1996. Adaptation of psychrophilic and psychrotrophic sulfate-reducing bacteria to permanently cold marine environments. Appl. Environ. Microbiol. 62:408–414.

Jackson, B. E., and M. J. McInerney. 2002. Anaerobic microbial metabolism can proceed close to thermodynamic limits. Nature 415:454–456.

Jacobi, C. A., B. Aßmus, H. Reichenbach, and E. Stackebrandt. 1997. Molecular evidence for association between the Sphingobacterium-like organism "Candidatus comitans" and the myxobacterium Chondromyces crocatus. Appl. Environ. Microbiol. 63:719–723.

Jaenicke, R. 1988. Molecular mechanisms of adaptation of bacteria to extreme environments. Forum Microbiol. 11:435–440.

Jannasch, H. W. 1967a. Enrichment of aquatic bacteria in continuous culture. Arch. Mikrobiol. 59:165–173.

Jannasch, H. W. 1967b. Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol. Oceanogr. 12:264–271.

Jannasch, H. W., and R. I. Mateles. 1974. Experimental bacterial ecology studied in continuous culture. Adv. Microb. Physiol. 11:165–212.

Jannasch, H. W., C. O. Wirsen, and C. D. Taylor. 1976. Undecompressed microbial populations from the deep sea. Appl. Environ. Microbiol. 32:360–367.

Jannasch, H. W., and C. D. Taylor. 1984. Deep-sea microbiology. Ann. Rev. Microbiol. 38:487–514.

Jannasch, H. W., C. O. Wirsen, and K. W. Doherty. 1996. A pressurized chemostat for the study of marine barophilic and oligotrophic bacteria. Appl. Environ. Microbiol. 62:1593–1596.

Janssen P. H., A. Schuhmann, E. Mörschel, and F. A. Rainey. 1997. Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial decent isolated by dilution culture from anoxic rice paddy soil. Appl. Environ. Microbiol. 63:1382–1388.

Janssen, P. H., P. S. Yates, B. E. Grinton, P. M. Taylor, and M. Sait. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl. Environ. Microbiol. 68: 2391–2396.

Jaspers, E., K. Nauhaus, H. Cypionka, and J. Overmann. 2001. Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. FEMS Microbiol. Ecol. 36:153–164.

Jones, A. K. 1982. The interaction of algae and bacteria. *In:* A. T. Bull and J. H. Slater (Eds.) Microbial Interactions and Communities. Academic Press. London, UK. 189–247.

Jones, S. L., P. Drouin, B. J. Wilkinson, and P. D. Morse. 2002. Correlation of long-rang membrane order with temperature-dependent growth characteristics of parent and a cold-sensitive, branched-chain-fatty-acid-deficient mutant of Listeria monocytogenes. Arch. Microbiol. 177:217–222.

Jørgensen, B. B. 1982. Ecology of the bacteria of the sulphur cycle with special reference to anoxic-oxic interface environments. Phil. Trans. R. Soc. Lond. B 298:543–561.

Jost, J. L., J. F. Drake, A. G. Fredrickson, and H. M. Tsuchiya. 1973. Interactions of Tetrahymena pyriformis, Escherichia coli, Azotobacter vinelandii and glucose in a minimal medium. J. Bacteriol. 113:834–840.

Jung, L., R. Jost, E. Stoll, and H. Zuber. 1974. Metabolic differences in Bacillus stearothermophilus grown at 55°C and 37°C. Arch. Microbiol. 95:125–138.

Kalmbach, S., W. Manz, and U. Szewyk. 1997. Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes. Appl. Environ. Microbiol. 63:4164–4170.

Kämpfer, P., F. A. Rainey, M. A. Andersson, E. L. Nurmiaho Lassila, U. Ulrych, H. J. Busse, N. Weiss, R. Mikkola, and M. Salkinoja-Salonen. 2000. Frigoribacterium faeni gen. nov., sp. nov., a novel psychrophilic genus of the family Microbacteriaceae. Int. J. Syst. Evol. Microbiol. 50:355–363.

Kane, M. D., L. K. Poulsen, and D. A. Stahl. 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide probes designed from environmentally derived 16S rRNA sequences. Appl. Environ. Microbiol. 59:682–686.

Kaneda, T. 1991. Iso-fatty and anteiso-fatty acids in bacteria-biosynthesis, function, and taxonomic significance. Microbiol. Rev. 55:288–302.

Kaprelyants, A. S., and D. B. Kell. 1993. Dormancy in stationary-phase cultures of Micrococcus luteus—flow cytometric analysis of starvation and resuscitation. Appl. Environ. Microbiol. 59:3187–3196.

Kaprelyants, A. S., G. V. Mukamolova, and D. B. Kell. 1994. Estimation of dormant Micrococcus luteus cells by penicillin lysis and by resuscitation in cell free spent culture medium at high dilution. FEMS Microbiol. Lett. 115:347–352.

Kaprelyants, A. S., G. V. Mukamolova, H. M. Davey, and D. B. Kell. 1996. Quantitative analysis of the physiological heterogeneity within starved cultures of Micrococcus luteus by flow cytometry and cell sorting. Appl. Environ. Microbiol. 62:1311–1316.

Karner, M., and J. A. Fuhrman. 1997. Determination of active bacterioplankton: A comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Appl. Environ. Microbiol. 63:1208–1213.

Kato, Y., R. M. Sakala, H. Hayashidani, A. Kiuchi, C. Kaneuchi, and M. Ogawa. 2000. Lactobacillus algidus sp. nov., a psychrophilic lactic acid bacterium isolated from vacuum-packaged refrigerated beef. Int. J. Syst. Evol. Microbiol. 50:1143–1149.

Keith, S. M., and R. A. Herbert. 1985. The application of compound bi-directional flow diffusion chemostats to the study of microbial interactions. FEMS Microbiol Ecol. 31:239–248.

King, D., and D. B. Nedwell. 1984. Changes in the nitrate-reducing community of an anaerobic saltmarsh sediment in response to seasonal selection by temperature. J. Gen. Microbiol. 130:2935–2941.

King, G. M. 1984. Utilization of hydrogen, acetate, and "non-competitive" substrates by methanogenic bacteria in marine sediments. Geomicrob. J. 3:275–306.

Kjaergaard, L., and B. B. Jørgensen. 1979. Redox potential as a state variable in fermentation systems. Biotechnol. Bioeng. Symp. 9:85–94.

Kjelleberg, S., B. A. Humphrey, and K. C. Marshall. 1982. Effect of interfaces on small, starved bacteria. Appl. Environ. Microbiol. 43:1166–1172.

Kjelleberg, S., K. B. G. Flardh, T. Nystrom, D. J. W. Moriarty. 1993. Growth limitation and stravation in bacteria. *In:* T. E. Ford (Ed.) Aquatic Microbiology: An Ecological Approach. Blackwell Scientific Publications. Oxford, UK. 298–320.

Kluyver, A. J., and H. J. L. Donker. 1926. Unity in biochemistry. Chemie der Zelle und Gewebe 13:134–190.

Knoblauch, C., and B. B. Jørgensen. 1999a. Effect of temperature on sulphate reduction, growth rate and growth yield in five psychrophilic sulphate-reducing bacteria from Arctic sediments. Environ. Microbiol. 1:457–467.

Knoblauch, C., B. B. Jørgensen, and J. Harder. 1999b. Community size and metabolic rates of psychrophilic sulfate-reducing bacteria in arctic marine sediments. Appl. Environ. Microbiol. 65:4230–4233.

Kogure, K., U. Simidu, and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25:415–420.

Kole, M. M., I. Draper, and D. F. Gerson. 1988. Protease production by Bacillus subtilis in oxygen controlled, glucose fed-batch fermentations. Appl. Microbiol. Biotechnol. 28:404–408.

Kolenbrander, P. E., and J. London. 1993. Adhere today, here tomorrow: Oral bacterial adherence. J. Bacteriol. 175:3247–3252.

Kolter, R., D. A. Siegele, and A. Tormo. 1993. The stationary phase of the bacterial life cycle. Ann. Rev. Microbiol. 47:855–874.

Krembs, C., A. R. Juhl, R. A. Long, and F. Azam. 1998. Nanoscale patchiness of bacteria in lake water studied with the spatial information preservation method. Limnol. Oceanogr. 43:307–314.

Kristjansson, J. R., P. Schönheit, and R. K. Thauer. 1982. Different Km values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: An explanation for the apparent inhibition of methanogenesis by sulfate. Arch. Microbiol. 131:278–282.

Krulwich, T. A., and A. A. Guffanti. 1983. Physiology of acidophilic and alkalophilic bacteria. Adv. Microb. Physiol. 24:173–214.

Krulwich, T. A., and A. A. Guffanti. 1989. Alkalophilic bacteria. Ann. Rev. Microbiol. 43:435–463.

Kuenen, J. G., and J. C. Gottschal. 1982a. Competition among chemolithotrophs and methylotrophs and their interactions with heterotrophic bacteria. *In:* A. T. Bull and J. H. Slater (Eds.) Microbial Interactions and Communities. Academic Press. London, UK. 1:153–187.

Kuenen, J. G., and W. Harder. 1982b. Microbial competition in continuous culture. *In:* R. G. Burns and J. H. Slater (Ed.) Experimental Microbial Ecology. Blackwell Scientific Publications. Oxford, UK. 342–367.

Kuenen, J. G., and L. A. Robertson. 1984. Competition among chemolithotrophic bacteria under aerobic and anaerobic conditions. *In:* M. J. Klug and C. A. Reddy (Eds.) Current Perspectives in Microbial Ecology. ASM Press. Washington, DC. 306–313.

Kuenen, J. G., L. A. Robertson, and H. van Gemerden. 1985. Microbial interactions among aerobic and anaerobic sulfur-oxidizing bacteria. Adv. Microb. Ecol. 8:1–59.

Kushner, D. J. 1978. Life in high salt and solute concentrations: Halophilic bacteria. *In:* D. J. Kushner (Ed.) Microbial Life in Extreme Environments. Academic Press. London, UK. 318–368.

Kuske, C. R., S. M. Barns, and J. D. Busch. 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographical regions. Appl. Environ. Microbiol. 63: 3614–3621.

Kuznetsov, S. I., G. A. Dubinina, and N. A. Laptev. 1979. Biology of oligotrophic bacteria. Ann. Rev. Microbiol. 33:377–387.

Laanbroek, H. J., A. J. Smit, G. Klein Nulend, and H. Veldkamp. 1979. Competition for L-glutamate between specialized and versatile Clostridium species. Arch. Microbiol. 120:61–66.

Laanbroek, H. J., and H. Veldkamp. 1982. Microbial interactions in sediment communities. Phil.Trans. R. Soc. Lond. B 297:533–550.

Laanbroek, H. J., H. J. Geerlings, A. A. C. M. Peynenburg, and J. Siesling. 1983. Competition for L-lactate between Desulfovibrio, Veillonella, and Acetobacterium species isolated from anaerobic intertidal sediments. Microb. Ecol. 9:341–354.

Laanbroek, H. J., H. J. Geerlings, L. Sijtsma, and H. Veldkamp. 1984. Competition for sulfate and ethanol among Desulfobacter, Desulfobulbus, and Desulfovibrio species isolated from intertidal sediments. Appl. Environ. Microbiol. 47:329–334.

Lange, W. 1971. Enhancement of algal growth in cyanophyta-bacteria systems by carbonaceous compounds. Can. J. Microbiol. 17:303–314.

Lange, R., and R. Hengge-Aronis. 1991. Identification of a central regulator of stationary-phase gene expression in Escherichia coli. Molec. Microbiol. 5:49–59.

Langworthy, T. A. 1978. Microbial life in extreme pH values. *In:* D. J. Kushner (Ed.) Microbial Life in Extreme Environments. Academic Press. New York, NY. 279–317.

Larsen, H. 1986. Halophilic and halotolerant microorganisms – an overview and historical perspective. FEMS Microbiol. Rev. 39:3–7.

Law, A. T., and D. K. Button. 1977. Multiple-carbon-source-limited growth kinetics of a marine coryneform bacterium. J. Bacteriol. 129:115–123.

Lee, I. H., A. G. Fredrickson, and H. M. Tsuchiya. 1976. Dynamics of mixed cultures of Lactobacillus plantarum and Propionibacterium shermanii. Biotechnol. Bioeng. 18:513–526.

Lee, N., P. H. Nielsen, K. H. Andrasen, S. Juretschko, J. L. Nielsen., K.-H. Schleifer, and M. Wagner. 1999. Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure: Function analyses in microbial ecology. Appl. Environ. Microbiol. 65:1289–1297.

Leedle, J. A. Z., and R. B. Hespell. 1980. Differential carbohydrate media and anaerobic replica plating technique in delineating carbohydrate utilizing subgroups in rumen bacterial populations. Appl. Environ. Microbiol. 39:709–719.

Legan, J. D., J. D. Owens, and G. A. Chilvers. 1987. Competition between specialist and generalist methylotrophic bacteria for an intermittent supply of methylamine. J. Gen. Microbiol. 133:1061–1073.

Legan, J. D., and J. D. Owens. 1988. Bacterial competition for methylamine: Computer simulation of a three-strain continuous culture supplied continuously or alternatively with two nutrients. FEMS Microbiol. Ecol. 53:307–314.

Lengeler, J. W., G. Drews, and H. G. Schlegel. 1999. Biology of the Prokaryotes. Thieme. New York, NY.

Li, L., C. Kato, Y. Nogi, and K. Horikoshi. 1998. Distribution of the pressure-regulated operons in dep-sea bacteria. FEMS Microbiol. Lett. 159:159–166.

Licht, T. R., T. Tolker-Nielsen, K. Holmstroslash;m, K. A. Krogfelt, and S. Molin. 1999. Inhibition of Escherichia coli precursor-16S rRNA processing by mouse intestinal contents. Environ. Microbiol. 1:23–32.

Liesack, W., P. H. Janssen, F. A. Rainey, N. L. Ward-Rainey, and E. Stackebrandt. 1997. Microbial diversity in soil: The need for a combined approach using molecular and cultivation techniques. *In:* J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington (Eds.) Modern Soil Microbiology. Marcel Dekker. New York, NY. 375–439.

Lins, U., and M. Farina. 1999. Organization of cells in magnetotactic multicellular aggregates. Microbiol. Res. 154: 9–13.

Little, B., S. Gerchakov, and L. Udey. 1987. A method for sterilization of natural seawater. J. Microbiol. Meth. 7:193–200.

Loewen, P. C., and R. Hengge-Aronis. 1994. The role of the sigma factor σ^6 (Kat F) in bacterial global regulation. Ann. Rev. Microbiol. 48:53–80.

Loewen, P. C., B. Hu, J. Stutinsky, and R. Sparling. 1998. Regulation in the rpoS regulon of Escherichia coli. Can. J. Microbiol. 44:707–717.

Lonhienne, T., C. Gerday, and G. Feller. 2000. Psychrophilic enzymes: Revisiting the thermodynamic parameters of activation may explain local flexibility. Biochim. Biophys. Acta 1543:1–10.

Loomis, W. D., and R. W. Durst. 1992. Chemistry and biology of boron. Biofactors 3:229–239.

Loveland-Curtze, J., P. P. Sheridan, K. R. Gutshall, and J. E. Brenchley. 1999. Biochemical and phylogenetic analyses of psychrophilic isolates belonging to the Arthrobacter subgroup and description of Arthrobacter psychrolactophilus, sp. nov. Arch. Microbiol. 171:355–363.

Lovitt, R. W., and J. W. T. Wimpenny. 1981. The gradostat: A bidirectional compound chemostat and its application in microbiological research. J. Gen. Microbiol. 127:261–268.

Lovley, D. R., D. F. Dwyer, and M. J. Klug. 1982. Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl. Environ. Microbiol. 43:1373–1379.

Lovley, D. R., J. D. Coates, E. L. Blunt-Harris, E. J. P. Phillips, and J. C. Woodward. 1996a. Humic substances as electron acceptors for microbial respiration. Nature 382: 445–448.

Lovley, D. R., J. C. Woodward, and F. H. Chapelle. 1996b. Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. Appl. Environ. Microbiol. 62:288–291.

Lovley, D. R., and E. L. Blunt-Harris. 1999a. Role of humic-bound iron as an electron transfer agent in dissimilatory Fe(III) reduction. Appl. Environ. Microbiol. 65:4252–4254.

Lovley, D. R., J. L. Fraga, J. D. Coates, and E. L. Blunt-Harris. 1999b. Humics as an electron donor for anaerobic respiration. Environ. Microbiol. 1:89–98.

Ludwig, W., S. H. Bauer, H. Bauer, I. Held, G. Kirchhof, R. Schulze, I. Huber, S. Spring, A. Hartmann, and K.-H. Schleifer. 1997. Detection and in situ identification of representatives of a widely distributed bacterial phylum. FEMS Microbiol. Lett. 153:181–190.

Lum, K. T., and P. D. Meers. 1989. Boric acid converts urine into an effective bacteriostatic transport medium. J. Infect. 18:51–58.

MacDonald, A. G. 1984. The effects of pressure on the molecular structure and physiological functions of cell membranes. Phil. Trans. R. Soc. Lond. B 304:47–68.

Macy, J. M., J. E. Snellen, and R. E. Hungate. 1972. Use of syringe methods for anaerobiosis. Am. J. Clin. Nutr. 25:1318–1323.

Madigan, M. T. 1998. Isolation and characterization of psychrophilic purple bacteria from Antarctica. *In:* G. A. Peschek, W. Löffelhardt, and G. Schmetterer (Eds.) The Phototrophic Prokaryotes. Kluwer Academic/Plenum. New York, NY. 699–706.

Madigan, M. T., J. M. Martinko, and J. Parker. 2000a. Biology of Microorganisms. Prentice-Hall International. Upper Saddle River, NJ.

Madigan, M. T., D. O. Jung, C. R. Woese, and L. A. Achenbach. 2000b. Rhodoferax antarcticus sp. nov., a moderately psychrophilic purple nonsulfur bacterium isolated from an Antarctic microbial mat. Arch. Microbiol. 173:269–277.

Mannisto, M. K., P. Schumann, F. A. Rainey, P. Kampfer, I. Tsitko, M. A. Tiirola, and M. S. Salkinoja-Salonen. 2000. Subtercola boreus gen. nov., sp. nov., and Subtercola frigoramans sp. nov., two new psychrophilic actinobacteria isolated from boreal groundwater. Int. J. Syst. Evol. Microbiol. 50:1731–1739.

Margulis, L. 1981. Symbiosis in Cell Evolution. Freeman. San Francisco, CA.

Marquis, R. E. 1976. High pressure microbial physiology. Adv. Microb. Physiol. 14:159–239.

Marquis, R. E., and P. Matsumura. 1978. Microbial life under pressure. *In:* D. J. Kushner (Ed.) Microbial Life in Extreme Environments. Academic Press. London, UK. 105–158.

Martin, G. A., and W. P. Hempfling. 1976a. A method for the regulation of microbial population density during continuous culture at high growth rates. Arch. Microbiol. 107:41–47.

Martin, S. E., R. S. Flowers, and Z. J. Ordal. 1976b. Catalase: Its effect on microbial enumeration. Appl. Environ. Microbiol. 32:731–734.

Maruyama, A., D. Honda, H. Yamamoto, K. Kitamura, and T. Higashihara. 2000. Phylogenetic analysis of psychrophilic bacteria isolated from the Japan Trench, including a description of the deep-sea species Psychrobacter pacificensis sp. nov. Int. J. Syst. Evol. Microbiol. 50:835–846.

Mason, C. A., and T. Egli. 1993. Dynamics of microbial growth in the decelerating and stationary phase of batch culture. *In:* S. Kjelleberg (Ed.) Starvation in Bacteria. Plenum Press. New York, NY. 81–98.

Matin, A., and H. Veldkamp. 1978. Physiological basis of the selective advantage of a Spirillum sp. in a carbon-limited environment. J. Gen. Microbiol. 105:187–197.

Matin, A. 1981. Regulation of enzyme synthesis as studied in continuous culture. *In:* P. H. Calcott (Ed.) Continuous Culture of Cells. CRC Press. Boca Raton, FL. 2:69–97.

Matin, A. 1990. Keeping a neutral cytoplasm: The bioenergetics of obligate acidophiles. FEMS Microbiol. Rev. 75:307–318.

Mazur, P. 1980. Limits to life at low temperatures and at reduced water contents and water activities. Orig. Life 10:137–159.

McInerney, M. J., M. D. Bryant, and N. Pfennig. 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. 122: 129–135.

McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton. 1981. Syntrophomonas wolfei gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl. Environ. Microbiol. 41:1029–1039.

Meers, J. L. 1973. Growth of bacteria in mixed cultures. CRC Crit. Rev. Microbiol. 2:139–184.

Megee 3rd, R. D., J. F. Drake, A. G. Fredrickson, and H. M. Tsuchiya. 1972. Studies in intermicrobial symbiosis. Saccharomyces cerevisiae and Lactobacillus casei. Can. J. Microbiol. 18:1733–1742.

Meldrum, F. C., B. R. Heywood, S. Mann, R. B. Frankel, and D. A. Bazylinski. 1993. Electron microscopy study of magnetosomes in a cultured coccoid magnetotactic bacterium. Proc. R. Soc. Lond. B 251:231–236.

Meyer, J. S., H. M. Tsuchiya, and A. G. Fredrickson. 1975. Dynamics of mixed populations having complementary metabolism. Biotechnol. Bioeng. 17:1065–1081.

Michels, P. A. M., J. P. J. Michels, J. Boonstra, and W. N. Konings. 1979. Generation of an electrochemical proton gradient in bacteria by the excretion of metabolic endproducts. FEMS Microbiol. Lett. 5:357–364.

Mikx, F. J. M., and J. S. van der Hoeven. 1975. Symbiosis of Streptococcus mutans and Veillonella alcalescens in mixed continuous cultures. Arch. Oral Biol. 20:407–410.

Miller, T. L., and M. J. Wolin. 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. 27:985–987.

Miura, Y., H. Tanaka, and M. Okazaki. 1980. Stability analysis of commensal and mutual relations with competitive assimilation in continuous mixed culture. Biotechnol. Bioeng. 22:929–948.

Mizunoe, Y., S. N. Wai, A. Takade, and S. Yoshida. 1999. Restoration of culturability of starvation-stressed and low-temperature-stressed Escherichia coli O157 cells by using H_2O_2-degrading compounds. Arch. Microbiol. 172:63–67.

Moench, T. T., and J. G. Zeikus. 1983. An improved preparation method for a titanium (III) media reductant. J. Microbiol. Meth. 1:199–202.

Moissl, C., C. Rudolph, and R. Huber. 2002. Natural communities of novel archaea and bacteria with a string-of-pearls-like morphology: Molecular analysis of the bacterial partners. Appl. Environ. Microbiol. 68:933–937.

Monod, J. 1942. Recherches sur la Croissance des Cultures Bacteriennes. Hermann. Paris, France.

Monod, J. 1950. La technique de culture continue: Théorie et applications. Ann. Inst. Pasteur 79:390–410.

Morita, R. Y. 1975. Psychrophilic bacteria. Bacteriol. Rev. 39:144–167.

Morita, R. Y. 1982. Starvation-survival of heterotrophs in the marine environment. Adv. Microb. Ecol. 6:171–178.

Morita, R. Y. 1986. Pressure as an extreme environment. *In:* R. A. Herbert and G. A. Codd (Eds.) Microbes in Extreme Environments. Academic Press. London, UK. 171–185.

Mossel D. A. A., A. Veldman, and I. Eelderink. 1980. Comparison of the effects of liquid medium repair and the incorporation of catalase in Macconkey type media on the recovery of Enterobacteriaceae sublethally stressed by freezing. J. Appl. Bacteriol. 49:405–419.

Mountfort, D. O., and M. P. Bryant. 1985. Isolation and characterization of an anaerobic syntrophic benzoate degrading bacterium from sewage sludge. Arch. Microbiol. 133:249–256.

Mountfort, D. O., and R. A. Asher. 1986. Isolation from a methanogenic ferulate degrading consortium of an

anaerobe that converts methoxyl groups of aromatic acids to volatile fatty acids. Arch. Microbiol. 144:55–61.

Mountfort, D. O., F. A. Rainey, J. Burghardt, H. F. Kaspar, and E. Stackebrandt. 1997. Clostridium vincentii sp. nov., a new anaerobic, saccharolytic, psychrophilic bacterium isolated from low-salinity pond sediment of the McMurdo ice shelf, Antarctica. Arch. Microbiol. 167:54–60.

Mountfort, D. O., F. A. Rainey, J. Burghardt, H. F. Kaspar, and E. Stackebrandt. 1998. Psychromonas antarcticus gen. nov., sp. nov., a new aerotolerant anaerobic, halophilic psychrophile isolated from pond sediment of the McMurdo ice shelf, Antarctica. Arch. Microbiol. 169:231–238.

Mukamolova, G. V., A. S. Kaprelyants, D. I. Young, M. Young, and D. B. Kell. 1998. A bacterial cytokine. Proc. Natl. Acad. Sci. USA 95:8916–8921.

Müller, R. H., and W. Babel. 1996. Measurement of growth at very low rates ($\mu \geq 0$), an approach to study the energy requirement for the survival of Alcaligenes eutrophus JMP 134. Appl. Environ. Microbiol. 62:147–151.

Munro, P. M., G. N. Flatau, R. L. Clément, and M. J. Gauthier. 1995. Influence of the RpoS (KatF) sigma factor on maintenance of viability and culturability of Escherichia coli and Salmonella typhimurium in seawater. Appl. Environ. Microbiol. 61:1853–1858.

Mur, L. R., H. J. Gons, and L. van Liere. 1977. Some experiments on the competition between green algae and blue-green bacteria in light-limited environments. FEMS Microbiol. Lett. 1:335–338.

Murray, D. R. 1989. Biology of Food Radiation. John Wiley and Sons. Chichester, UK.

Myers, J., and L. B. Clark. 1944. Culture conditions and the development of the photosynthetic mechanism. II: An apparatus for the continuous culture of Chlorella. J. Gen. Physiol. 28:103–112.

Nakamura, I., K. Ogimoto, and H. Izumu. 1995. ATP-dependent calcium release from binding site in Streptococcus bovis: Bound versus free pools. J. Gen. Appl. Microbiol. 41:389–398.

Nakasone, K., A. Ikegami, C. Kato, R. Usami, and K. Horikoshi. 1998. Mechanisms of gene expression controlled by pressure in deep-sea microorganisms. Extremophiles 2:149–154.

Nedwell, D. B. 1984. The input and mineralization of organic carbon in anaerobic aquatic sediments. Adv. Microb. Ecol. 7:93–131.

Nedwell, D. B., and M. Rutter. 1994. Influence of temperature on growth rate and competition between two psychrotolerant Antarctic bacteria: Low temperature diminishes affinity for substrate uptake. Appl. Environ. Microbiol. 60:1984–1992.

Neidhardt, F. C., and H. E. Umbarger. 1996. Chemical composition of Escherichia coli. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella, 2nd ed. ASM Press. Washington, DC. 13–16.

Nelson, D. C., B. B. Jørgensen, and N. P. Revsbech. 1986. Growth pattern and yield of a chemoautotrophic Beggiatoa sp. in oxygen-sulfide microgradients. Appl. Environ. Microbiol. 52:225–233.

Neuhard, J., and R. A. Kelln. 1996. Biosynthesis and conversions of pyrimidines. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella, 2nd ed. ASM Press. Washington, DC. 580–599.

Nichols, D. S., A. R. Greenhill, C. T. Shadbolt, T. Ross, and T. A. McMeekin. 1999. Physicochemical parameters for growth of the sea ice bacteria Glaciecola punicea ACAM 611T and Gelidibacter sp. strain IC158. Appl. Environ. Microbiol. 65:3757–3760.

Niehaus, F., K. Hantke, and G. Unden. 1991. Iron content and FNR-dependent gene regulation in Escherichia coli. FEMS Microbiol. Lett. 84:319–324.

Nielsen, P. H., M. A. de Muro, and J. L. Nielsen. 2000. Studies on the in situ physiology of Thiothrix spp. present in activated sludge. Environ. Microbiol. 2:389–398.

Nogales, B., R. Guerrero, and I. Esteve. 1997. A heterotrophic bacterium inhibits growth of several species of the genus Chlorobium. Arch. Microbiol. 167:396–399.

Nogales, B., E. R. B. Moore, W. R. Abraham, and K. N. Timmis. 1999. Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. Environ. Microbiol. 1:199–212.

Norland, S., K. M. Fagerbakke, and M. Heldal. 1995. Light element analysis of individual bacteria by X-ray microanalysis. Appl. Environ. Microbiol. 61:1357–1362.

Norris, J. R., and D. W. Ribbons. 1970a. Methods in Microbiology. Academic Press. New York, NY. 3a:1–506.

Norris, J. R., and D. W. Ribbons. 1970b. Methods in Microbiology. Academic Press. New York, NY. 3b:1–369.

Notley, L., and T. Ferenci. 1996. Induction of RpoS-dependent functions in glucose-limited continuous culture: What levels of nutrient limitation induces the stationary phase of Escherichia coli. J. Bacteriol. 178:1465–1468.

Novick, A., and L. Szilard. 1950. Description of the chemostat. Science 112:715–716.

Nozhevnikova, A. N., M. V. Simankova, S. N. Parshina, and O. R. Kotsyurbenko. 2001. Temperature characteristics of methanogneic archaea and acetogenic bacteria isolated from cold environments. Water Sci. Technol. 44:41–48.

Nurmikko, V. 1956. Biochemical factors affecting symbiosis among bacteria. Experientia 12:245–249.

Oerther, D. B., J. Pernthaler, A. Schramm, R. Amann, and L. Raskin. 2000. Monitoring precursor 16S rRNAs of Acinetobacter spp. in activated sludge wastewater treatment systems. Appl. Environ. Microbiol. 66:2154–2165.

Ohmura, N., N. Matsumoto, K. Sasaki, and H. Saiki. 2002. Electrochemical regenration of Fe(III) to support growth on anaerobic iron respiration. Appl. Environ. Microbiol. 68:405–407.

Okada, T., K. Ueyama, S. Niya, H. Kanazawa, M. Futai, and T. Tsuchiya. 1981. Role of inducer exclusion in preferential utilization of glucose over melibiose in diauxic growth of Escherichia coli. J. Bacteriol. 146:1030–1037.

Oliver, J. D. 1995. The viable but non-culturable state in the human pathogen Vibrio vulnificus. FEMS Microbiol. Lett. 133:203–208.

Olsen, R. A., and L. R. Bakken. 1987. Viability of soil bacteria: Optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. Microb. Ecol. 13:59–74.

Oltmann, L. F., G. S. Schoenmaker, W. N. M. Reijnders, and A. H. Stouthamer. 1978. Modification of the pH auxostat culture method for the mass cultivation of bacteria. Biotechnol. Bioeng. 20:921–925.

Orcutt, K. M., U. Rasmussen, E. A. Webb, J. B. Waterbury, K. Gundersen, and B. Bergman. 2002. Characterization of Trichodesmium spp. by genetic techniques. Appl. Environ. Microbiol. 68:2236–2245.

Oremland, R. S. 1988. Biogeochemistry of methanogenic bacteria. *In:* A. J. B. Zehnder (Ed.) Biology of Anaerobic

Microorganisms. John Wiley and Sons. New York, NY. 641–706.

Oren, A. 1986. Intracellular salt concentrations of the halophilic eubacteria Haloaerobium praevalens and Halobacteroides halobius. Can. J. Microbiol. 32:4–9.

Oren, A. 1999. Bioenergetic aspects of halophilism. Microbiol. Molec. Biol. Rev. 63:334–348.

Ostrowski, M., R. Cavicchioloi, M. Blaauw, and J. C. Gottschal. 2001. Specific growth rate plays a critical role in hydrogen peroxide resistance of the marine oligotrophic ultramicrobacterium Sphingomonas alaskensis strain RB2256. Appl. Environ. Microbiol. 67:1292–1299.

Ott, J. A., R. Novak, F. Schiemer, U. Hentschel, M. Nebelsiek, and M. Polz. 1991. Tackling the sulfide gradient: A novel strategy involving marine nematodes and chemoautotrophic ectosymbionts. Marine Ecol. 12:261–279.

Otto, R., J. Hugenholtz, W. N. Konings, and H. Veldkamp. 1980. Increase of molar growth yield of Streptococcus cremoris for lactose as a consequence of lactate consumption by Pseudomonas stutzeri in mixed culture. FEMS Microbiol. Lett. 9:85–88.

Ouverney, C. C., and J. A. Fuhrman. 2000. Marine planktonic Arhcaea take up amino acids. Appl. Environ. Microbiol. 66:4829–4833.

Overmann, J., and N. Pfennig. 1989. Pelodictyon phaeoclathratiforme sp. nov., a new brown-colored member of the Chlorobiaceae forming net-like colonies. Arch. Microbiol. 152:401–406.

Overmann, J., S. Lehmann, and N. Pfennig. 1991. Gas vesicle formation and buoyancy regulation in Pelodictyon phaeoclathratiforme (Green sulfur bacteria). Arch. Microbiol. 157:29–37.

Overmann, J., and N. Pfennig. 1992. Continuous chemotrophic growth and respiration of Chromatiaceae species at low oxygen concentrations. Arch. Microbiol. 158:59–67.

Overmann, J., J. T. Beatty, and K. J. Hall. 1996. Purple sulfur bacteria control the growth of aerobic heterotrophic bacterioplankton in a meromictic salt lake. Appl. Environ. Microbiol. 62:3251–3258.

Overmann, J., C. Tuschak, J. Fröstl, and H. Sass. 1998. The ecological niche of the consortium "Pelochromatium roseum." Arch. Microbiol. 169:120–128.

Overmann, J., and H. van Gemerden. 2000. Microbial interactions involving sulfur bacteria: Implications for the ecology and evolution of bacterial communities. FEMS Microbiol. Rev. 24:591–599.

Overmann, J. 2002a. Phototrophic consortia: A tight cooperation between non-related eubacteria. *In:* J. Seckbach (Ed.) Symbiosis. Mechanisms and Model Systems. Kluwer. Dordrecht, The Netherlands. 239–255.

Overmann, J., and K. Schubert. 2002b. Phototrophic consortia: Model systems for symbiotic interrelations between prokaryotes. Arch. Microbiol. 177:201–208.

Padan, E., and S. Schuldinger. 1986. Intracellular pH regulation in bacterial cells. Meth. Enzymol. 125:337–352.

Paerl, H. 1978. Role of heterotrophic bacteria in promoting N_2-fixation by Anabaena in aquatic habitats. Microb. Ecol. 4:215–231.

Paerl, H. W. 1982. Interactions with bacteria. *In:* N. G. Carr and N. G. Whitton (Eds.) The Biology of Cyanobacteria. University of California Press. Los Angeles, CA. 441–461.

Parkes, R. J., and E. Senior. 1988. Multistage chemostats and other models for studying anoxic ecosystems. *In:* J. W. T. Wimpenny (Ed.) Handbook of Laboratory Model Systems for Microbial Ecosystems. CRC Press. Boca Raton, FL. 1:51–71.

Pastan, I., and R. Perlman. 1969. Repression of β-galactosidase synthesis by glucose in phosphotransferase mutants of Escherichia coli. Repression in the absence of glucose phosphorylation. J. Biol. Chem. 244:5836–5842.

Pasteur, L. 1862. Mémoire sur les corpuscules qui existent dans l'atmosphère: Examen de la doctrine des générations spontanées. Ann. Chémie Physique 64:5–110.

Pernthaler J., T. Posch, K. Simek, J. Vrba, A. Pernthaler, F. O. Glöckner, U. Nübel, R. Psenner, and R. Amann. 2001. Predator-specific enrichment of Actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture. Appl. Environ. Microbiol. 67:2145–2155.

Pfennig, N. 1980. Syntrophic mixed cultures and symbiotic consortia with phototrophic bacteria: A review. *In:* G. Gottschalk, N. Pfennig, and Werner (Eds.) Anaerobes and Anaerobic Infections. Fischer. Stuttgart, Germany. 127–131.

Pfennig N., Trüper HG. 1989. Anoxygenic phototrophic bacteria. *In*: Staley JT, Bryant MP, Pfennig N, Holt JG (eds) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 3:1635–1709.

Pfennig, N. 1993. Reflections of a microbiologist, or how to learn from the microbes. Ann. Rev. Microbiol. 47:1–29.

Pietzsch, O. 1967. Ein Nährboden zur Schwärmhemmung und gleichzeitigen Unterscheidung der Proteuskeime von Salmonellen. Fleischwirtschaft 1:31–32.

Pinhassi, J., U. L. Zweifel, and Å. Hagström. 1997. Dominant marine bacterioplankton species found among colony-forming bacteria. Appl. Environ. Microbiol. 63:3359–3366.

Pirt, S. J. 1974. The theory of fed batch culture with reference to the penicillin fermentation. J. Appl. Chem. Biotechnol. 24:415–4224.

Pirt, S. J. 1975. Principles of Microbe and Cell Cultivation. Blackwell. Oxford, UK.

Ploug, H., H. P. Grossart, F. Azam, and B. B. Jørgensen. 1999. Photosynthesis, respiration, and carbon turnover in sinking marine snow from surface waters of Southern California Bight: Implications for the carbon cycle in the ocean. Marine Ecol. Prog. Ser. 179:1–11.

Poindexter, J. S., and E. R. Leadbetter. 1986. Enrichment cultures in bacterial ecology. *In:* J. S. Poindexter and E. R. Leadbetter (Eds.) Bacteria in Nature. Plenum Press. New York, NY. 2:229–260.

Poindexter, J. S. 1992. Dimorphic protshecate bacteria: The genera Caulobacter, Asticacaulis, Hyphomicrobium, Pedomicrobium, Hyphomonas, and Thiodendron. *In:* A. Balows, H. G. Tr¨per, M. Dworkin, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 2176–2196.

Porter, J. R. 1976. Antony van Leeuwenhoek: Tercentenary of is discovery of bacteria. Microbiol. Rev. 40:260–269.

Pörtner, R., and H. Märkl. 1998. Dialysis cultures. Appl. Microbiol. Biotechnol. 50:403–414.

Postgate, J. R., and J. R. Hunter. 1963. Acceleration of bacterial death by growth substrates. Nature 198:273–280.

Postgate, J. R., and J. R. Hunter. 1964. Accelerated death of Aerobacter aerogenes starved in the presence of growth-limiting substrates. J. Gen. Microbiol. 34:459–473.

Powell, E. O. 1958. Criteria for the growth of contaminants and mutants in continuous culture. J. Gen. Microbiol. 18:259–268.

Pratuangdejkul, J., and S. Dharmsthiti. 2000. Purification and characterization of lipase from psychrophilic Acinetobacter calcoaceticus LP009. Microbiol. Res. 155:95–100.

Price, B. 2000. A habitat for psychrophiles in deep Antarctic ice. Proc. Natl. Acad. Sci. USA 97:1247–1251.

Pringault, O., and R. de Wit, and P. Caumette. 1996. A benthic gradient chamber for cultering phototrophic sulfur bacteria on reconstituted sediments. FEMS Microbiol. Ecol. 20:237–250.

Puskas, A., E. P. Greenberg, S. Kaplan, and A. L. Schaefer. 1997. A quorum-sensing system in the free-living photosynthetic bacterium Rhodobacter sphaeroides. J. Bacteriol. 179:7530–7537.

Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murrell. 2000. Stable-isotope probing as a tool in microbial ecology. Nature 403:646–649.

Ratkowsky, P. A., R. R. Lowry, T. A. McMeekin, A. N. Stokes, and R. E. Chandler. 1983. Model for bacterial culture growth rate throughout the entire biokinetic temperature range. J. Bacteriol. 154:1222–1226.

Reddy, G. S., R. K. Aggarwal, G. I. Matsumoto, and S. Shivaji. 2000. Arthrobacter flavus sp. nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antarctica. Int. J. Syst. Evol. Microbiol. 50:1553–1561.

Reed, R. H., and A. E. Walsby. 1985. Changes in turgor pressure in response to increases in external NaCl concentation in the gas-vacuolate cyanobacterium Microcystis sp. Arch. Microbiol. 143:290–296.

Reichenbach, H. 1984. Myxobacteria: A most peculiar group of social prokaryotes. *In:* E. Rosenberg (Ed.) Myxobacteria: Development and Cell Interactions. Springer-Verlag. New York, NY. 1–50.

Reusse, U., and A. Meyer. 1972. Der "Pril-Mannit-Agar" in der Salmonellen-Diagnostik. Zbl. Bakt. I. Abt. Orig. 219:555–557.

Revsbech, N. P., and B. B. Jørgensen. 1986. Microelectrodes: Their use in microbial ecology. Adv. Microb. Ecol. 9:293–352.

Rhee, G. Y. 1972. Competition between an algae and an aquatic bacterium for phosphate. Limnol. Oceanogr. 17:505–514.

Ricica, J., and P. Dobersky. 1981. Complex systems. *In:* P. H. Calcott (Ed.) Continuous Cultures of Cells. CRC Press. Boca Raton, FL. 1:63–96.

Robinson, J. A., and J. M. Tiedje. 1984. Competition between sulfate-reducing and methanogenic bacteria for H_2 under resting and growing conditions. Arch. Microbiol. 137:26–32.

Roeßler, M., and V. Müller. 1998. Quantitative and physiological analyses of chloride dependence of growth of Halobacillus halophilus. Appl. Environ. Microbiol. 64:3813–3817.

Rose, A. H., and L. M. Evison. 1965. Studies on the biochemical basis of the minimum temperature for growth of certain psychrophilic and mesophilic microorganisms. J. Gen. Microbiol. 38:131–141.

Rosenberg, E., K. H. Keller, and M. Dworkin. 1977. Cell-density dependent growth of Myxococcus xanthus on casein. J. Bacteriol. 129:770–777.

Roslev, P., and N. Iversen. 1999. Radioactive fingerprinting of microorganisms that oxidize atmospheric methane in different soils. Appl. Environ. Microbiol. 65:4064–4070.

Roszak, D. B., and R. R. Colwell. 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51:365–379.

Ruger, H. J., D. Fritze., and C. Sproer. 2000. New psychrophilic and psychrotolerant Bacillus marinus strains from tropical and polar deep-sea sediments and emended description of the species. Int. J. Syst. Evol. Microbiol. 50:1305–1313.

Russel, N. J. 1984. Mechanisms of thermal adaptation in bacteria: Blueprints for survival. TIBS: Trends in Biological Sciences 9:108–112.

Russel, N. J., and N. Fukunaga. 1990. A comparison of thermal adaptation of memebrane lipids in psychrophilic and thermophilic bacteria. FEMS Microbiol. Rev. 74: 171–182.

Russel, N. J. 2000. Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles 4:83–90.

Sambanis, A., and A. G. Fredrickson. 1987. Long-term studies of ciliate-bacterial interactions: Use of a chemostat fed with bacteria grown in a separate chemostat. J. Gen. Microbiol. 133:1619–1630.

Sánchez, O., H. van Gemerden, and J. Mas. 1996. Description of a redox-controlled sulfidostat fro the growth of sulfide-oxidizing phototrophs. Appl. Environ. Microbiol. 62:3640–3645.

Sandaa, R.-A., V. Torsvik, Ø. Enger, F. L. Daae, T. Castberg, and D. Hahn. 1999. Analysis of bacterial communitiues in heavy metal-contaminated soils at different levels of resolution. FEMS Microbiol. Ecol. 30:237–251.

Sass, A., H. Sass, M. J. L. Coolen, H. Cypionka, and J. Overmann. 2001. Microbial communities in the chemocline of a hypersaline deep-sea basin (Urania Basin, Mediterranean Sea). Appl. Environ. Microbiol. 67:5392–5402.

Schauer, N. L., D. P. Brown, and J. G. Ferry. 1982. Kinetics of formate metabolism in Methanobacterium formicicum and Methanospirillum hungatei. Appl. Environ. Microbiol. 44:549–554.

Shindler, D. B., R. M. Wydro, and D. J. Kushner. 1977. Cell-bound cations in the moderately halophilic bacterium Vibrio costicola. J. Bacteriol. 130:698–703.

Schink, B. 1991. Syntrophism among prokaryotes. *In:* A. Balows, H. G. Trüper, M. Dworkin, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 276–299.

Schink, B. 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Molec. Biol. Rev. 61:262–280.

Schink, B., and M. Friedrich. 2000. Phosphite oxidation by sulphate reduction. Nature 406:37.

Schink, B., V. Thiemann, H. Laue, and M. W. Friedrich. 2002. Desulfotignum phosphitoxidans sp. nov., a new marine sulfate reducer that oxidizes phosphite to phosphate. Arch. Microbiol. 177:381–391.

Schlegel, H. G., and H. W. Jannasch. 1967. Enrichment cultures. Ann. Rev. Microbiol. 21:49–70.

Schlesner, H. 1986. Pirellula marina sp. nov., a budding peptidoglycanless bacterium from brackish water. Syst. Appl. Microbiol. 8:177–180.

Schmid, M., S. Schmitz-Esser, M. Jetten, and M. Wagner. 2001. 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: Implications for phylogeny and in situ detection. Environ. Microbiol. 3:450–459.

Schmidt, G. B., C. L. Rosano, and C. Hurwitz. 1971. Evidence for a magnesium pump in Bacillus cereus T. J. Bacteriol. 105:150–155.

Schober, A., R. Günther, A. Schwienhorst., M. Döring, and B. F. Lindemann. 1993. Accurate high-speed liquid han-

dling of very small biological samples. BioTechniques 15:324–329.

Scholten, J. C. M., and R. Conrad. 2000. Energetics of syntrophic propionate oxidation in defined batch and chemostat cultures. Appl. Environ. Microbiol. 66:2934–2942.

Schopfer, P., and A. Brennicke. 1999. Pflanzenphysiologie, 5th ed. Springer-Verlag. New York, NY.

Schramm, A., D. de Beer, J. C. van den Heuvel, S. Ottengraf, and R. Amann. 1999. Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: Quantification by in situ hybridization and the use of microsensors. Appl. Environ. Microbiol. 65:3690–3696.

Schulten, H. R., B. Plage, and M. Schnitzer. 1991. A chemical structure for humic substances. Naturwissenschaften 78:31–312.

Schulz, E., H.-D. Lüdemann, and R. Jaenicke. 1976. High-pressure equilibrium studies on the dissociation-association of E. coli ribosomes. FEBS Lett. 64:40–43.

Schut, F., E. J. de Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button. 1993. Isolation of typical marine bacteria by dilution culture: Growth, maintenance, and characteristics of isolates under laboratory conditions. Appl. Environ. Microbiol. 59:2150–2160.

Schut, F., M. Jansen, T. M. Pedro Gomes, J. C. Gottschal, W. Harder, and R. A. Prins. 1995. Substrate uptake and utilization by a marine ultramicrobacterium. Microbiology 141:351–361.

Schut, F., R. A. Prins, and J. C. Gottschal. 1997. Oligotrophy and pelagic marine bacteria: Facts and fiction. Aquat. Microb. Ecol. 12:177–202.

Scott, D. T., D. M. McKnight, E. L. Blunt-Harris, S. E. Kolesar, and D. R. Lovley. 1998. Quinone moieties act as electron acceptors in the reduction of humic substances by humic-reducing microorganisms. Environ. Sci. Technol. 32:2984–2989.

Seitz, A. P., T. H. Nielsen, and J. Overmann. 1993. Physiology of purple sulfur bacteria forming macroscopic aggregates in Great Sippewissett Salt Marsh, Massachusetts. FEMS Microbiol. Ecol. 12:225–236.

Sekiguchi, Y., H. Takahashi, Y. Kamagata, A. Ohashi, and H. Harada. 2001. In situ detection, isolation, and physiological properties of a thin filamentous microorganism abundant in methanogenic granular sludges: A novel isolate affiliated with a clone cluster, the green non-sulfur bacteria, subdivision I. Appl. Environ. Microbiol. 67:5740–5749.

Shilo, M. 1984. Bdellovibrio as a predator. *In:* M. J. Klug and C. A. Reddy (Eds.) Current Perspectives in Microbial Ecology. ASM Press. Washington, DC. 334–339.

Shockey, W. L., and B. A. Dehority. 1989. Comparison of two methods for enumeration of anaerobe numbers on forages and evaluation of ethylene oxide treatment for forage sterilization. Appl. Environ. Microbiol. 55:1766–1768.

Siebert, J., and P. Hirsch. 1988. Characterization of 15 selected coccal bacteria isolated from Antarctic rock and soil samples from the McMurdo-Dry Valleys (South-Victoria Land). Polar Biol. 9:37–44.

Simankova, M. V., O. R. Kotsyurbenko, E. Stackebrandt, N. A. Kostrikina, A. M. Lysenko, G. A. Osipov, and A. N. Nozhevnikova. 2000. Acetobacterium tundrae sp. nov., a new psychrophilic acetogenic bacterium from tundra soil. Arch. Microbiol. 174:440–447.

Simek, K., J. Vrba, J. Pernthaler, T. Posch, P. Hartman, J. Nedoma, and R. Psenner. 1997. Morphological and compositional shifts in an experimental bacterial community influenced by protists with contrasting feeding modes. Appl. Environ. Microbiol. 63:587–595.

Sitnikov, D. M., J. B. Schineller, andT. O. Baldwin. 1996. Control of cell division in Escherichia coli: Regulation of transcription of ftsQA involves both rpoS and SdiA-mediated autoinduction. Proc. Natl. Acad. Sci. USA 93:336–341.

Skerman, V. D. B. 1968. A new type of micromanipulator and microforge. J. Gen. Microbiol. 54:287–297.

Skulachev, V. P. 1987. Bacterial sodium transport: Bioenergetic functions of sodium ions. *In:* B. P. Rosen and S. Silver (Eds.) Ion Transport in Prokaryotes. Academic Press. New York, NY. 131–164.

Slater, J. H., and A. T. Bull. 1978. Interactions between microbial populations. *In:* A. T. Bull and P. M. Meadow (Eds.) Companion to Microbiology. Longman. London, UK. 181–206.

Slonczewski, J., and J. W. Foster. 1996. pH-regulated genes and survival at extreme pH. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella, 2nd ed. ASM Press. Washington, DC. 1539–1549.

Sly, L. I., and V. Arunpairojana. 1987. Isolation of manganese-oxidizing Pedomicrobium cultures from water by micromanipulation. J. Microbiol. Meth. 6:177–182.

Soendergaard, M., B. Riemann, and N. O. G. Jörgensen. 1985. Extracellular organic carbon (EOC) released by phytoplankton and bacterial production. Oikos 45:323–332.

Somero, G. N. 1992. Adaptations to high hydrostatic pressure. Ann. Rev. Physiol. 54:557–577.

Spring, S., R. Amann, W. Ludwig, K.-H. Schleifer, H. van Gemerden, and N. Petersen. 1993. Dominating role of an unusual magnetotactic bacterium in the micraerobic zone of a freshwater sediment. Appl. Environ. Microbiol. 59:2397–2403.

Spring, S., R. Schulze, J. Overmann, and K.-H. Schleifer. 2000. Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: Molecular and cultivation studies. FEMS Microbiol. Rev. 24:573–590.

Stal, L. J., H. van Gemerden, andW. E. Krumbein. 1985. Structure and development of a benthic marine microbial mat. FEMS Microbiol. Ecol. 31:111–125.

Staley, J. T., J. A. Fuerst, S. J. Giovannoni, and H. Schlesner. 1992. The order Planctomycetales and the genera Planctomyces, Pirellula, gemmata and Isosphaera. *In:* A. Balows, M. Dworkin, W. Harder, K.-H. Schleifer, and H. G. Trüper (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 3710–3731.

Stetter, K. O. 2001. Genus III: Pyrolobus. *In:* D. R. Boone and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology. Springer-Verlag. New York, NY. 1:186–197.

Stevens, T. O. 1995. Optimization of media for enumeration and isolation of aerobic heterotrophic bacteria from the deep terrestrial subsurface. J. Microbiol. Meth. 21:293–303.

Stewart, C. S., and M. P. Bryant. 1988. The rumen bacteria. *In:* P. N. Hobson (Ed.) The Rumen Microbial Ecosystem. Elsevier Applied Science. London, UK. 21–75.

Stolp, H., and M. P. Starr. 1963. Bdellovibrio bacteriovorus gen. et sp. n., a predatory ectoparasitic and bacteriolytic microorganism. Ant. v. Leeuwenhoek 29:217–248.

Stolp, H., and M. P. Starr. 1965. Bacteriolysis. Ann. Rev. Microbiol. 19:79–104.

Stringfellow, W. T., and M. D. Aitken. 1994. Comparative physiology of phenanthrene degradation by two dissimilar pseudomonads isolated from a creosote-contaminated soil. Can. J. Microbiol. 40:432–438.

Suh, D. H., T. C. Becker, J. A. Sands, and B. S. Montenecourt. 1988. Effects of temperature on xylanase secretion by Trichoderma reesei. Biotechnol. Bioeng. 32:821–825.

Suzuki, K., J. Sasaki, M. Uramoto, T. Nakase, and K. Komagata. 1997a. Cryobacterium psychrophilum gen. nov., sp. nov., nom. rev., comb. nov., an obligately psychophilic actinomycete to accomodate "Curtobacterium psychrophilum," Inoue and Komagata 1976. Int. J. Syst. Evol. Microbiol. 47:474–478.

Suzuki, M. T., M. S. Rappé, Z. W. Haimberger, H. Winfield, N. Adair, J. Ströbel, and S. J. Giovannoni. 1997b. Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. 63:983–989.

Sweerts, J. P. R. A., and D. de Beer. 1989. Microelectrode measurements of nitrate gradients in the littoral and profundal sediments of a meso-eutrophic lake (Lake Vechten, The Netherlands). Appl. Environ. Microbiol. 55:754–757.

Swift, S. T., I. Y. Najita, K. Ohtaguchi, and A. G. Fredrickson. 1982. Continuous culture of the ciliate Tetrahymena pyriformis on Escherichia coli. Biotechnol. Bioeng. 24:1953–1964.

Szewzyk, U., and B. Schink. 1989. Degradation of hydroquinone, gentisate, and benzoate by a fermenting bacterium in pure or defined mixed culture. Arch. Microbiol. 15:541–545.

Talamoto, S., K. Yamada, and Y. Ezura. 1994. Producing of bacteriolytic enzymes during the growth of a marine bacterium Alteromonas sp. no. 8-R. J. Gen. Appl. Microbiol. 40:499–508.

Tang, W.-C., J. C. White, and M. Alexander. 1998. Utilization of sorbed compounds by microorganisms specifically isolated for that purpose. Appl. Microbiol. Biotechnol. 49:117–121.

Tappe, W., C. Tomaschewski, S. Rittershaus, and J. Groeneweg. 1996. Cultivation of nitrifying bacteria in the retentostat, a simple fermenter with internal biomass retention. FEMS Microbiol. Ecol. 19:47–52.

Tappe, W., A. Laverman, M. Bohland, M. Braster, S. Rittershaus, J. Groeneweg, and H. W. van Versefeld. 1999. Maintenance energy demand and starvation recovery synamics of Nitrosomonas europaea and Nitrobacter winogradskyi cultivated in a retentostat with complete biomass retention. Appl. Environ. Microbiol. 65:2471–2477.

Teather, R. M. 1982. Maintenance of laboratory strains of obligately anaerobic rumen bacteria. Appl. Environ. Microbiol. 44:499–501.

Tempest, D. W., F. Herbert, and P. J. Phipps. 1967. Studies on the growth of Aerobacter aerogenes at low dilution rates in a chemostat. *In:* E. O. Powell, C. G. T. Evans, R. E. Strange, and D. W. Tempest (Eds.) Micobial Physiology and Continuous Culture. HMSO. London, UK. 240–254.

Tempest, D. W. 1970a. The continuous culture in microbial research. Adv. Microb. Physiol. 4:223–250.

Tempest, D. W. 1970b. The place of continuous culture in microbial resaerch. Adv. Microb. Physiol. 4:223–250.

Tempest, D. W., and O. M. Neyssel. 1978. Eco-physiological aspects of microbial growth in aerobic nutrient limited environments. Adv. Microb. Ecol. 2:105–153.

Thauer, R. K., B. Käufer, and G. Fuchs. 1975. The active species of "CO_2" utilized by reduced ferredoxin: CO_2 oxidoreductase from Clostridium pasteurianum. Eur. J. Biochem. 27:282–290.

Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anarobic bacteria. Bacteriol. Rev. 41:100–180.

Thiele, J. H., and J. G. Zeikus. 1988a. Control of interspecies electron flow during anaerobic digestion: Significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. Appl. Environ. Microbiol. 54:20–29.

Thiele, J. H., M. Chartrain, and J. G. Zeikus. 1988b. Control of interspecies electron flow during anaerobic digestion: Role of floc formation in syntrophic methanogenesis. Appl. Environ. Microbiol. 54:10–19.

Thompson, L. A., D. B. Nedwell, M. T. Balba, I. M. Banat, and E. Senior. 1983. The use of multiple vessel, open flow systems to investigate carbon flow in anaerobic microbial communities. Microb. Ecol. 9:189–199.

Torella, F., and R. Y. Morita. 1981. Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. Appl. Environ. Microbiol. 41:518–527.

Torsvik, V., J. Goksør, F. L. Daae, R. Sørheim, J. Michalsen, and K. Salte. 1994. Use of DNA analysis to determine the diversity of microbial communities. *In:* K. Ritz, J. Dighton, and K. E. Giller (Eds.) Beyond the Biomass: Compositional and Functional Analysis of Soil Microbial Communities. British Society of Soil Science/John Wiley. Chichester, UK. 39–48.

Trimbur, D. E., K. R. Gutshall, P. Prema, and J. E. Brenchley. 1994. Characterization of a psychrotrophic Arthrobacter gene and its cold-active β-galactosidase. Appl. Environ. Microbiol. 60:4544–4552.

Trüper, H. G., and N. Pfennig. 1971. Family of phototrophic Green Sulfur Bacteria: Chlorobiaceae Copeland, the correct family name; rejection of Chlorobacterium Lauterborn; and the taxonomic situation of the consortium-forming species. Int. J. Syst. Bacteriol. 21:8–10.

Tuschak, C., J. Glaeser, and J. Overmann. 1999. Specific detection of green sulfur bacteria by in situ hybridization with a fluorescent labeled oligonucleotide probe. Arch. Microbiol. 171:265–272.

Uphoff, H. U., A. Felske, W. Fehr, and I. Wagner-Döbler. 2001. The microbial diversity in picoplankton enrichment cultures: A molecular screening of marine isolates. FEMS Microbiol. Ecol. 35:249–258.

Urakawa, H., K. Kita-Tsukamoto, S. E. Steven, K. Ohwada, and R. R. Colwell. 1998. A proposal to transfer Vibrio marinus (Russell 1891) to a new genus Moritella gen. nov. as Moritella marina comb. nov. FEMS Microbiol. Lett. 165:373–378.

Urbach, E., K. L. Vergin, and S. J. Giovannoni. 1999. Immunochemical detection and isolation of DNA from metabolically active bacteria. Appl. Environ. Microbiol. 65:1207–1213.

Vancanneyt, M., F. Schut, C. Snauwaert, J. Goris, J. Swings, and J. C. Gottschal. 2001. Sphingomonas alaskensis sp. nov., a dominant bacterium from a marine oligotrophic environment. Int. J. Syst. Evol. Microbiol. 51:73–79.

Van den Ende, F. P. L., A. M. Laverman, and H. van Gemerden. 1996. Coexistence of aerobic chemotrophic and

anaerobic phototrophic sulfur bacteria under oxygen limitations. FEMS Microbiol. Ecol. 19:141–151.

Van den Ende, F. P., J. Meier, and H. van Gemerden. 1997. Syntrophic growth of sulfate-reducing bacteria and colorless sulfur bacteria during oxygen limitation. FEMS Microbiol. Ecol. 23:65–80.

van der Meer, M. T. J., S. Schouten, B. E. van Dongen, W. I. C. Rijpstra, G. Fuchs, J. S. Sinninghe Damsté, J. W. de Leeuw, and D. M. Ward. 2001. Biosynthetic controls on the 13C contents of organic components in the photoautotrophic bacterium Chloroflexus aurantiacus. J. Biol. Chem. 276:10971–10976.

Van der Wielen, P. W. J. J., L. J. A. Lipman, F. van Knapen, and S. Biesterveld. 2002. Competitive exclusion of Salmonella enterica serovar enteritidis by Lactobacillus crispatus and Clostridium lactatifermentans in a sequencing fed-batch culture. Appl. Environ. Microbiol. 68:555–559.

van Gemerden, H. 1974. Coexistence of organisms competing for the same substrate: An example among purple sulfur bacteria. Microb. Ecol. 1:104–110.

van Gemerden, H., C. S. Tughan, R. De Witz, and R. A. Herbert. 1989. Laminated microbial ecosystems on sheltered beaches in Scapa Flow, Orkney Islands. FEMS Microbiol. Ecol. 62:87–102.

van Gemerden, H., and J. Mas. 1995. Ecology of phototrophic sulfur bacteria. *In:* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Phototrophic Bacteria. Kluwer Academic Publishers. Dordrecht, The Netherlands. 49–85.

Van Iterson Jr., G., L. E. Den Dooren de Jong, and A. J. Kluyver. 1940. Martinus Willem Beyerinck, His Life and Work. Martinus Nijhoff. The Hague, The Netherlands.

Van Niel, C. B. 1930. Contributions to Marine Biology. Stanford University Press. Stanford, CA. 161–169.

Van Niel, C. B. 1967. Prefatory chapter. The education of a microbiologist: Some reflections. Ann. Rev. Microbiol. 21:1–30.

Van Verseveld, H. W., W. R. Chesbro, M. Braster, and A. H. Stouthamer. 1984. Eubacteria have 3 growth modes keyed to nutrient-flow—consequences for the concept of maintenance and maximal growth yield. Arch. Microbiol. 137:176–184.

Varon, M., and M. Shilo. 1980. Ecology of aquatic Bdellovibrios. Adv. Aquat. Microbiol. 2:1–48.

Veldkamp, H. 1965. Enrichment cultures – history and prospects. *In:* H. G. Schlegel and E. Kröger (Eds.) Anreicherungskultur und Mutantenauslese: Zentralbl. Bakteriol. Parasitenkd. Infektionskrankh. Hygiene I. Abteilung. Gustav Fischer Verlag. Stuttgart, Germany. Supplement 1:1–13.

Veldkamp, H., and H. W. Jannasch. 1972. Mixed culture studies with the chemostat. J. Appl. Chem. Biotechnol. 22:105–123.

Veldkamp, H., and J. G. Kuenen. 1973. The chemostat as a model system for ecological studies. Bull. Ecol. Res. Comm. 17:347–355.

Veldkamp, H. 1977. Ecological studies with the chemostat. Adv. Microb. Ecol. 1:59–94.

Veldkamp, H., H. van Gemerden, W. Harder, and H. J. Laanbroek. 1984. Microbial competition. *In:* M. J. Klug and C. A. Reddy (Eds.) Current Perspectives in Microbial Ecology. ASM Press. Washington, DC. 279–290.

Vester, F., and K. Ingvorsen. 1998. Improved most-probable-number method to detect sulfate-reducing bacteria with natural media and a radiotracer. Appl. Environ. Microbiol. 64:1700–1707.

Visscher, P. T., R. A. Prins, and H. van Gemerden. 1992a. Rates of sulfate reduction and thiosulfate consumption in a marine microbial mat. FEMS Microb. Ecol. 86:283–294.

Visscher, P. T., F. P. van den Ende, B. E. M. Schaub, and H. van Gemerden. 1992b. Competition between anoxygenic phototrophic bacteria and colorless sulfur bacteria in a microbial mat. FEMS Microbiol. Ecol. 101:51–58.

Vobis, G. 1992. The genus,Actinoplanes and related genera. *In:* A. Balows, H. G. Trüper, M. Dworkin, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 1029–1060.

Wachenheim, D. E., and R. B. Hespell. 1984. Inhibitory effects of titanium (III) citrate on enumeration of bacteria from rumen contents. Appl. Environ. Microbiol. 48:444–445.

Walderhaug, M. O., D. C. Dosch, and W. Epstein. 1987. Potassium transport in bacteria. *In:* B. P. Rosen and S. Silver (Eds.) Ion Transport in Prokaryotes. Academic Press. New York, NY. 85–130.

Walker, G. C. 1996. The SOS response of Escherichia coli. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella. ASM Press. Washington, DC. 1:1400–1416.

Wang, X., P. A. J. de Boer, and L. I. Rothfield. 1991. A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of Escherichia coli. EMBO J. 10:3363–3372.

Ward, D. M., M. M. Bateson, R. Weller, and A. L. Ruff-Roberts. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol. 12:219–286.

Warthmann, R., H. Cypionka, and N. Pfennig. 1992. Photoproduction of H2 from acetate by syntrophic cocultures of green sulfur bacteria and sulfur-reducing bacteria. Arch. Microbiol. 157:343–348.

Waterbury, J. B., C. B. Calloway, and R. D. Turner. 1983. A cellulolytic nitrogen-fixing bacterium cultured from the gland of Deshayes in shipworms (Bivalvia: Terebrenidae). Science 221:1401–1403.

Waterbury, J. B. 1991. The cyanobacteria—isolation, purification, and identification. *In:* A. Balows, H. G. Trüper, M. Dworkin, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 149–196.

Watson, T. G. 1969. Steady state operation of a continuous culture at maximum growth rate by control of carbon-dioxide production. J. Gen. Microbiol. 59:83–89.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463–464.

Weimer, P. J., and J. G. Zeikus. 1977. Fermentation of cellulose and cellobiose by Clostridium thermocellum in the absence and presence of Methanobacterium thermoautotrophicum. Appl. Environ. Microbiol. 33:289–297.

Weise, W., and G. Rheinheimer. 1978. Scanning electron microscopy and epifluorescence investigation of bacterial colonization of marine sand sediments. Microb. Ecol. 4:175–188.

Welch, T. J., A. Farewell, F. C. Neidhardt, and D. Bartlett. 1993. Stress response of Escherichia coli to elevated hydrostatic pressure. J. Bacteriol. 175:7170–7177.

Westermann, P., B. K. Ahring, and R. A. Mah. 1989. Temperature compensation in Methanosarcina barkeri by modulation of hydrogen and acetate affinity. Appl. Environ. Microbiol. 55:1262–1266.

White, R. H. 1984. Hydrolytic stability of biomolecules at high temperatures and its implications for life at 250°C. Nature 310:430–432.

Whitesides, M. D., and J. D. Oliver. 1997. Resuscitation orf Vibrio vulnificus from the viable but non-culturable state. Appl. Environ. Microbiol. 63:1002–1005.

Widdel, F., G.-W. Kohring, and F. Mayer. 1983. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III: Characterization of the filamentous gliding Desulfonema limicola gen. nov. sp. nov., and Desulfonema magnum sp. nov. Arch. Microbiol. 134:286–294.

Widdel, F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria. *In:* A. J. B. Zehnder (Ed.) Biology of Anaerobic Microorganisms. John Wiley and Sons. New York, NY. 469–585.

Widdel, F., and F. Bak. 1992. Gram-negative mesophilic sulfate-reducing bacteria. *In:* A. Balows, H. G. Trüper, M. Dworkin, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3352–3378.

Wiegel, J., and L. G. Ljungdahl. 1986. The importance of thermophilic bacteria in biotechnology. CRC Crit. Rev. Biotechnol. 3:39–107.

Wiegel, J. 1990. Temperature spans for growth: Hypothesis and discussion. FEMS Microbiol. Rev. 75:1565–170.

Wilkinson, T. G., H. H. Topiwala, and G. Hamer. 1974. Interactions in a mixed bacterial population growing on methane in continuous culture. Biotechnol. Bioeng. 16:41–59.

Winogradsky, S. 1949. Microbiologie du Sol: Oeuvres Complèetes. Marson. Paris, France.

Wirsen, C. O., and H. W. Jannasch. 1978. Physiological and morphological observations on Thiovulum sp. J. Bacteriol. 136:765–774.

Wirsen, C. O., H. W. Jannasch, S. G. Wakeham, and E. A. Cannel. 1987. Membrane lipids of a psychrophilic and barophilic deep-sea bacterium. Curr. Microbiol. 14:319–322.

Wirsen, C. O., and S. J. Molyneaux. 1999. A study of deep-sea natural microbial populations and barophilic pure cultures using a high-pressure chemostat. Appl. Environ. Microbiol. 65:5314–5321.

Wolfe, R. S., and N. Pfennig. 1977. Reduction of sulfur by Spirillum 5175 and syntrophism with Chlorobium. Appl. Environ. Microbiol. 33:427–433.

Wolin, M. J. 1982. Hydrogen transfer in microbial communities. *In:* A. T. Bull and J. H. Slater (Eds.) Microbial Interactions and Communities. Academic Press. London, UK. 1:323–356.

Wolter, K. 1982. Bacterial incorporation of organic substances released by natural phytoplankton populations. Marine Ecol. Prog. Ser. 7:287–295.

Xu, H. S., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R. R. Colwell. 1982. Survival and viability of nonculturable Escherichia coli and Vibrio cholerae in the estuarine and marine environment. Microbiol. Ecol. 8:313–323.

Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero. 1982. Living with water stress: Evolution of osmolyte systems. Science 217:1214–1227.

Yayanos, A. A. 1978. Recovery and maintenance of live amphipods at a pressure of 580 bars from an ocean depth of 5700 meters. Science 200:1056–1059.

Yayanos, A. A. 1986. Evolutional and ecological implications of the properties of deep-sea barophilic bacteria. Proc. Natl. Acad. Sci. USA 83:9542–9546.

Yoshida, F., T. Yamane, and K. Nakamoto. 1973. Fed-batch hydrocarbon fermentation with colloidal emulsion feed. Biotechnol. Bioeng. 15:257–270.

Yumoto, I., K. Yamazaki, M. Hishinuma, Y. Nodasaka, K. Nakajima, N. Inoue, and K. Kawasaki. 2001. Pseudomonas alcaliphila sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. Int. J. Syst. Evol. Microbiol. 51:349–355.

Yumoto, I., A. Nakamura, H. Iwata, K. Kusumoto, Y. Nodasaka, and H. Matsuyama. 2002. Dietzia psychralcaliphila sp. nov., a novel, facultatively psychrophilic alkaliphile that grows on hydrocarbons. Int. J. Syst. Evol. Microbiol. 52:85–90.

Zehnder, A. J. B., and K. Wuhrman. 1976. Titanium (III) citrate as a non-toxic oxidation-reduction buffering system for the culture of obligate anaerobes. Science 194:1165–1166.

Zehnder, A. J. B., and W. Stumm. 1988. Geochemistry and biogeochemistry of anaerobic habitats. *In:* A. J. B. Zehnder (Ed.) Biology of Anaerobic Microorganisms. John Wiley and Sons. New York, NY. 1–38.

Zengler, K., H. H. Richnow, R. Rosselló-Mora, W. Michaelis, and F. Widdel. 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401:266–269.

Zinder, S. H., and M. Koch. 1984. Non-aceticlastic methanogenesis from acetate: Acetate oxidation by a thermophilic syntrophic coculture. Arch. Microbiol. 138:263–272.

ZoBell, C. E., and F. H. Johnson. 1949. The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. J. Bacteriol. 57:179–189.

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CHAPTER 1.6

Prokaryotes and Their Habitats

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Prokaryotes are well recognized as essential members of the biosphere. They inhabit all possible locations in which life exists from those offering ideal conditions for growth and reproduction to those representing extreme environments at the borderline of abiotic conditions.

The ubiquity of microorganisms is based on three major properties: their small size for easy dispersal by air and water, their metabolic versatility and flexibility, and their ability to tolerate unfavorable conditions. A predominant population is commonly composed of species able to grow under the particular conditions of a habitat. Many other species may also be present but in low numbers of individuals. As a rule, ecosystems of indistinct physicochemical and nutritional characteristics, such as many soils or sea water, which neither suppress nor specifically support microbial growth, usually carry low numbers of microorganisms but a high diversity of species. In contrast, ecosystems of strong environmental characteristics, such as acid mine waters, salt brines, and hot springs, commonly contain high cell numbers of very few species.

Experimental enrichment procedures bring about the predominance of certain species by controlling the supply of specific nutrients or the use of certain physicochemical conditions. If the growth conditions of a particular microorganism are known and reproducible, enrichment and isolation usually pose no problem. But if the particular requirements for growth of an organism are unknown, isolation procedures may be difficult to discover (Pfennig, 1961; Schlegel and Pfennig, 1961). For that reason, a number of organisms long known from microscopical observations, such a *Thiovulum* or *Achromatium*, have not yet been isolated in pure culture. Furthermore, organisms that have hitherto unknown growth characteristics and that are too small and inconspicuous for easy microscopical recognition have often escaped detection. An excellent example is *Desulfuromonas acetoxidans* (Pfennig and Biebl, 1976).

In characterizing an ecosystem microbiologically, it is important to distinguish between 1) organisms introduced incidentally by air, soil runoff, etc., physiologically just making the best of it, and 2) organisms typically adapted to the particular habitat and not occurring in any other except in the form of survival stages. An example of the former is *Escherichia coli*, as frequently found in polluted waters. An example of the latter is the above-mentioned *Thiovulum* sp., whose need for dissolved oxygen and hydrogen sulfide at the same time requires a high motility combined with chemotactic orientation in an aquatic oxic/anoxic interface.

Although their morphological differentiation is limited, prokaryotes have evolved a number of structural and chemical mechanisms that enable them to inhabit various extreme environments. The presence of a specified pigment, for instance, protects a cell against detrimental radiation or may provide for the absorption of light energy at specific wavelengths encountered in deeper water. Some filamentous cyanobacteria show a certain degree of cell differentiation, a feature that permits the fixation of elemental nitrogen concomitantly with oxygenic photosynthesis in oligotrophic environments. More importantly, however, the metabolic versatility of the prokaryotes, which reflects the development of metabolism during the evolution of life, enables them to live in many parts of the biosphere, including several where eukaryotes are not able to exist.

The vegetative microbial cell, with its relatively large reactive surface, responds quickly to changing physicochemical conditions of its immediate surrounding. As a consequence, the effective habitat of a microorganism is its microhabitat, the immediate surrounding of the cell in a compatible scale of space and time as determined by the radius of its metabolic action and interaction.

Naming microorganisms for their occurrence in certain characteristic macrohabitats, for example, soil and water bacteria, is of limited use. The two apparently very different habitats, soil and water, can be characterized as representing different proportions of the two phases, solid surface and water. The continuum of habitats ranges from highly arid desert soil with no or firmly bound pore water to offshore pelagic sea water containing a minimum of suspended particulate matter. Within the range of suitable physicochemical conditions, the abundance of microorganisms in an ecosystem is determined by the availability of the required energy and carbon sources and essential nutrients. All the more or less specific environments—e.g., the surface of leaves or skin, intestinal tracts, and symbiotically or parasitically invaded tissues—conform to this general description.

The concept of microenvironments eliminates the sharp dividing lines between aquatic, terrestrial, and even medical microbiology. Indeed, in ecological research the distinction between these academic disciplines is now more and more deemphasized by encompassing them under the label of environmental or biogeochemical microbiology. This chapter does not try to cover all the habitats of all organisms treated in this Handbook; the individual habitats and their characteristics are considered chapter by chapter for single species or physiological groups of prokaryotes. This chapter reviews the versatility of prokaryotic metabolism in relation to a few principles that determine the distribution of prokaryotes in nature.

This chapter was taken unchanged from the second edition.

The principal methods for the enrichment and isolation of the major metabolic types of microorganisms were discovered within a relatively short time. Details of the techniques developed by Winogradsky and Beijerinck are dispersed through the journals. Their collected papers (Beijerinck, 1921–1940; Winogradsky, 1949) are treasure troves for microbiologists; only one contemporary compilation exists (Stockhausen, 1907). Since the enrichment principles and methods were the subject of a symposium (Schlegel, 1965), several reviews have appeared (Aaronson, 1970; Schlegel and Jannasch, 1967; Veldkamp, 1970).

The aim of this Handbook is to encourage biologists to continue and intensify the search for bacteria in their natural environments, define habitats and ecological niches, and understand the flux of matter and energy through the biosphere. One may remember that in many soil and water samples there are more kinds of bacteria present than we can cultivate. Furthermore, much data on the flux of carbon and of trace gases through ecosystems cannot yet be accounted for by the bacteria cultivated so far. The gaps need to be filled by laboratory and field studies.

Ecological Terminology

Ecosystems

"The ultimate aim of ecology is to understand the relationships of all organisms to their environment" (Hungate, 1962). The principles of ecology were developed independently for the sciences dealing with plants, animals, and microorganisms. Identical terms are used to designate ecological units and parameters when dealing with macro- and microorganisms, but the implications of these terms are slightly different. The differences sometimes lead to considerable confusion (Whittaker, Levin, and Root, 1973). Furthermore, microorganisms, especially bacteria, exhibit properties not encountered in higher forms of life. The ecology of microorganisms has, therefore, been studied separately, and emphasis has been given to those microorganisms and their habitats that live under conditions not tolerated by the majority of the higher forms of life (Alexander, 1971, 1977; Hungate, 1962; Kushner, 1978). The recognition of the principles of microbial ecology is not yet complete and requires studies in many areas of experimental science (Atlas and Bartha, 1987; Brock, 1986a, b, 1987; Stetter, 1986, 1989; Megusar and Gantar, 1986). Therefore, the usage of terms in microbial ecology is not uniform and partially deviates from that used for the ecology of macroorganisms. A short survey of the present usage of some terms found in this chapter is presented below.

The ecology of microorganisms is concerned with the relationships between different species of microorganisms and between microorganisms and the environment. The basic unit of ecology is the community or biocoenosis. The community has to be considered in relation to the physical and chemical characteristics of the site. Both the biotic components—the community—and the abiotic, physicochemical components make up the ecosystem. The abiotic component of the ecosystem is frequently referred to as the environment, although when speaking about a particular member of the community, for example a species or its population, the term environment is often used to designate both the physicochemical and the biotic components of the ecosystem. In this case the term environment is used synonymously with "habitat."

Ecosystems differ from each other with respect to their extension. Ecosystems may be as large as a pond, a lake, a river, or a fir forest, or they may be as small as the rumen, the intestinal tract of an earthworm, or the rhizosphere of a plant. Some ecosystems, such as a cultivated field or the human skin, are characterized by weak (normal) environmental factors. Other ecosystems, such as hot springs or solar salt ponds, are characterized by strong environmental factors which cause a strict selection among potential inhabitants.

Habitats

Within an ecosystem a habitat for each species can be defined. The habitat is the location or dwelling place of a particular organism. For each microorganism, at least one habitat can be defined where it lives and grows and from which it may be recovered and isolated. Within a distinct ecosystem, a microorganism usually has only one kind of habitat. However, a microorganism may have two or more habitats, each in a different ecosystem. For example, many rhizobia are able to grow in the soil as well as intracellularly in root nodules; opportunist pathogenic bacteria such as *Pseudomonas aeruginosa*, *P. pseudomallei*, and *Erwinia* live in aquatic habitats as well as in mammalian hosts; and luminescent bacteria are met as free-living forms as well as living symbiotically in light organs of fish and invertebrates. Some bacteria are associated with both plant and animal hosts. A particular species of cellulolytic bacteria or of methanogenic bacteria may have its habitat in a lake bottom sediment, in the rumen, and in the sewage-sludge fermentor. In other words: the habitat designates the street and house number of a particular organism; some organisms may have several addresses.

When a species or a particular group of microorganisms is discussed, the word habitat is often used to denote an ecosystem, i.e., the entirety of the biotic and abiotic factors to which the particular microorganism is exposed in its dwelling place. This meaning of the term is in general usage in bacterial ecology. Since the operational area of many bacteria is small, the

term "microhabitat" is frequently used synonymously with "microecosystem" or with "microenvironment."

Inhabitants of an Ecosystem

Each ecosystem harbors a variety of diverse microorganisms. Two principal categories of microorganisms are distinguished: 1) autochthonous or indigenous (resident) microorganisms and 2) zymogenous, allochthonous, or nonindigenous (transient) microorganisms. This concept of two large groups of microorganisms, which are different with respect to their function, was first suggested by Winogradsky (1925). This concept, originally developed in soil microbiology (Winogradsky, 1925, 1926, 1947, 1949), has been adopted in general ecology (Alexander, 1971; Savage, 1977a). Autochthonous microorganisms in the strict sense are those that are native to a distinct soil (e.g., humic soil) and are always present. The population of autochthonous bacteria does not depend on exterior organic matter or on periodic increases of nutrients. Instead it reflects a more or less constant flux of nutrients. Indigenous microorganisms are known for many ecosystems, such as humus soil, the intestinal tract of animals, the rumen, the skin, and plant leaves.

The terms zymogenous, allochthonous, or nonindigenous refer to microorganisms that are dependent on occasional increases of nutrient concentrations or the occurrence of unusual substrates. As aliens and transients, they may persist in the absence of their required substrate or substrate concentration in dormant or starvation-resistant cell stages.

The ecosystem may be occupied by metabolically highly specialized organisms that are restricted to a distinct environment. These microorganisms are easily recognized as belonging to the indigenous flora of the ecosystem. Various obligately parasitic bacteria are specialized to certain animals or plants or even to certain tissues; some bacteria have only been isolated from hot springs, from sewage, or from other extreme environments. At the opposite extreme are the ubiquitous bacteria that are able to grow in distinctly different environments. Examples are soil and water bacteria that occasionally become opportunistic pathogens in animals (*Pseudomonas aeruginosa*, *P. pseudomallei*) or that may switch from plants to animals (*Erwinia*).

Ecological Niches

The term ecological niche was originally used in the sense of an area or location, the properties of which enabled an organism to live there. The functional concept of the niche led to some confusion. The different meanings were discussed by Whittaker, Levin, and Root (1973). Now "ecologists use the term ecological niche to mean the role that the organisms play in the ecosystem" (Odum, 1977). The ecological niche means the function of a species in a community of species in the ecosystem. Each species or strain of a bacterium has particular nutritional requirements, kinetic properties, biochemical abilities, and structural particularities; furthermore, it has distinct degrees of tolerance to environmental conditions. The ability or inability to fulfill a particular function in a distinct ecosystem is dependent on those properties.

For example, many cellulose-hydrolyzing bacteria are not highly specialized. They occupy a broad niche. However, in some ecosystems they may be restricted to cellulose utilization only, because they cannot compete with other organotrophic bacteria in performing other functions such as glucose fermentation. In these ecosystems they occupy a narrow niche. This description agrees with the general observation that the actual distribution of a species or population is usually more limited than its predicted one. In other words, real niches are always smaller than potential niches. Secondary factors determine the predominance of one species among those which could potentially occupy the niche. In the rumen, for example, among the cellulolytic bacteria only those that are able to hydrolyze cellulose under anaerobic conditions and to generate energy for growth by fermentation will be suited to fulfill the function of cellulose hydrolysis. Furthermore, cellulose degradation in the rumen requires tolerance to the particular environmental conditions in the rumen, such as temperatures up to 39–40°C, presence of various fatty acids, enzymes, ammonia, gases, etc. Finally, the function of the cellulolytic population of the rumen is influenced by the activities of other members of the community. In some rumen bacteria the metabolic processes are either impeded or modified if a product of their fermentation, hydrogen, is not removed by methanogenic bacteria. In this case, interspecies hydrogen transfer may be involved, as demonstrated for the S-organism and strain MoH of *Methanobacterium omelianskii* (Reddy, Bryant, and Wolin, 1972a), *Ruminococcus albus* and *Vibrio succinogenes* (Iannotti et al., 1973), *Ruminococcus flavefaciens* and *Methanobacterium ruminantium* (Latham and Wolin, 1977), and *Clostridium thermocellum* and *Methanobacterium thermoautotrophicum* (Winfrey and Zeikus, 1977). Therefore, the organism fulfilling a certain function in a particular ecosystem is determined by many physical and chemical factors and by all of the organisms constituting the community.

Studies on bacteria in pure culture attempt to define their entire ecological potential. The conclusions derived from pure-culture studies can be used to describe the potential niche of the particular bacterium. In nature, the predicted niche may be narrowed by competition. For example, it is possible to grow a pure culture of *Chromatium vinosum* in the laboratory in a medium containing malate as the sole electron donor (Thiele, 1968; van Niel, 1932, 1936). In its natural habitat, *Chromatium vinosum* would not dominate on this medium; members of the Rhodospirillaceae would outgrow the Chromatiaceae.

Finding the explanation for the dominance of a particular species in the habitat requires nutritional and kinetic pure-culture studies. For example, in aquatic environments where hydrogen sulfide is generated in the bottom sediment, bright purple or salmon-colored suspensions of the phototrophic bacterium *Chromatium okenii* or *Thiospirillum jenense* are often seen. Microscopic inspection reveals the presence of several small species of the Chromatiaceae in addition to the large species. Both types evidently coexist in their habitat. However, when samples of these natural enrichments are used for inoculating enrichment cultures in the laboratory, the dominant large bacterial species are overgrown by small species of the same physiological group, such as *C. vinosum* (Pfennig, 1965; Schlegel and Pfennig, 1961). The dominance of the large species in the natural habitat was not understood until Van Gemerden (1974) studied the competition of a representative of the large species, *C. weissei*, and a representative of the small species, *C. vinosum*, for the same substrate, hydrogen sulfide, in chemostat culture.

The dominance of *C. weissei* in the natural habitat turned out to depend upon the diurnal rhythm of illumination. The two species differ from each other with respect to the affinity (the K_s of intact cells) for hydrogen sulfide and to the rate of its uptake and oxidation to sulfur globules stored intracellularly. The maximal rate of hydrogen sulfide uptake (on the basis of total cell number) by *C. weissei* is about two and a half times higher than that by *C. vinosum*. In contrast, *C. vinosum* has a lower K_s for hydrogen sulfide and has a higher maximum growth rate. On the basis of experimental evidence, the dominance or the coexistence of *C. weissei* in the natural habitat can be explained: when both strains are growing in continuous light, most of the hydrogen sulfide will be consumed by *C. vinosum* due to its high growth rate and high affinity for hydrogen sulfide. In the dark, hydrogen sulfide accumulates; on illumination the major amount of the accumulated hydrogen sulfide is oxidized by *C. weissei* and sulfur is stored intracellularly, allowing at least moderate growth for a few hours. Under intermittent illumination both species can coexist. The example demonstrates that competition in the natural habitat can be based on kinetic properties. It further emphasizes the necessity of pure-culture studies for understanding the ecological niches of some microorganisms.

The importance of the substrate affinity, expressed in the K_s constant, can be easily demonstrated by growing mixed bacterial populations in the chemostat at varying dilution rates. At high dilution rates with substrate excess, the fast-growing bacteria are favored. At low dilution rates, when the substrate limits growth, the organism endowed with high substrate affinity (low K_s) will successfully compete. Continuous culture enrichment procedures with natural populations are based on these species-specific substrate affinities (Jannasch, 1967). Competition studies were conducted with psychro- and mesophilic bacteria (Harder and Veldkamp, 1971), a spirillum, and a rod-shaped bacterium in phosphate-limited medium (Kuenen et al., 1977). Such studies have been used to describe the characteristics of prokaryotes living in habitats where growth is largely limited by carbon compounds utilized by a large number of competing species. The use of the chemostat and the kinetics of microbial growth at characteristic ecological niches have been repeatedly reviewed (Jannasch and Mateles, 1974; Veldkamp, 1976; Veldkamp and Jannasch, 1972).

Understanding the ecological niche of a bacterial species is necessary to design enrichment culture conditions. Elective enrichment requires knowing more about a bacterium than its basic features. The knowledge of general growth conditions is not sufficient. For elective enrichment, only those properties can be considered which allow successful competition within a mixed population. The determining feature for occupying the actual narrow niche may be as obvious as in the case of the dinitrogen-fixing *Azotobacter*, abundantly growing and dominating in a sample of soil to which sucrose has been added without providing a source of combined nitrogen. The determining feature, however, may still be hidden, as in the case of enriching for green sulfur bacteria (Chlorobiaceae), which compete successfully at low light intensities but succumb to purple sulfur bacteria at high light intensities (Biebl and Pfennig, 1978; Pfennig and Cohen-Bazire, 1967).

Bacterial Metabolism as an Ecological Determinant

Many ecosystems, both natural and artificial, are almost exclusively occupied by prokaryotes. Environments characterized by the absence of

eukaryotes are the strictly anaerobic regions of freshwater lakes and of marine estuary sediments and any organic material fermented or decomposed under the exclusion of air (sauerkraut, silage, biodigestion with methane production). Prokaryotic cells have also adapted almost exclusively to certain extreme conditions of temperature, acidity, and salinity.

The exclusiveness of bacteria is based on unique metabolic capabilities either absent from or only rudimentarily present in eukaryotes. The uniqueness of bacteria pertains to their modes of energy conversion, to their wide range of growth substrates, and to their tolerance toward extreme environmental conditions.

Modes of Energy Conversion

All forms of life use energy for maintenance as well as for biosynthesis of cell material. Biochemical energy is generated by metabolic reactions. Energy sources are organic or inorganic substrates that are taken up from the environment. In the cell they are converted via a series of metabolic pathways. These pathways fulfill two essential functions: they provide precursors for the macromolecular cell constituents and they make energy available for biosynthetic and other energy-requiring processes.

The principle of "unity in biochemistry" (Kluyver and Donker, 1925, 1926) is one of the few lasting dogmas of this century. Although the original concept referred to the basic energy relationships of organisms, it is now clear that "unity" expresses the assumption that the biochemistry of all organisms on this planet follows a few basic principles, for example, the uniformity of cell constituents, the universality of ATP as the principal carrier of biological energy, the universality of the genetic code, and the distribution of the degradative pathways, the respiratory chain, and the basic mechanisms of cellular energy conversion. Even the main metabolic pathways are almost identical in all organisms. Only a few groups of bacteria have modified patterns of central metabolic routes. The metabolic pathways arose during evolution. The biochemical apparatus that is typical for aerobic organisms developed when oxygen became available.

The prokaryotes developed billions of years before the atmosphere became aerobic and before carbohydrates became abundant products of primary biomass production. They may be regarded as relics of the evolution of life. During their early evolution, the prokaryotes learned to exploit a multitude of sources of energy and cell carbon different from the predominant present-day nutrient sources. The morphological uniformity of prokaryotes contrasts astonishingly with their versatility in substrate utilization, peripheral metabolic pathways, and modes of energy conversion. The most outstanding metabolic capacities, which are either restricted to only a few eukaryotes or completely lacking among eukaryotes, are anaerobic growth, use of inorganic electron donors for growth, fixation of molecular nitrogen, and the utilization of methane and a few polymers.

Anaerobic Growth

The ability to grow indefinitely under anaerobic conditions is almost exclusively confined to prokaryotes (see The Anaerobic Way of Life in Volume 2). In the majority of eukaryotes, anaerobic energy generation is only a transient process occurring during intense activity and exposure to hypoxic environments (Bennett, 1978). There are a few exceptions; among the protozoa two ciliate groups, holotrichs and entodiniomorphs, live in the rumen (Hungate, 1975). *Entamoeba*, *Diplomonas*, and *Trichomonas* lack mitochondria and grow under strictly anaerobic conditions (Bauchop, 1971; Müller, 1975; Steinbüchel, 1986; Williams, 1986; Stumm and Zwart, 1986; Hobson, 1988). Among the helminths there are various facultative anaerobes, such as *Ascaris lumbricoides*, *Trichuris vulpis*, *Trichinella spiralis* larvae, various tapeworms, and *Schistosoma mansoni* (Fairbairn, 1970; Hochachka and Mustafa, 1972; Hochachka and Somero, 1973). The question of whether fungi are able to grow anaerobically has now been definitely settled. An early statement ["One of the major metabolic differences between fungi and bacteria is that there are no anaerobic moulds either obligate or facultative" (Foster, 1949)] has been shown to be wrong by experimental evidence for anaerobic growth of species of *Mucor* (Bartnicki-Garcia and Nickerson, 1962) and *Fusarium* (Gunner and Alexander, 1964), *Aqualinderella fermentans* (Held, 1970; Held et al., 1969), and *Neocallimastix frontalis* (Mountfort and Asher, 1983; Orpin and Joblin, 1988). The latter belongs to the Chytridiomycetes and ferments polysaccharides, cellulose included, with the formation of lactic and acetic acids and H_2 and CO_2.

The contribution of eukaryotes to anaerobic decomposition appears to be negligibly small. The presence of various protozoa, fungi, and lower metazoa in the anaerobic layers of coastal marine sediments, especially within the sulfide system, and their disappearance when the sediment becomes oxidized suggest that the number of eukaryotes able to live under anaerobic conditions is large (Fenchel, 1969; Fenchel and Riedl, 1970; Schroff and Schöttler, 1977; Zebe, 1977).

The forms of anaerobic metabolism are briefly discussed here because of the uniqueness of

anaerobic metabolism in prokaryotes and its function in whole ecosystems. The energy for bacterial growth under anaerobic conditions can be provided by three fundamental types of anaerobic energy generation: 1) anoxygenic photosynthesis, 2) anaerobic respiratory energy generation, and 3) fermentative energy generation.

Anoxygenic photosynthesis is the light-driven process that anaerobic phototrophic bacteria use to generate energy (Table 1). Unlike green plants, these bacteria are not able to use water as the ultimate reductant; consequently they do not evolve oxygen and they require the presence of other reduced compounds such as organic acids, alcohols, carbohydrates, hydrogen sulfide, sulfur, or hydrogen (Gromet-Elhanan, 1977; Jones, 1977). Two major groups are different by their pigmentation: the purple and the green bacteria. On the basis of their source of reducing power, the purple and green bacteria are subdivided into sulfur and nonsulfur bacteria. The purple and green sulfur bacteria can use reduced sulfur compounds and oxidize them via elemental sulfur to sulfate. The nonsulfur purple bacteria require reduced organic compounds or, when using hydrogen sulfide, oxidize it to sulfur or sulfate. In contrast to the purple sulfur bacteria they lack the ability to oxidize elemental sulfur. The green bacteria comprise two groups, the *Chlorobium* group and the *Chloroflexus* group. The chlorobia use hydrogen sulfide as reductant and comprise strictly anaerobic bacteria; the latter are facultative and versatile (Pfennig, 1979; Trüper, 1976). The majority of phototrophic bacteria require light for growth; a few species can equally well obtain energy via aerobic respiration.

Anaerobic respiratory energy conversion is similar to aerobic respiration with respect to the electron donors, which may be either organic or inorganic compounds. Oxygen serves as the ultimate electron acceptor in aerobic respiratory energy generation, but anaerobic respiration depends on the presence of inorganic compounds, which, under anaerobic conditions, are reduced. The physiological groups listed in Table 2 are differentiated with respect to the electron acceptors used and to respective end products of the respiratory process. Each of these physiological groups comprise strains and species belonging to various taxonomic groups.

Fermentative energy generation depends on organic compounds serving as electron donors and as electron acceptors. These compounds are usually two different metabolites derived from sugar by cleavage or, in a few cases, derived from two different compounds. Fermentation is accompanied by the production of more or less reduced compounds, such as alcohols, organic acids, ammonia and hydrogen and of carbon dioxide as the oxidized product.

The fermentative bacteria are usually separated into groups on the basis of one or several fermentation products which reflect their metabolic pathways (Table 3).

Table 1. Groups of anaerobic phototrophic bacteria.

Bacterial group	Typical species	Growth: Anaerobically in light	Growth: Aerobically in dark	Photosynthetic electron donors	Sulfur deposited
Nonsulfur purple bacteria	*Rhodospirillum rubrum*	+	(+)	H_2, organic, (S^{2-})	(extracellularly)[1]
Purple sulfur bacteria	*Chromatium okenii*	+	–	S^{2-}, S^0, $S_2O_3^{2-}$, H_2	Intracellularly
Green sulfur bacteria	*Chlorobium limicola*	+	–	S^{2-}, S^0, $S_2O_3^{2-}$, H_2	Extracellularly
Chloroflexus group	*Chloroflexus aurantiacus*	+	+	Organic (S^{2-})	(extracellularly)
Heliobacterium	*Heliobacterium chlorum*	+	–	Organic	—

Parentheses indicate substrates used by only a few strains or species.

Table 2. Physiological groups of bacteria able to grow under anaerobic conditions using external electron acceptors for electron transport ("erobic respiration").

Bacterial group	Typical species	Metabolic process	Electron acceptor	Reduction products(s)
Denitrifiers	*Pseudomonas denitrificans*	Nitrate respiration	NO_3^-	N_2, N_2O, NO_2^-
Sulfate reducers	*Desulfovibrio vulgaris*	Sulfate respiration	SO_4^{2-}	S^{2-}
Sulfur reducers	*Desulfuromonas acetoxidans*	Sulfur respiration	S^0	S^{2-}
Methanogenic bacteria	*Methanobacterium thermoautotrophicum*	Carbonate respiration	CO_2	CH_4
Acetogenic bacteria	*Acetobacterium woodii*	Carbonate respiration	CO_2	CH_3—COOH
Succinogenic bacteria	*Wolinella succinogenes*	Fumarate respiration	Fumarate	Succinate
Iron reducers	*Pseudomonas* GS-15	Iron respiration	Fe^{3+}	Fe^{2+}

Table 3. Groups of fermentative bacteria able to grow under anaerobic conditions, and their fermentation products.

Fermentation characterizing bacterial groups	Typical species	Substrate	Fermentation product	
			Major	Minor
Ethanol fermentation	*Zymomonas mobilis*	Glucose	Ethanol	CO_2
Lactate fermentation:				
Homofermentative	*Lactobacillus casei*	Glucose	Lactate	
Heterofermentative	*Leuconostoc mesenteroides*	Glucose	Lactate	Ethanol, CO_2
Heterofermentative	*Bifidobacterium bifidum*	Glucose	Acetate	Lactate
Butyrate fermentation	*Clostridium butyricum*	Glucose	Butyrate	Acetate + H_2 + CO_2
	Clostridium acetobutylicum	Glucose	Butyrate, butanol	Acetone, 2-propanol
	Clostridium kluyveri	Ethanol + acetate	Butyrate	Caproate, H_2
Homoacetate fermentation	*Clostridium aceticum*	Fructose	Acetate	
Propionate and succinate fermentation	*Propionibacterium pentosaceum*	Sugars, lactate	Propionate	Succinate
	Veillonella alcalescens	Lactate	Propionate	Acetate, H_2, CO_2
	Bacteroides ruminicola	Sugars	Propionate	
Mixed acid and butanediol fermentation	*Escherichia coli*	Glucose	Lactate, ethanol, acetate	Formate, H_2 + CO_2, succinate
	Enterobacter aerogenes	Glucose	2,3-Butanediol, ethanol	Formate, H_2 + CO_2
Nitrogenous compounds fermentation	*Clostridium tetanomorphum*	Glutamate	Butyrate	Acetate, CO_2, NH_3
	Clostridium sticklandii	Lysine	Butyrate	Acetate, NH_3
	Clostridium oroticum	Orotate	Acetate	CO_2, NH_3

Table 4. Groups of bacteria able to use inorganic electron donors for growth ("chemolithoautotrophs").

Bacterial group	Typical species	Metabolic process	Electron donor	Electron acceptor	Carbon source	Product
Hydrogen-oxidizing bacteria	*Alcaligenes eutrophus*	H_2 oxidation	H_2	O_2	CO_2	H_2O
Carbon monoxide-oxidizing bacteria	*Pseudomonas carboxydovorans*	CO oxidation	CO	O_2	CO_2	CO_2
Ammonium-oxidizing bacteria	*Nitrosomonas europaea*	Ammonium oxidation	NH_4^+	O_2	CO_2	NO_2^-
Nitrite-oxidizing bacteria	*Nitrobacter winogradskyi*	Nitrite oxidation	NO_2^-	O_2	CO_2	NO_3^-
Sulfur-oxidizing bacteria	*Thiobacillus thiooxidans*	Sulfur oxidation	S, $S_2O_3^{2-}$	O_2	CO_2	SO_4^{2-}
Iron-oxidizing bacteria	*Thiobacillus ferrooxidans*	Iron oxidation	Fe^{2+}	O_2	CO_2	Fe^{3+}
Methanogenic bacteria	*Methanobacterium thermoautotrophicum*	Methanogenesis	H_2	CO_2	CO_2	CH_4
Acetogenic bacteria	*Acetobacterium woodii*	Acetogenesis	H_2	CO_2	CO_2	CH_3-COOH

Growth with Inorganic Electron Donors

The ability to use inorganic compounds as electron donors for growth, called lithotrophy, is exclusively restricted to prokaryotes (see The Chemolithotrophic Prokaryotes; The H_2-Metabolizing Prokaryotes; The Colorless Sulfur Bacteria; and Oxidation of Inorganic Nitrogen Compounds as an Energy Source all in Volume 2). The electrons are used for electron transport phosphorylation, either aerobically with oxygen or anaerobically with inorganic compounds (nitrate, sulfate, carbonate) as the ultimate electron acceptors. The electrons also serve to reduce carbon dioxide, which is the common carbon source for a physiological group of bacteria called chemolithoautotrophs. These bacteria are usually separated into groups on the basis of their electron donors (Table 4). In memory of S.N. Winogradsky, who developed the concept of chemolithotrophy and autotrophy in 1887–1891, a symposium on lithoautotrophy was held in 1987 (Schlegel and Bowien, 1989).

Fixation of Molecular Nitrogen

Only prokaryotes are able to fix molecular nitrogen (dinitrogen = N_2) and to grow in the absence of a source of combined nitrogen (see Chapter 22). Dinitrogen fixation requires the presence of a special enzyme system, nitrogenase, and particular environmental conditions. The ability to fix dinitrogen is distributed among many species of oxygenic and anoxygenic phototrophic bacteria, chemoautotrophic and chemoheterotrophic bacteria, and both aerobic

and anaerobic bacteria—in short, in all major physiological groups. There is almost no correlation with the taxonomic unit. A recent symposium considered all aspects of research in this area (Bothe et al., 1988).

Range of Organic Substrates for Growth

The bacteria as a whole are considered to be omnipotent with respect to their substrate spectrum. All natural (biosynthetic) compounds are subject to degradation by microorganisms. There are a few compounds that can be utilized exclusively by prokaryotes. Among the low-molecular-weight compounds, methane is oxidized only by highly specialized bacteria, the methylotrophs. Some of these bacteria are obligate methylotrophs, being able to use methane, methanol, and dimethyl ether as substrates.

No macromolecular compounds are used only by prokaryotes. Cellulose, hemicelluloses, pectins, xylanes, galactan, mannans, chitin, and others are subject to degradation by bacteria and fungi; some polymers are hydrolyzed by protozoa and a few metazoa. Under anaerobic conditions, however, the degradation of polysaccharides is almost exclusively confined to prokaryotes.

Extremes of Environmental Conditions Allowing Bacterial Growth and Survival

Room or body temperature, the oxygen partial pressure of the atmosphere, neutrality of pH, and abundant nutrients supporting luxurious growth of the kinds of organisms studied in the laboratory are generally considered to be "normal" conditions for growth of microorganisms. Any conditions substantially deviating therefrom are regarded as being extreme. The ecologically minded biologist will define those conditions under which the greatest species diversity develops as normal. Environmental conditions different from the norm have an elective effect on organisms; as a rule, the species diversity decreases with the increase of environmental adversity. The greater the severity of an adverse environmental factor, the smaller is the number of species of actively growing microorganisms. In addition, among the organisms adapted to extreme conditions the abundance of physiological types is restricted. However, this trend of limitation of species and number of individuals may not be as pronounced as is presently assumed. It may just reflect reluctance to examine extreme environments and to isolate new organisms, many of which cannot easily be grown in laboratory cultures.

In general, the microorganisms that tolerate and grow under the most extreme conditions are obligately adapted to their particular environment and fail to grow at lower intensities of the same environmental factor. Such an organism has acquired the ability to grow in one extreme environment at the expense of its ability to grow in others. Less rigid or extreme environmental conditions are tolerated by a greater number of organisms; some of these are obligately bound to these conditions and others grow there facultatively. There are several examples of low numbers of species and of high specialization in habitats of extreme conditions, such as low and high temperature, high salt concentrations, low moisture, low pH, low nutrient concentrations, and others. Much attention has been given during recent years both to microorganisms that either tolerate or are dependent on extreme conditions and to their habitats. Life in extreme environments was the subject of several symposia, and emphasis was paid to the microorganisms, to the regulatory or molecular mechanisms that make life under adverse conditions possible, and to the possible existence of life on other planets (Alexander, 1971, 1976; Brock, 1969; Ellwood et al., 1980; Heinen, 1974; Hochachka and Somero, 1973; Kushner, 1971, 1978; Shilo, 1979; Brock, 1986a, b; Stetter, 1986, 1989; Larsen, 1986; Tindall and Trüper, 1986).

Thanks to the amount of attention that the "extremophiles" and their habitats have received during recent years, there is no need to add another exhaustive review. However, it is likely that more as yet unidentified microorganisms representing various metabolic groups are present in extreme environments. They can only be discovered by experimental approaches based on new ideas on possibly existing organisms. The following short survey is meant to encourage relevant research.

Low Temperatures

The temperature of the natural environments of water and soil bacteria is distinctly lower than their optimum growth temperature. The average soil temperature in the temperature climate zone is 12°C, and 90% of the ocean water is 5°C or colder. Many mesophilic bacteria do not find their optimum growth temperature in their natural habitats; they are, however, able to tolerate the seasonal fluctuations of temperature, which in the summer can easily span 30°C or more. This tolerance is lacking in psychrophilic bacteria. As defined by Morita (1975), "the psychrophiles are those organisms having an optimum growth temperature of 15°C or lower, a maximal temperature for growth at about 20°C and a minimal temperature for growth at 0°C or lower." Many

bacteria that have been isolated from Arctic and Antarctic Ocean waters and sediments have optimal growth temperatures of about 10°C but do not survive exposure to 20°C. True psychrophiles will, therefore, be found only in habitats that never become warmer than 20°C. At temperatures below 10°C, psychrophiles do have selective advantage as demonstrated in continuous culture experiments (Harder and Veldkamp, 1968, 1971). One of the ecological niches of psychrophiles in the ocean is chitin degradation. The vast majority of psychrophilic bacteria are members of *Pseudomonas*, *Flavobacterium*, *Achromobacter*, and *Alcaligenes* (Rose, 1968).

Food preservation added another habitat for psychrophilic bacteria (Schmidt-Lorenz, 1967). However, the majority of bacteria that grow on food near the freezing point are apparently facultative psychrophiles or psychrotrophs that have temperature maxima for growth above 20°C, but are able to grow at low temperatures also. With respect to growth at temperatures down to –18°C, the capacities of prokaryotes do not seem to exceed those of the eukaryotes.

Microbial life at low temperatures has been comprehensively reviewed and discussed on the basis of ecological aspects (Baross and Morita, 1978) and molecular aspects (Inniss, 1975; Inniss and Ingraham, 1978).

High Temperatures

Growth at temperatures higher than about 60°C is restricted to the prokaryotes. Habitats with such temperatures include piles of self-heating hay, compost, or other organic materials (Hussain, 1973), circulating hot or cooling water in industrial plants, and hot springs and other geothermal sources.

The ecology of hot springs has been especially well studied and reviewed (Brock, 1967, 1970, 1978, 1986; Castenholz, 1969, 1979). The runoff of a hot spring is an ideal location to study the upper temperatures for continual growth of organisms. As in most extreme habitats, the species diversity decreases with increasing severity of the environmental factor. When the temperature gradient rises from 50 to 70°C, thermophilic bacteria such as the yellow-pigmented *Thermus aquaticus* (Brock and Freeze, 1969), which has an optimum temperature for growth at 70°C and a maximum at 79°C, can be found. In many runoff channels, thick mats consisting of the cyanobacterium *Synechococcus lividus* and the phototrophic, gliding, filamentous bacterium *Chloroflexus aurantiacus* are present (Pierson and Castenholz, 1974) (see also Chapter 207). In these mats, several layers of *Chloroflexus* are covered by a thin surface layer containing a mixture of *Synechococcus* and *Chloroflexus* (Bauld and Brock, 1973; Madigan and Brock, 1977).

Several strains of the facultative autotroph *Sulfolobus acidocaldarius,* with a growth pH range of 0.9–5.8 and temperature optimum of 70–75°C, and of an extreme thermophile growing at 85°C have been isolated from acid, hot, aqueous and soil habitats (Brock et al., 1971, 1972; Fliermans and Brock, 1972). Thermophilic *Thiobacillus*-type bacteria growing at 60 or 75°C were found in Icelandic thermal areas (Le Roux, Wakerley, and Hunt, 1977). The habitats par excellence of these bacteria are volcanic hot springs where magmatic hydrogen sulfide is oxidized to elemental sulfur and sulfuric acid. Thermophilic iron- and sulfur-oxidizing bacteria may be involved in metal leaching from low-grade ore (Brierley, C.L., 1977; Brierley and Lockwood, 1977; Brierley, J.A., 1978; Golovacheva and Karavaiko, 1978; Levi and Linkletter, 1989; Hughes and Poole, 1989).

Yellow-pigmented *Thermus* strains have also been isolated from Icelandic hot springs (Pask-Hughes and Williams, 1977). Nonpigmented thermophilic bacteria, otherwise similar to *Thermus aquaticus*, were found in laundry heaters (Brock and Boylen, 1973), hot tap water (Pask-Hughes and Williams, 1975), and a stream receiving hot-water effluents (Ramaley and Hixson, 1970) (see also The Family Chloroflexaceae in Volume 7). The range between 50 and 70°C is occupied by a multitude of bacteria, among them members of the genera *Bacillus*, *Thermoactinomyces* (Cross, 1968), *Methanobacterium* (Zeikus and Wolfe, 1972), *Methylococcus*, and others. Several thermophilic bacteria find excellent growth conditions in canned foods (Gillespy and Thorpe, 1968) and sugar (Scarr, 1968).

Our knowledge of microbial life at high temperatures has been greatly extended by studies of continental and marine hot springs of volcanic origin. A considerable number of organisms have been isolated that cover a range for optimal growth at 85–105°C and are generally termed extreme thermophiles or hyperthermophiles (Stetter, 1986; Stetter et al., 1990) (see also Thermoproteales; The Order Thermococcales; The Genus *Archaeoglobus*; and Thermoplasmatales, all in Volume 3). These organisms are mainly archaebacteria and appear to be so well adapted to these high temperatures that they do not grow below 60°C, and some of them do not grow below 80°C. Acidic terrestrial sulfotaric environments have primarily yielded acidophilic, hyperthermophilic autotrophs of the order Sulfolobales (see Thermoproteales in Volume 3) that aerobically oxidize H_2S and S°, with the exception of *Acidianus,* which is able to grow anaerobically on H_2 and S° forming H_2S (Huber

et al., 1987a). The same is true for some neutrophilic isolates that exist in neutral and anaerobic zones of terrestrial sulfotaras and are members of the genera *Thermoproteus* and *Pyrobaculum* (Zillig et al., 1981, 1982; Stetter and Zillig, 1985). A facultatively autotrophic isolate (*Pyrobaculum islandium*) grows by sulfur respiration of organic matter (Huber et al., 1987a).

Another habitat that has recently yielded a number of extremely thermophilic bacterial isolates are the deep-sea hydrothermal hot vents found at depths from 1,800–3,700 m. The high pressures make possible the presence of liquid water at temperatures far beyond the 1-atm boiling point of water, i.e., at a depth of 2,500 m, extruding vent water will not boil below approximately 460°C. This habitat provides an ideal situation to look for the upper temperature limit of prokaryotic life. Early indications that growth might occur at 250°C (Baross and Deming, 1983) have not yet been confirmed, although active growth at 110°C has been found in a culture of a new group of methanogenic archaebacteria (*Methanopyrus,* Huber et al., 1989). While this organism grows with its optimum doubling time of about 1 h just below 100°C, isolates of the genus *Pyrodictium* (Stetter et al., 1983) grow optimally at 105°C. *Pyrodictium,* as well as a large number of physiologically diverse hyperthermophilic archaebacteria, were isolated from a shallow marine hot spring (Stetter, 1986) and an erupting volcano (Huber et al., 1990).

Using $^{35}SO_4^{2-}$, hyperthermophilic sulfate reduction has been demonstrated in sediment cores freshly collected from a hydrothermal vent field (Jørgensen et al., 1990), and the organisms responsible (*Archaeoglobus profundus*, Burggraf et al., 1990) could be isolated at the same time. This particular vent field, located in the Gulf of California at a depth of 2,000 m, is overlayed by 3–400 m of organic-rich sediment (Jannasch, 1989). In the upper 60 cm of these sediments, downward temperature gradients of 3–180°C have been measured, overlayed by ambient bottom water with a temperature of 2.1°C. In these sediments, methanogenic hyperthermophiles of the genus *Methanococcus* (Jones et al., 1983, 1989; Zhao et al., 1988) appear to be common in addition to those of the above-mentioned *Methanopyrus.* The majority of the newly described hyperthermophiles were found in both the deep and the shallow marine hot vents. This includes the genera *Pyrococcus*, *Pyrodictium*, and *Staphylothermus* and the only eubacterial genus of extreme thermophiles, *Thermotoga* (Fiala et al., 1986; Belkin et al., 1986; Huber et al., 1986; Jannasch et al., 1988; Windberger et al., 1989). Some of them depend on the reduction of elemental sulfur, while others conduct an unknown type of fermentation.

Although many thermophilic water and soil bacteria were reported to be present only in samples of thermal habitats, experience indicates that they are much more widespread. The isolation of thermophilic, hydrogen-oxidizing bacteria from hot springs and the failure to isolate them from other places indicated a very narrow distribution of these highly specialized bacteria (Goto, Kodama, and Minoda, 1977). Surprisingly, attempts to isolate thermophilic, hydrogen-oxidizing bacteria from cold lake sediments (Aragno, 1978; Schenk and Aragno, 1979) and from the oxidation ponds of a sugar factory (H.G. Schlegel, unpublished) were successful. The search for thermophiles among other highly specialized bacteria resulted in equally encouraging findings, for example, thermophilic, nitrifying bacteria (Golovacheva, 1976), iron-oxidizing bacteria (Brierley, J.A., 1978), sulfur-oxidizing bacilli (Golovacheva and Karavaiko, 1978), and carbon monoxide-oxidizing bacteria (O. Meyer, unpublished). These recent findings indicate that the ability to grow at temperatures higher than 50°C is not confined to only a few metabolic types.

Research in the ecology of thermophilic microorganisms has recently yielded spectacular results; they are summarized by Brock (1978), Tansey and Brock (1978), Brock (1986), and Castenholz (1979).

Water Stress

Without water, life is not possible. The normal environment of microorganisms is an aqueous solution. The water body may differ in size, and the water may be free or adsorbed to external or internal surfaces of materials. The availability of water to microorganisms is a function not only of the water content of a material, but also of solution and adsorptive factors. In order to compare solutions and solid materials with respect to available water, the parameters "water activity" (a_w, ranging from 0 to 1.0) and "relative humidity" (expressed as a percentage) are used. These parameters express the ratio of water in the vapor phase to the amount of water the air would contain when vapor-saturated at a given temperature. Another parameter, the "water potential," is based on the free energy of molecules in water and makes differentiation of the matric and solute components of the system easy (Brown, 1978; Griffin and Luard, 1979; Smith, 1978, Griffin, 1981).

Microbial growth is possible in the range of water activity between 0.998 and 0.6 (Duckworth, 1975; Mossel, 1975; Mossel and Ingram, 1955). Bacteria are not very successful at extracting water from environments of lowest water activities. Fungi lead the list of organisms arranged with respect to the lowest water activity

at which they can grow. The osmotolerant yeast, *Saccharomyces rouxii*, and the osmophilic mold, *Xeromyces bisporus*, are able to grow down to $a_w = 0.60$; even *Aspergillus glacus* can survive at $a_w = 0.80$. In contrast, the majority of bacteria need water activities higher than 0.98 (which is the a_w of sea water at 25°C); only a few bacteria grow at 0.95 to 0.91. The halophilic bacteria are exceptions, growing at $a_w = 0.75$. The data are in accordance with the results of measurements on the microbial degradation of plant residues and straw mixed with field soil. If the soil was kept in equilibrium with pure water, the rate of carbon dioxide evolution was maximal. With decreasing humidity, the activity of bacteria decreased and reached zero at 92% relative humidity.

One has to distinguish between two effects of humidity, that on the activity and that on the viability of microorganisms. Some bacterial species are more resistant to drying than others. After some weeks of air-drying in the laboratory, the remaining viable population consists of bacteria that are able to form endospores, cysts, and other resting stages resistant to desiccation (Boylen, 1973; Robinson, Salonius, and Chase, 1965). However, under certain conditions even vegetative cells survive (Clark, 1967). Survival of vegetative cells depends on the type of soil, the velocity of the drying process, and the water activity. Several reports indicate that processes like nitrification, sulfur oxidation, and nonsymbiotic nitrogen fixation proceeded after rewetting without any soil reinoculation. Apparently, the endospores of some bacilli can survive for hundreds of years. On the basis of viable counts made on soil granules from the roots of plants gathered and dried in 1640 (Herbarium, Royal Botanical Gardens, Kew), "one can estimate that a ton of dry soil would still contain a few viable spores even after 1,000 years" (Sneath, 1962).

Salinity

High salt concentrations represent a special case of low water activity. Sea water ($a_w = 0.98$) is not tolerated by the majority of bacteria living in soil and in fresh water. Ecosystems containing salt (sodium chloride) at saturating concentrations are inhabited by only a few organisms. Many bacteria (Brison, Courtois, and Denis, 1974) as well as the flagellated alga *Dunaliella viridis* and the brine shrimp *Artemia salina* can tolerate such high salt concentrations, but only members of the prokaryotes find their optimum growth conditions there. The extreme halophiles have their best growth at 20–30% salt, moderate halophiles at 5–20%, and slight halophiles at 2–5% (Dundas, 1977; Larsen, 1967, 1971, 1973, 1986; Tindall and Trüper, 1986).

Extremely halophilic bacteria, such as *Halobacterium cutirubrum*, *H. salinarium*, and *Halococcus morrhuae*, are distributed in evaporation ponds for the production of solar salt and occasionally on salted fish and hides. They are easily recognized by their red color caused by carotenoids. Moderately halophilic bacteria live in similar habitats, namely salt brines and mud, and are often found in curing brines for meat, fish, and vegetables. About a dozen different bacteria are reported to find their optimum growth conditions in the range of 5–20% sodium chloride. The majority of the bacteria inhabiting the sea and marine mud require 2–5% sodium chloride; some of these slight halophiles fail to grow at lower salt concentrations.

The general aspects of halophilism and life in high salt concentrations have been recently discussed by Kushner (1978); Lanyi (1979); Brock (1979); Bayley and Morton (1978, 1979); and Csonka (1989).

From the ecological point of view, salinity plays a more important role than the water potential suggests. The following differences between freshwater and saline ecosystems cannot be overemphasized. As long as a saline water body or a water-soaked sediment is aerobic, the water potential may be the dominating factor among the selective environmental conditions for the growth of bacteria. The sequence of dominance of selective factors changes as soon as the location becomes anaerobic. Many saline waters, either marine or salt-polluted inland waters, contain sulfate; in the absence of oxygen, sulfate is the preferred terminal electron acceptor in anoxic environments and gives rise to the generation of hydrogen sulfide. The concentration of hydrogen sulfide may then exert a selective pressure on the bacterial flora in the location and govern the composition of the anaerobic food chain. In many freshwater lakes, the sulfate concentration is just high enough to provide sufficient hydrogen sulfide for securing the redox potential necessary for methanogenes to grow, but in marine ecosystems, such as sublittoral and estuarine sediments, the concentration of sulfate and, in consequence, that of hydrogen sulfide exceed the threshold where methanogenesis is possible (Cappenberg, 1974a,b; Winfrey and Zeikus, 1977).

Environments of Extreme pH Values

Hydrogen and hydroxyl ions are the most mobile of all ions. The concentration of these ions affects the growth of microorganisms either directly or indirectly via its influence on the ionic state and the availability of many inorganic ions and metabolites to the cells. The majority of bacteria prefer neutral or slightly alkaline conditions.

Acid Environments The hydrogen ion concentrations of the ocean and the major part of the land vary only within a narrow range, allowing growth of both fungi and bacteria. Only volcanic lakes and soils of recently claimed land have drastically reduced pH values. An extreme aquatic habitat of low pH is represented by the drainage of coal mines and coal mine refuse piles; the acidity is due to the oxidation of pyritic minerals, reduced iron, and sulfur compounds associated with coal (Colmer, Temple, and Hinkle, 1950; Leathen, Braley, and McIntyre, 1953). Sulfuric acid produced by thiobacilli causes a decrease of the pH down to 2.0 or even 0.7. Iron- and sulfur-oxidizing bacteria are present wherever mine water enters a stream. *Thiobacillus ferrooxidans*, involved in metal-leaching processes (Tuovinen and Kelly, 1972), can tolerate even pH 1.0, as well as ions of copper, cobalt, zinc, nickel, and iron up to extremely high concentrations (Torma, 1977). Organic acids are excreted in laboratory cultures (Schnaitman and Lundgren, 1965); their ecological significance is unknown. In acidic water, most Gram-positive, aerobic and anaerobic, heterotrophic bacteria die quickly, while yeasts and molds predominate (Marchlewitz and Schwartz, 1961). Among the heterotrophic bacteria only *Bacillus*, *Pseudomonas*, *Achromobacter* (Tuttle, Randles, and Dugan, 1968), *Flavobacterium acidurans* (Millar, 1973), and other slime-forming bacteria (Dugan, MacMillan, and Pfister, 1970) were encountered. Waters of acid hot springs are further habitats of acidophilic bacteria, such as thermophilic bacteria related to *Sulfolobus acidocaldarius* (Brierley and Brierley, 1973; Brock et al., 1971; De Rosa, Gambacorta, and Bu'lock, 1975) and, at lower temperatures, of mesophilic thiobacilli.

Many lakes, bogs, pine forests, and tea soils are slightly acidic, with pH values between 3 and 5.5. Bacterial life in these locations is scarce and, especially under anoxic conditions, degradation of plant polymers is slow. Nitrification proceeds slowly in acidic soils, and the question of whether nitrification in these areas is partly due to autotrophic nitrifying bacteria (Bhuiya and Walker, 1977) and to heterotrophs is still open (Focht and Verstraete, 1977). Acid bogs apparently offer the marginal conditions under which some rarely encountered bacteria, such as *Planctomyces*, *Bactoderma*, *Caulobacter*, and *Microcyclus* (Hirsch and Pankratz, 1970), as well as other stalked and prosthecate bacteria, can thrive (Henrici and Johnson, 1935; Schmidt, 1971).

Alkaline Environments The environment where human or animal urine undergoes a urea fermentation cannot be ignored because of the penetrating odor of ammonia. The causative agents of this noticeable process were first studied by Pasteur in 1862 and designated *Torule ammoniacale.* The ureolytic bacterium, *Bacillus pasteurii*, is an alkalophile that requires pH values of 8.5 or higher and high ammonia concentrations (Wiley and Stokes, 1963). *Sporosarcina ureae* grows at lower alkalinities (pH 8.5) (Mazanec, Kocur, and Martinec, 1965).

The isolation of alkaliphilic bacteria, able to grow in culture media of pH 10–11, is apparently rather easy. Such variants growing up to pH 11 were isolated after adapting *Bacillus circulans* (Chislett and Kushner, 1961). *Bacillus alcalophilus* growing at pH 8–10 was isolated from human feces (Vedder, 1934) and from dried sewage sludge (Boyer, Ingle, and Merver, 1973). Several *Bacillus* strains originated from projects on the production of alkali-tolerant enzymes and were isolated from indigo balls and plain soil (Ohta et al., 1975).

The Szik (salt and alkali) soils with pH values of 8–9 in the Hungarian lowlands have been extensively studied with regard to soil ecology and agriculture; however, their bacterial flora was not characterized in detail (Bokor, 1933). Alkali soils in India were reported to contain only few microorganisms, the total count decreasing drastically with increasing pH (Rupela and Tauro, 1973). With these exceptions, alkaline environments have not attracted much attention. Investigations of strictly alkaline springs in northern California led to the isolation of an anaerobic sporeformer and an aerobic, pigmented bacterium. The latter grew between pH 8 and 11.4 with the optimum within the range of pH 9–9.5 (Souza and Deal, 1977; Souza et al., 1974). The lakes of the Wadi el Natrun in Egypt are most interesting aquatic habitats, with pH values ranging from 9 to 11. Topographically and geochemically described by Schweinfurth and Lewin in 1898, these shallow salt pans harbor a rich population of halophilic and phototrophic microorganisms. Sodium is the major cation of the saturated brine (98%); the major anions are chloride, sulfate, and carbonate/bicarbonate (56, 26, and 17%, respectively; Jannasch, 1957). Species of the genus *Ectothiorhodospira* and other phototrophic bacteria have been isolated from these and other alkaline waters (Grant, Mills, and Schofield, 1979; Imhoff and Trüper, 1977). Alkaline lakes such as those in Ethiopia and Anatolia, with ambient values of pH 10–11, are also worth study.

Life at extreme pH values has been discussed by Langworthy (1978), Horikoshi and Akiba (1982), and Krulwich and Guffanti (1983).

Oxygen

When considering oxygen as an environmental factor, at first glance the anoxic environment appears to be the extreme one. However, if the peculiarities of generating biochemical energy

under anaerobic conditions are not considered, the anoxic environment is not difficult for life. Oxygen causes troubles for living cells. It plays a dual role: it acts as an effective electron acceptor, making energy conversion with high efficiency possible; however, oxygen can be considered toxic to life processes that depend on slow and thoroughly controlled oxidations. The toxic effect is intensified in light. Oxygen is reduced by univalent or single electron steps, and by the uptake of one electron, the superoxide anion radical (or simply the superoxide) is formed, which by further reactions gives rise to hydrogen peroxide and to the hydroxyl radical; this radical is the most reactive and, therefore, the most detrimental product. From the onset of photosynthetic oxygen production in the early atmosphere of this planet, it was the major task of biochemical evolution to develop an enzyme system for tetravalent reduction of oxygen, cytochrome oxidase, which does not release any toxic intermediates, and to develop elaborate detoxification systems (Fridovich, 1974, 1975, 1976). Although forms of life are still susceptible to oxygen toxicity (Gottlieb, 1971), there now exist a variety of defense mechanisms in different organisms (Hassan and Fridovich, 1979; Morris, 1975, 1976, 1978, 1979).

The rise of the oxygen level in the early atmosphere may have started not only the evolution of aerobic organisms, but also that of modestly oxygen-tolerant, anaerobic, fermentative bacteria as well. There is a wide spectrum of degrees of oxygen tolerance, starting with the strict anaerobes such as *Bacteroides*, *Butyrivibrio*, *Fusobacterium*, *Megasphaera*, *Peptococcus*, *Ruminococcus*, *Selenomonas*, *Succinivibrio*, *Methanobacterium*, *Methanococcus*, *Methanospirillum*, and *Methanosarcina* and continuing to the moderately oxygen-tolerant clostridia (*C. tetani*, *C sporogenes*) and the highly oxygen-tolerant clostridia (*C. perfringens*, *C. acetobutylicum*) and to the majority of the lactic acid bacteria, which can grow in the presence of air (Loesche, 1969; Morris and O'Brien, 1971; O'Brien and O', Morris, 1971; Uesugi and Yajima, 1978).

Although the obligately anaerobic bacteria have in common a hypersensitivity to oxygen, they are apparently not restricted to places like mud and intestinal tracts but can also be found in seemingly aerobic locations. The wide distribution of *Clostridium* can be explained by their possession of oxygen-insensitive spores. However, nonsporeforming, strictly anaerobic bacteria are similarly widely dispersed, perhaps because under field conditions the anaerobes are associated with oxygen-consuming bacteria that keep the local oxygen concentration low. These apparent discrepancies caution one not to overemphasize the results of pure culture studies when extrapolating to the behavior of microorganisms in their natural environment.

In some cases, the oxygen concentration is the deciding factor in the ability of the organism to develop potential metabolic activities. The oxygen concentration may determine whether various nitrogen-fixing bacteria can occupy a niche in an environment low in combined nitrogen. In contrast to members of the *Azotobacter* group, which fix nitrogen in the presence of air (Mulder and Brotonegoro, 1974), the nitrogen-fixing, hydrogen-oxidizing bacterium *Xanthobacter autotrophicus* (Berndt et al., 1976; Wiegel and Schlegel, 1976; Wiegel et al., 1978) is able to grow with molecular nitrogen as the sole nitrogen source only at oxygen concentrations below 2% oxygen (by volume), while in the presence of combined nitrogen the bacterium grows under air. Other nitrogen-fixing bacteria, such as *Azospirillum lipoferum* (Okon, Albrecht, and Burris, 1976), *Aquaspirillum fasciculus* (Strength et al., 1976), and *Methylosinus* species (De De Bont and Mulder, 1974), respond to oxygen in a similar manner (see also The Genus *Azospirillum* and The Genus *Herbaspirillum* in Volume 5). A number of aerobic marine bacteria have been demonstrated to be microaerophilic; they survive but do not grow in air-saturated seawater medium (Jannasch, 1977).

Whether there are differences among microorganisms with respect to their tolerance to oxygen at higher concentrations than that of air-saturated water is not known. Many strictly aerobic bacteria that form colonies from single cells on petri dishes exposed to air can tolerate gas mixtures up to 40% oxygen by volume but fail to grow at 50%. Also, 100% oxygen is usually considered to suppress growth; however, many cyanobacteria that form blooms are certainly tolerant to high oxygen concentrations. During photosynthesis in the sun, blooms of cyanobacteria and algal mats near the water surface, as well as the lawn of submerged water plants like *Elodea canadensis*, *Chara fragilis*, or *Ranuculus aquaticus* in ditches, shallow ponds, and estuaries, are covered with bubbles of oxygen. This growth indicates that the water body must contain higher concentrations of oxygen than in equilibrium with air and that the epiphytic and other bacteria are exposed to oxygen concentrations supersaturated with respect to oxygen. It is not known whether bacteria exist able to grow at these oxygen concentrations of almost 100%. Furthermore, it would be interesting to know whether among the variety of bacteria a continuum of thresholds of oxygen tolerance exists or whether there are certain discontinuities of threshold concentrations for certain species or metabolic types, e.g., at 2, 20, 40, and 100% oxygen and whether correlations between oxygen-tolerance thresholds and habitats are encountered.

Low-Nutrient Environments

The most commonly cited low-nutrient environments are desert soils, oligotrophic lakes, and, most prominently, the oceans. Considering suspended detrital matter, decaying plankton organisms, and fecal pellets, isolated high-nutrient habitats abound in various parts of the sea. However, if this amount is divided by the vast volume of the ocean, which amounts to about 90% of the biosphere, the dissolved organic carbon in sea water is rarely more than 1 mg/liter. Particulate organic carbon commonly amounts to 10–20% of the total organic carbon. A large fraction of the latter is "refractory," that is, unavailable for microbial attack (Barber, 1968; Menzel and Ryther, 1970). The description of low-nutrient habitats is, therefore, more realistically based on the flux of nutrients across the ecosystem rather than on the standing nutrient concentrations (Hirsch, 1979).

Many prokaryotes will survive in such conditions, as amply documented by the fact that poor soils and natural waters have often been used as a source for the isolation of microorganisms. Low-nutrient habitats may be extreme with respect to stress exerted toward some vegetative cells, but these conditions are hardly selective.

Vegetatively growing cells may be carried into the ocean from land run-off or be exposed to low nutrient levels after separation from relatively nutrient-rich particles. These cells may adopt one of a variety of strategies in order to cope with starvation conditions. Two general strategies have been pinpointed whereby: 1) the survival of the individual cell is achieved by the most efficient use of the limited amount of food available or 2) the survival of the species is achieved by producing the maximum amount of progeny in the form of dormant stages capable of immediate growth and multiplication at the renewed availability of nutrients.

In terms of population dynamics studied in continuous culture, the "K-strategists" have been described as organisms adapted to highly efficient growth or uptake at low substrate concentrations, as indicated by a low substrate saturation constant ($K[s]$). In contrast, μ_{max}-strategists (μ_{max} = maximum growth rate, in non-prokaryotes also called r-strategists) will outgrow the former in the presence of relatively high nutrient concentrations (Koch, 1979).

Early continuous culture studies on the synthesis and activity of prokaryotic enzyme systems led to the discovery of catabolic derepression by Gorini (1960) in the presence of extremely low concentrations of the growth-limiting substrate. Subsequently, the mechanisms of adaptation to low-nutrient environments have been intensively studied (Tempest and Neijssel, 1976, 1979). These studies resulted in the discovery of the high-affinity mechanism for assimilating ammonia into glutamate via glutamine synthetase and glutamate synthase (Tempest, Meers, and Brown, 1970, 1973) and of dual mechanisms for the assimilation of glycerol: the glycerol kinase route at carbon-limiting conditions and the glycerol dehydrogenase route in glycerol-sufficient environments (Neijssel et al., 1975). Matin (1979) reported the onset of a multiple substrate utilization technique as a response to decreasing dilution rates and pools of intracellular metabolites. Adaptation of distinctive species to low-nutrient environments is well illustrated by a comparison with respect to starvation and survival of a freshwater *Spirillum* sp., apparently belonging to the oligotrophic flora, with a *Pseudomonas* sp. adapted to environments richer in nutrients (Matin and Veldkamp, 1978). The spirillum accumulated poly-β-hydroxybutyric acid (PHB) during carbon-limited growth in the chemostat; the stored amount was highest at the lowest dilution rate examined. After growth at D = 0.03 to 0.05 h^{-1}, the spirillum was much more resistant to starvation than the pseudomonad that did not accumulate PHB (Matin et al., 1979). The survival value of storage materials and the regulation of their synthesis in various types of bacteria may assist the understanding of adaptation to low-nutrient environments.

One of the most important characteristics of low-nutrient habitats is the strong influence of solid surfaces in the colonizing of microbial films and layers (Marshall, 1976). In a summary of work done on the effect of inert particulate material suspended in low-nutrient aquatic habitats, Jannasch and Pritchard (1972) included studies using the chemostat for the enrichment of bacterial strains that exhibit a tendency for specific attachment mechanisms. In Caulobacteriales, typical low-nutrient organisms, Poindexter (1979) postulates a morphological-physiological mechanism that regulates an efficient metabolic response to low-nutrient conditions, including the role of holdfast organelles (see also Dimorphic Prosthecate Bacteria in Volume 5).

Life under conditions of low-nutrient concentrations was a topic of a Dahlem Konferenz (Jannasch, 1979; Koch, 1979; Poindexter, 1979; Rittenberg, 1979) and an international symposium on microbial ecology (Hattori et al., 1989).

Resting Stages and Survival

In order to survive under adverse environmental conditions, a few bacterial groups are able to form resting cells, which are more resistant to deleterious environmental conditions than their vegetative cells. The transformation occurs when the metabolic activities decline because of nutrient depletion or transfer to a growth-limiting environment. The resting cells are characterized

by thick, frequently multilayered walls. Typical is endospore formation by the genera *Bacillus* and *Clostridium*. Many endospores are extremely resistant to heat, desiccation, radiation, and chemical agents. The formation of exospores is, as far as is known, restricted to the methane-utilizing bacterium, *Methylosinus trichosporium*; the thermoresistant exospores are formed by a process similar to budding of vegetative cells (Whittenbury, Davies, and Davey, 1970).

The rod-shaped cells of the genus *Azotobacter* frequently turn into spherically shaped cells called cysts. Unlike endospores, the vegetative rod as a whole is transformed into a cyst. These cysts share with endospores structural rigidity and resistance to desiccation and to ultraviolet radiation; however, they lack heat resistance (Sadoff, 1975). Similarly, myxobacteria (*Myxococcus*) turn into spherical cells called myxospores (Sadoff, 1973; Voelz and Dworkin, 1962). In the genus *Methylocystis*, the rodlike cells round up and turn into desiccation-resistant cysts similar to those of *Azotobacter* species. *Methylomonas* and *Methylococcus* form spherical cells that are neither thick walled nor resistant to desiccation (Whittenbury, Davies, and Davey, 1970). A modest resistance to desiccation is shown by *Arthrobacter* coccoid cells (Boylen, 1973; Ensign and Wolfe, 1964; Veldkamp, van den Berg, and Zevenhuizen, 1963).

Bacteria that are not able to transform into resting stages are less apt to survive under adverse environmental conditions. Their resistance, however, varies from species to species; it may be high as in mycobacteria or arthrobacters, which survive desiccation for several weeks, or low as in *Neisseria gonorrhoea* or *Treponema pallida*, which respond to desiccation by immediate death. Furthermore, maintenance of the viability of vegetative cells under a mild environmental stress is a function of a multitude of factors, such as growth conditions, content of storage materials, speed of transition from growth to resting conditions, and many others (Strange, 1976). Many observations on the effect of drying and rehydration of vegetative bacteria have been made during long-term preservation in culture collections (Bousfield and MacKenzie, 1976; Lapage et al., 1970; Martin, 1964; Miller and Simons, 1962). These observations indicate that the survival of bacteria depends on many factors and may vary among species and environments. Many aspects were reviewed by Kjelleberg et al. (1987) and Matin et al. (1989).

Light as an Extreme Environment

The well-known predominance of pigmented colonies on nutrient-agar plates exposed for some time to air, compared to plates inoculated with soil, indicates the selective action of sunlight and air and the survival value of pigments. Yellow, orange, and red pigments point to members of the genera *Micrococcus*, *Corynebacterium*, *Mycobacterium*, and *Nocardia*, which contain carotenoids. In nonphototrophic bacteria there is a good negative correlation between the presence of carotenoids in the cell and the anaerobic way of life; carotenoids are rarely found in strict anaerobes except the phototrophic bacteria. Selective effects similar to those observed in air are exerted in several sunlight-exposed environments, such as leaf surfaces, fresh waters, and salt brine. The radiation resistance of pigmented bacteria has been studied with *Halobacterium salinarium* (Dundas and Larsen, 1962), *Rhodospirillum rubrum*, *Corynebacterium poinsettiae* (Cohen-Bazire and Stanier, 1958), and other microorganisms.

Resistance to radiation also seems to be positively correlated with a high GC content of the cellular DNA; this correlation agrees with the conclusion that inactivation of cells by ultraviolet light is mainly due to thymine dimerization (Singer and Ames, 1970). The hypothesis was in accordance with the distribution of GC contents of the purple and green bacteria, the majority of which possess DNAs with 60–70 mol% GC (Mandel et al., 1971). However, in contrast to the anoxygenic phototrophic bacteria, the cyanobacteria show a markedly different mean DNA base composition, the majority of species having only 40–50 mol% GC (Herdman et al., 1979). Cyanobacteria obviously need solar radiation and are more exposed to ultraviolet light than most bacteria. One would have expected them to possess a high GC content, if the hypothesis of Singer and Ames holds true. Solar radiation should, therefore, not be considered as the selective factor that led to the great divergence of DNA base composition among prokaryotes. The effect of high irradiation, mainly of ultraviolet-light damage to DNA and its repair, has been recently reviewed by Nasim and James (1978).

The bactericidal action of diffuse sunlight has not gained much attention. The growth-retarding and lethal action of wavelengths of light not absorbed by DNA has been shown for *Serratia marcescens* (Swart-Füchtbauer and Rippel-Baldes, 1951), *Nitrobacter winogradskyi* (Müller-Neuglück and Engel, 1961), and *Nitrosomonas europaea* (Schön and Engel, 1962). The inhibitory effect is apparently most pronounced and therefore easily recognizable with slowly growing bacteria. Further studies were reviewed by Jagger (1983) and Cornax et al. (1990).

Light exerts its killing effect on microorganisms primarily in the presence of oxygen; in its absence, the effect is smaller by several orders of magnitude. The killing effect is apparently due to photooxidation reactions that are mediated by singlet oxygen. This highly toxic oxygen type is

produced by photosensitized activation of triplet oxygen. Protection against single oxygen is readily provided by carotenoids that possess more than seven conjugated double bonds. These carotenoids are able to quench singlet oxygen as well as excited photosensitizers very effectively. Experimental results and a literature review "on the mechanisms for coping with the stress of photosensitized oxidations" have been presented by Krinsky (1979).

The information on the tolerance to intense solar radiation is still scarce. The generalizations on the protective effect of carotenoids just outlined are mainly based on studies of anaerobic, phototrophic bacteria. Among the aerobic bacteria only those containing carotenoids were considered. There are many more bacteria characterized by remarkable pigments different from carotenoids, such as pyocyanine *Pseudomonas aeruginosa*), prodigiosin (*Serratia marcescens*) and related pigments (Gerber, 1975), indochromes, violaceine, xanthomonadines, and others. It would be interesting to know the ecological significance of these pigments in the natural habitats of the bacteria containing them.

The sensitivity of bacteria and protozoa to visible light and oxygen can be increased by the addition to the medium of sensitizing pigments such as methylene blue, toluidine blue, eosin, or acridine orange. Although this photodynamic effect has long been known and is advantageously applied on animal farms to keep the microbial number in drinking water low (Acher and Juven, 1977), the ecological significance of photosensitization by natural pigments liberated by autolysis of part of the population is far from clear. Carotenoid pigments also exert a protective effect against photosensitized killing, as has been shown for *Micrococcus luteus* (Anwar et al., 1977; Matthews and Sistrom, 1959; Prebble and Huda, 1977).

Surfaces as Habitats

The microbiologist dealing with homogeneous suspensions of bacteria for physiological studies usually pays little attention to the growth of bacteria on surfaces. In nature, however, the growth of bacteria as a surface film may be more frequent and important than growth in homogeneous suspensions (Marshall, 1976, 1984; Fletcher and Marshall, 1982) (see also Planktonic Versus Sessile Life of Prokaryotes in Volume 2). Three kinds of interfaces have to be considered: 1) liquid-solid interfaces, such as stationary objects in a river, the tidal zone, and ponds; the inner surfaces of the mouth and the intestinal tract; the outer surfaces of rocks piled up for bacterial leaching of ore or of rocks stacked in columns for waste-water purification in trickling filters; food particles in the digestive system; and silt particles in rivers or marine environments; 2) liquid-gas interfaces, such as the water surface of a pond and gas bubbles in the ocean; and 3) liquid-liquid interfaces, such as oil droplets in an aqueous solution.

Liquid-Solid Interfaces

Adhesion to surfaces is an important ecological determinant in many ecosystems. The colonization of a suitable liquid-solid interface may be the prerequisite for exploitation of a habitat. Many organisms that inhabit rushing rivers stick to solid surfaces. In open ecosystems, sessile animals as well as bacteria take advantage of being fixed to a privileged location. In a running river that receives organic waste from upstream, slow-growing bacteria could not compete with fast-growing bacteria if they were not attached to stationary objects at a location where food is still available in concentrations sufficient for growth. Growth in locations of this kind is restricted to those bacteria that can attach themselves to a solid surface. Thick plaits of *Sphaerotilus natans* were found in rivers where waste water of sugar factories was discharged (Cohn, 1881; Demoll and Liebmann, 1952; Dondero, 1961, 1975; Kolkwitz, 1904–1906). A similar habitat for mass development is held by *Leptothrix ochracea* in field drains (Dondero, 1975), by *Crenothrix polyspora* in water wells (Völker, Schweisfurth, and Hirsch, 1977; Wolfe, 1960), and by *Toxothrix trichogenes* in iron springs (Krul, Hirsch, and Staley, 1970; van Veen, Mulder, and Deinema, 1978; see The Genera *Leptothrix* and *Sphaerotilus* in Volume 5). The trickling filters of waste-disposal plants resemble the river habitat. In some of these habitats, *Sphaerotilus*, *Caulobacter*, *Asticcacaulis*, *Zoogloea ramigera*, and other stalked, prosthecate, or slime-forming bacteria have been found in addition. In many of these habitats, the bacteria form thick, slimy layers on rocks and pebbles.

In the marine environment, *Leucothrix mucor* holds a similar position to *Sphaerotilus* in fresh water. *L. mucor* forms sessile filaments and grows as a bacterial epiphyte on seaweed, such as filamentous red and green algae living in habitats where water flows and aeration is good (Kelly and Brock, 1969; Raj, 1977). The filament is attached to the seaweed by a holdfast (Harold and Stanier, 1955) that is part of the sheath (Pringsheim, 1957). By scanning electron microscopy (Fig. 1), bacterial lawns were revealed that consisted of long and short end-attached bacteria, including *Leucothrix mucor* and flexibacteria (Cundell, Sleeter, and Mitchell, 1977). *Thiothrix nivea* was observed to grow in tufts attached to

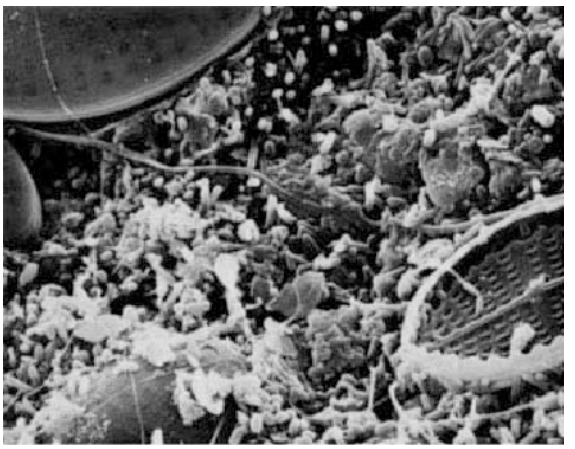


Fig. 1. Microflora on the surface of the brown alga *Ascophyllum nodosum.* Scanning electron micrograph. (Courtesy of Ralph Mitchell.)

pebbles in the runoff of a sulfur spring in Seattle, Washington (Bland and Staley, 1978).

In rivers and sea water, small, floating particles such as silt, clay, or detritus particles have a remarkable growth-promoting effect on microorganisms. At low nutrient concentrations, which are suboptimal for efficient growth in homogeneous suspension, the addition of suspended particles favors the growth of bacteria (Heukelekian and Heller, 1940) due to adsorption of nutrients to the particle surface. The attachment of bacteria to dense suspensions of silt particles, as is characteristic for certain river waters, has a marked effect on growth and oxygen uptake (Jannasch, 1955). The degree of attachment was affected by the concentration of the dissolved organic substrate (Jannasch, 1958; Jannasch and Pritchard, 1972). Dense growth of bacteria on suspended particles may lead to anoxic microenvironments, as demonstrated by model experiments on denitrification (Jannasch, 1960, 1978).

The ability to attach to surfaces may be considered as a means to escape low-nutrient environments. Interfaces play an eminent role in the transport and accumulation of nutrients, such as polysaccharides or proteins. Not only do these nutrients accumulate at solid-liquid interfaces, but they even accumulate at liquid-gas interfaces, and gas bubbles serve as vectors for nutrients (Marshall, 1979).

Bacteria are able to stick to solid surfaces in several ways. The outer layers of the bacterial cell envelope (Costerton, Ingram, and Cheng, 1974) evidently play a special role. The cells are either attached by a sheath as in the case of *Sphaerotilus*, *Leptothrix*, and *Leucothrix*; by holdfast substances, which are apparently a gum-like outer layer of the cell envelope and consist of polymers (Umbreit and Pate, 1978); or by nonflagellar, filamentous appendages such as fimbriae or pili. The attachment of bacteria to solid surfaces by polymers is apparently widely distributed.

As mentioned above, at high flow rates of a nutrient medium, sessile bacteria are able to exploit the nutritional opportunities of the habitat better than nonattached forms. Cells that do not stick to the surfaces of stationary objects are washed out into another ecosystem; the nutrient exchange is not facilitated by the movement of the medium past the cell. The ability to adhere to surfaces confers a selective advantage on certain metabolic types. Studies on the fine structure of extracellular polysaccharide fibers (Cagle, 1975) gave rise to a generalized view of the ability of bacterial cells to adhere to surfaces. The fiber network that extends from the bacterial surface, called bacterial "glycocalyx," mediates adhesion not only to abiotic surfaces, but also to other cells, either host or prey.

Adhesion is a way for a bacterium to enter a habitat. The adhesion of the causative agent of caries, *Streptococcus mutans*, to the enamel surface of the tooth, the adhesion of *Vibrio cholerae* to intestinal cells, and other bacterium-host relationships are examples for the potential role of glycocalyx formation in the establishment of infectious diseases (Costerton, Geesey, and Cheng, 1978; Costerton et al., 1987). Several unidentified, segmented filamentous bacteria attached to the epithelium of the mucosa of the small bowels of mice have been revealed by scanning electron microscopy (Blumershine and Savage, 1978).

For utilization of some solid substrates, a close contact is necessary between the bacterium and its substrate. *Cytophaga* cells adhere closely to cellulose fibers, the rods being aligned with the orientation of the microfibrils. Cellulose degradation by *Cytophaga* and *Sporocytophaga* requires direct contact between the bacteria and the cellulose fibers (Berg, van Hofsten and Pettersson, 1972; Stanier, 1942). The attachment of rumen bacteria to plant particles (Akin, 1976; Akin and Amos, 1975) is well documented. *Ruminococcus* species were shown to adhere strongly to cotton cellulose fibers and to the cell walls of leaf sections of rye grass, evidently by means of their prominent glycoprotein coats (Latham et al., 1978; Minato and Suto, 1978; Patterson et al., 1975). Further examples concern the adherence of starch-digesting bacteria to starch grains, of chitin-hydrolyzing bacteria to chitin, of *Sulfolobus* to sulfur globules (Weiss, 1973), and of a thermophilic sulfur oxidizer to sulfide minerals (Golovacheva, 1979). Knowledge about the specific mechanisms of attachment of bacteria to solid substrates suggests methods for their enrichment.

Specific interactions between the bacterial cell coat and the plant cell wall apparently precede the invasion of rhizobia into the root hairs of their host plants. Phytohemagglutinins or lectins (Liener, 1976) of the plant may be involved in the recognition process by binding only the corresponding bacteria and not bacteria that infect other legumes (Dazzo, Yanke, and Brill, 1978; Kato, Maruyama, and Nakamura, 1979; Marx, 1977).

Fimbriation is apparently another means of bacterial attachment to submerged objects (Hirsch and Pankratz, 1970). The possession of filamentous, nonflagellar appendages seems to be distributed mainly among members of the Enterobacteriaceae and Pseudomonadaceae but is not restricted to members of these families (Ottow, 1975). The ecological importance of fimbriae or pili may consist in their initiating both attachment to solid surfaces as well as contact with other members of the community. The solid surface may be a substrate of low solubility, such as sulfur oxidized by *Sulfolobus* (Brierley, C.L., 1978; Weiss, 1973) or *Thiobacillus* A2 (Korhonen, Nurmiaho, and Tuovinen, 1978). Attachment to surfaces has not been studied as well as the phenomenon called star formation. The formation of stars, rosettes, or cellular aggregates was originally observed when strains of *Agrobacterium tumefaciens* and *Rhizobium* were studied (Stapp and Bortels, 1931; Stapp and Knösel, 1954). Since both *Agrobacterium tumefaciens* and rhizobia normally invade plant tissues and must attach to the surface of the plant beforehand, the connection between attachment, important in the natural habitat, and the formation of aggregates as observed in slide cultures under the microscope is obvious. The formation of the frequently very regular rosette-like aggregates in pure cultures of bacteria, such as *Pseudomonas "rhodos"* (Marx and Heumann, 1962), *Pseudomonas "echinoides"* (Heumann and Marx, 1964; Mayer, 1971; Mayer and Schmitt, 1971), and *Agrobacterium "luteum"* (Ahrens, Moll, and Rheinheimer, 1968) may be regarded as a consequence of the presence of polar fimbriae and slime in these bacteria. Similar aggregates are formed on the surface of liquid media (Ahrens, Moll, and Rheinheimer, 1968).

Aggregation may be either specific, as in the cases of bacterial conjugation, host cell infection, and growth at special surfaces, or may be due to a rather nonspecific process (Fletcher and Loeb, 1979), when extracellular polymers such as complex polysaccharides and polyamino acids are excreted or are exposed at the cellular surface under varying physiological conditions, especially during the stationary growth and the death phases and at low-nutrient concentrations. The formation of microbial aggregates is of considerable importance in the soil, in floc formation, in activated sludge treatment, and in industrial biomass production (Harris and Mitchell, 1973).

Microbial adhesion has recently grown into a self-contained field of research (see Planktonic Versus Sessile Life of Prokaryotes in Volume 2). The phenomenon is studied on the physiological (Costerton et al., 1981, 1985; Berkeley et al., 1980), biochemical (Kefford et al., 1982), molecular (Switalski et al., 1989), and biotechnological level (Savage and Fletcher, 1985; Characklis and Marshall, 1990).

Liquid-Gas Interfaces

The liquid-gas interface is a unique habitat in many respects. Depending on the ecosystem, this environment harbors a diverse bacterial flora, sometimes called the bacterial neuston. Common to the liquid-air interfaces are: 1) direct exposure to the oxygen of the air; 2) accumulation of hydrophobic substances either originating from the air or from the water body; and 3) high light intensities.

The surface film of lakes and ponds formed in summer during calm weather harbors bacteria such as *Pseudomonas*, *Caulobacter*, *Hyphomicrobium*, *Nevskia*, *Flavobacterium*, *Alcaligenes*, *Micrococcus*, and others (Babenzien, 1965, 1967; Hirsch, 1974; Hirsch and Pankratz, 1970). Bacteria that contain gas vacuoles may dominate under certain conditions (Walsby, 1978).

The surface layer of the ocean is a separate environment (Zaitsev, 1971). It has gained special attention because oil pollution has increased and wide areas of the ocean are occasionally covered with petroleum (Bartha and Atlas, 1977). A considerable proportion of organic substances accumulating in the marine neuston is released from planktonic organisms; fatty acids, lipids, polysaccharides, hydrocarbons, and proteins have been identified in the surface film (Wangersky, 1976). The marine neuston is the habitat of various marine bacteria (Bezdek and Carlucci, 1972).

The surface film of water baths in laboratories offers a special habitat for bacteria that either utilize traces of conventional substrates inadvertently added to the water or that utilize volatile or gaseous substrates present in laboratory air (Leifson, 1962). The gum formed within several weeks contains *Bacteriogloea*, *Hypomicrobium*, *Caulobacter*, *Mycobacterium*, and *Norcardia*.

Gradients as Habitats

In nature, the distribution of nutrients or environmental factors is generally patchy. In many ecosystems vertical gradients of concentrations

of various nutrients exist. The nutrients produced in sediments diffuse into the aqueous layer and, in the absence of mixing by convection or currents, form a concentration gradient. Such gradients are to be expected either in a microscale in sediments, soil, or shallow water bodies or in a macroscale in lakes or oceans. The gradients may either be of substances used as nutrients or of physical factors such as redox potential, temperature, and radiation. Of primary importance for growth and distribution of microorganisms are the concentration gradients of organic acids, hydrogen sulfide, carbon dioxide, and oxygen.

The major amount of organic matter that is decomposed by microorganisms in nature consists of fresh plant litter. Another fraction enters the soil or sediments of aquatic ecosystems after partial degradation by herbivorous animals. Only a comparatively small fraction of the plant tissue is degraded by animals; the major part, cellulose and lignin, is released and becomes the major substrate of the microbial food chain.

Terrestrial and aquatic ecosystems differ greatly with respect to further degradation of the primary biomass and the cellulose-rich detritus derived therefrom. On the land, the solid organic matter stays on or near the surface of the soil and is decomposed mainly aerobically. Only part of the organic matter is transported into the soil by animals as vectors and is degraded by microorganisms either aerobically or anaerobically, depending on aeration and moisture content. Undoubtedly, diffusion processes and concentration gradients play a role in the soil. However, due to the heterogeneous distribution of organic matter, the basic features of anaerobic and aerobic food chains cannot easily be recognized in terrestrial systems.

In contrast, in a stagnant body of water the solid organic matter produced in the surface layers sinks, either immediately or after passage through a short, animal food chain, to the bottom of the water body. There microbial degradation occurs. Due to its low solubility in water, the oxygen may soon be exhausted in the course of the initial aerobic degradation of organic substances. In highly productive waters and in the absence of convection, the deeper layers become anoxic, which results in anaerobic microbial degradation processes. These processes give rise to the production of various soluble or gaseous substances such as organic acids, hydrogen sulfide, hydrogen, methane, carbon dioxide, and ammonia. These products freely diffuse upwards, each forming its own concentration gradient.

The chemical conditions may be further diversified by the liberation of ions from the sediment. These upward gradients are overlayerd by the downward gradients of temperature, light, and oxygen. Thus, due to the gradients of various nutrients and other environmental factors, a stagnant water body offers a variety of diverse living conditions for microorganisms. The study of concentration profiles and of the zonation of aquatic ecosystems and the assignment of the microorganisms to the particular conditions of their habitat are a major concern of microbial ecology. Several lakes have been studied as model systems (Clark and Walsby, 1978b; Gorlenko, Dubinina, and Kuznezow, 1977; Kuznezow, 1959, 1977). The stratified lake will be presented here as an example. The principles derived from these studies can be easily applied to zonations and processes occurring in waterlogged soils, tundras, and rice fields. They apply with slight variation also in shallow ponds, sediments, and shallow mats in the runoffs of hot sulfur springs.

Gradients of a Macroscale: The Stratified Lake

Many lakes are temporarily or permanently stratified (Figs. 2 and 3). Stratification occurs when a dense, either cold or saline (salt-rich) water body is overlayered by a less dense, either warm or salt-free water body. The stratification patterns vary with different climate zones. In temperate climates, normal stratification occurs in freshwater lakes more than 10 m in depth.

The holomictic stratified lake may serve as an example of the stratification and gradient-forming process (Overbeck, 1972; Pfennig, 1979; Sorokin, 1970). After total circulation in winter time, the warming of the surface layers during the summer yields a less dense upper layer (epilimnion) of circulating, aerated water and a dense, cold, stagnant bottom layer (hypolimnion). The intermediate layer (metalimnion) is characterized by a temperature gradient (the thermocline) and, after oxygen deprivation and anaerobic decomposition of organic matter in the hypolimnion, by a chemical gradient (chemocline) also. In the epilimnion, which is exposed to sunlight, biomass is produced by phototrophic cyanobacteria, diatoms, and green algae; allochthonous matter from the surroundings usually adds to the total organic material. Part of the biomass usually sinks to the hypolimnion and to the bottom of the lake where it is degraded. Degradation is accompanied by oxygen consumption, resulting in a decrease in oxygen concentration and, finally, in anoxic conditions, first in the sediment, then in the bottom layer. Continued anaerobic degradation results in the production of organic fermentation products as well as hydrogen, methane, hydrogen sulfide, and carbon dioxide, which diffuse upwards forming concentration gradients. Methane,

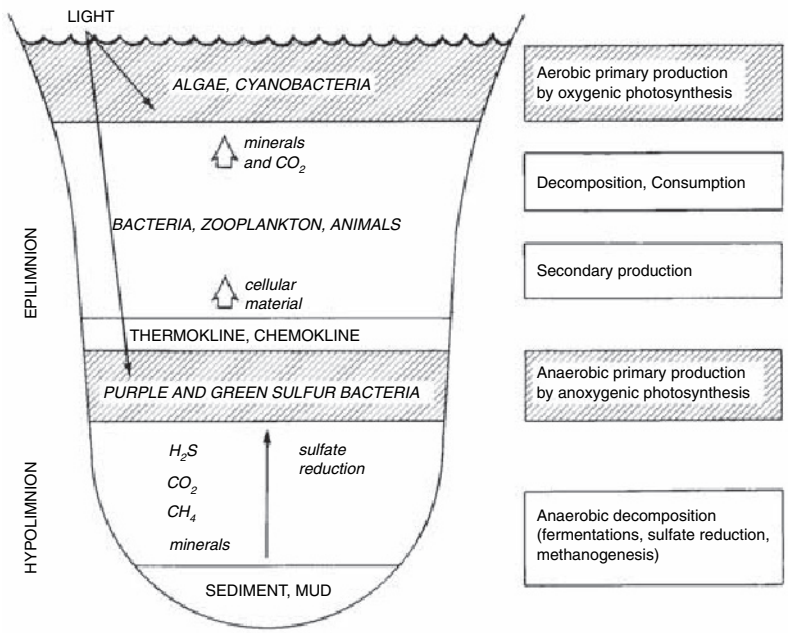


Fig. 2. Diagram of production, consumption, and decomposition in an aquatic ecosystem with a chemocline.

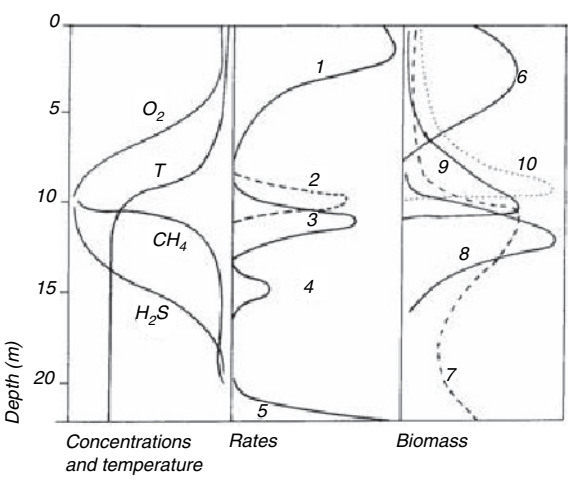


Fig. 3. Idealized vertical profile of a freshwater lake in the temperate climate zone in summer, showing concentrations, conversion rates, and biomass. The figure is based on drawings and data of Sorokin (1970), Gorlenko, Dubinina, and Kuznezow (1977), and Overbeck (1972). Symbols: T, temperature; (1–5, conversion rates) 1, CO_2 fixation in the light (oxygenic photosynthesis); 2, CO_2 fixation in the dark; 3, CO_2 fixation in the light (anoxygenic photosynthesis); 4 and 5, sulfate reduction; 6, biomass of algae and cyanobacteria; 7, total bacterial biomass; 8, biomass of phototrophic bacteria; 9, biomass of protozoa; 10, biomass of Copepoda and Cladocera.

which is the major carbonaceous substance released from the sediment, is discharged in the form of gas bubbles. Part of the methane is dissolved in the water as it moves upwards and is oxidized by methane-utilizing aerobic bacteria. The quick removal of oxygen from the hypolimnion is primarily due to the quick dispersal of methane and the growth of methane-oxidizing bacteria. Finally, the hypolimnion becomes totally anoxic.

As soon as anaerobic conditions have been established in the bottom layer or in the total hypolimnion, the anaerobic conversion processes, which started with the degradation of solid material, mainly cellulose, in the sediment, continue in the water body. Primary soluble fermentation products, hydrogen gas included, are used to reduce sulfate to hydrogen sulfide. The major amount of hydrogen sulfide originates from sulfate reduction in the free water. Simultaneously, nitrate is reduced to nitrite, which transiently accumulates and forms a distinct layer, and then to nitrogen.

The upper part of the epilimnion is the zone of primary production by the oxygenic phototrophic organisms, such as photosynthetic higher plants, algae, diatoms, flagellates, green algae, and cyanobacteria. These primary producers are accompanied by bacteria, protozoa, and metazoa that consume photosynthetized biomass, which results in secondary production and release of cellulosic detritus (Fenchel and Jøorgensen, 1977). The thermally stratified lake is a very common habitat for cyanobacteria that contain gas vacuoles. *Oscillatoria rubescens*, *O. agardhii*, *Aphanizomenon flosaquae*, and *Microcystis aeruginosa* form stable populations near the bottom of the epilimnion (Clark and Walsby, 1978a,b).

The chemocline and the hypolimnion are the favored habitats of the anaerobic prokaryotes. Provided hydrogen sulfide is present and the light intensity allows, anoxygenic phototrophic

bacteria develop underneath the chemicline and form a second layer of primary biomass production (Sorokin, 1970). The upper hypolimnion just below the chemocline is the habitat of the purple and green sulfur bacteria, among which the brown forms dominate (Fig. 2). They produce biomass by anoxygenic photosynthesis from carbon dioxide and hydrogen sulfide. Bacteria that are either buoyant by gas vacuoles or motile by flagellation dominate. The buoyancy of *Lamprocystis*, *Amoebobacter*, *Thiodictyon*, *Thiopedia*, *Pelodictyon*, and *Ancalochloris* is apparently just sufficient to allow them to float in the heavy cold water beneath but insufficient to float in the light warm water above the thermocline. Among the motile purple sulfur bacteria (Chromatiaceae), the large *Chromatium* species (*C. okenii*, *C. weissei*, *C. warmingii*, *C. buderi*) and *Thiospirillum* live in this layer.

Further details on the ecology of photosynthetic bacteria with emphasis on the distribution in stratified lakes were presented in a comprehensive review (Pfennig, 1979). The efficiency of biomass production by anoxygenic photosynthesis is remarkable. Part of the biomass that by slight turbulences reaches the upper chemocline is grazed by copepods, cladocera, and protozoa and transported into upper aerobic regions. Photosynthesis by the purple and green sulfur bacteria is accompanied by sulfate production. The sulfate produced is immediately reduced to hydrogen sulfide. As documented by measurements of the sulfate-reducing activity and cell counts of sulfate-reducing bacteria in various depths, the activity of sulfate reduction has two maxima: one in the hypolimnion bottom layers and one below the chemocline (Sorokin, 1970). While the reduction power for the first process originates from the sediment, sulfate reduction below the chemocline seems to be supplied by indigenous solutes released from cells within the region, either by excretion or by decay.

The metalimnion is a layer of high biological activity. Because of its richness in inorganic nutrients as compared to the epilimnion, it is inhabited by a few aerobic photosynthetic prokaryotes that can tolerate hydrogen sulfide and anaerobic conditions. One of them is *Oscillatoria limnetica*, which has been isolated from the sulfide-rich layers of Solar Lake (Elat, Israel) and studied in detail (Cohen, Padan, and Shilo, 1975; Padan, 1979a,b). It is able to use hydrogen sulfide as an electron donor in a photosystem I-driven reaction and to photoreduce carbon dioxide; hydrogen sulfide is oxidized to elemental sulfur. Once the system has been induced, *O. limnetica* can grow under anaerobic conditions (Oren and Padan, 1978; Oren and Shilo, 1979). After they return to the oxic zones, the cells switch from anoxygenic to oxygenic photosynthesis. This study of *O. limnetica* explained the predominance of cyanobacteria in sulfide-containing microaerophilic habitats or in habitats of frequently alternating conditions (Padan, 1979).

Because of the oxidation of hydrogen sulfide by the purple sulfur bacteria in the light and diffusion of oxygen from the epilimnion, the chemocline moves downward several meters during the daytime. Diurnal vertical fluctuations of the redox discontinuity layer obviously represent a major selective factor for the organisms occupying this habitat.

Gradients of a Microscale: The Sediments

Among the various types of sediments, only the coastal marine sediment and the lacustrine sediment will be discussed here. In principle, the degradative and biosynthetic processes that occur in sediments are similar to those in stratified lakes. The organic material that supplies the energy for life processes in the sediment is mainly allochthonous. Either leaves or large algae are buried in the sand of coastal marine areas or detritus is incorporated into the mud.

If sulfate is present in excess, as in marine ecosystems, the anaerobic food chain yields mainly hydrogen sulfide and gives rise to the activities of bacteria involved in the sulfur cycle. Habitats of this sort gained early attention by the smell of hydrogen sulfide and the bright red layers and bloom of purple sulfur bacteria and have repeatedly been described (see Bavendamm, 1924). There are numerous kinds of sulfureta, most of them estuaries, limans, salt marshes, tidal flats, and swamps.

In typical sulfureta of littoral marine areas, buried organic material is covered by sand and the water layer is 10–20 cm deep (Fenchel, 1969). Hydrogen sulfide production may be high. The sulfide concentration in a few centimeters' depth of these sulfide systems may easily reach 10 mM H_2S (Fenchel and Riedl, 1970). The diffusing hydrogen sulfide may give rise to emission to the atmosphere during the night. During the day the emission of hydrogen sulfide amounts to only 4% of that during the night (Hansen, Ingvorsen and Jørgensen, 1978). The diurnal fluctuations of the hydrogen sulfide concentration indicate the significant role of the purple sulfur bacteria in the photic zones of these coastal areas. The bacteria respond to the exhaustion of sulfide and the alternation of the oxygen tension by chemotactic (aerotactic) movements and disappear in the course of illumination into the sediments. The movements parallel the diurnal vertical migrations observed in stratified lakes. Depending on the particular environmental condition, the ecological niche of sulfide oxidation may be occupied by colorless sulfur bacteria such as

Thiovulum, *Macromonas*, *Achromatium*, and *Beggiatoa*, or by phototrophic purple sulfur bacteria.

A characteristic ecosystem, with a very steep gradient from the anoxic hydrogen sulfide-rich zone to the oxic layer, has been described as the "Farbstreifen-Sandwatt" (Hoffmann, 1942); the name was coined due to the succession of green, red, and black layers of the sand of marine coastal areas. The phenomenon is restricted to localities with a high level of ground water, sand of grain diameter to provide capillary ascension almost up to the sand surface, sufficient organic material and sulfate in the lower sediment layers, and high light intensities to penetrate the uppermost layers of sand. The green layer contains cyanobacteria exclusively, demonstrating their tolerance to hydrogen sulfide compared to green algae as emphasized by Padan (1979). The red layer underneath is an almost monospecific culture of purple sulfur bacteria, which is in direct contact with the black zone. These observations confirm the ecological significance of the complementary spectral absorption of green plants and purple bacteria (Buder, 1919) and are in accordance with measurements showing that long wavelengths penetrate in sand further than short wavelengths (Fenchel and Staarup, 1971), in contrast to water (Pfennig, 1967).

Multilayered microbial communities in aquatic ecosystems resembling the "Farbstreifen-Sandwatt" have been observed in areas of hot springs and in solar ponds. Various kinds of mats have been described and studied in detail (Cohen and Rosenberg, 1989).

A particular environment is apparently required by the sulfide-oxidizing, nonphotosynthetic bacteria such as *Beggiatoa*, *Thiothrix*, *Achromatium*, *Macromonas*, *Thiovulum*, *Thiospira*, and a few others. Their habitats appear to be areas where the gradients of both hydrogen sulfide and oxygen are overlapping or, as in running waters, where the hydrogen sulfide-providing sediment is covered by running water containing oxygen.

Due to the instability of hydrogen sulfide in the presence of oxygen, a habitat requiring the simultaneous presence of both compounds will be small and transient. The coexistence of hydrogen sulfide and oxygen has already been convincingly demonstrated by Beijerinck (1895). He introduced a solution of hydrogen sulfide containing a small amount of oxygen into an anaerobic suspension of luminous bacteria—precautions to exclude the access of air were taken. Luminescence occurred, indicating that the hydrogen sulfide solution contained free oxygen and that the affinity of the bacteria for oxygen is high. Hydrogen sulfide and oxygen will be simultaneously available for a long period of time only in areas to which both compounds are continuously supplied. Observations in nature and when attempting to grow isolates of colorless sulfur-oxidizing bacteria are in agreement with this idea (Bland and Staley, 1978; la Rivière, 1963, 1965; Strohl and Larkin, 1979; The Colorless Sulfur Baeteria in Volume 2 and The Genus *Beggiatoa* and *Thioploca* in Volume 6). In a study on the chemolithotrophic nature of *Thiovulum* species (Wirsen and Jannasch, 1978), the difficulty of obtaining pure cultures is ascribed to the instability of the oxygen/hydrogen sulfide environment. The individual cells of *Thiovulum* aggregate in "veils" by extruding slime threads, a possible means of stabilizing an oxygen/hydrogen sulfide interface for a period of time (la Rivière, 1963).

Careful studies using microelectrodes for the measurement of oxygen and hydrogen sulfide have been carried out on *Beggiatoa* populations at natural oxic/anoxic interfaces of estuarine sediments (Jørgensen, 1982; Jørgensen and Revsbech, 1983; see also Chapter 166). In *Beggiatoa* "plates" prepared in artificial agar-gelled interfaces or O_2/H_2S gradient cultures, chemoautotrophy was demonstrated (Nelson and Jannasch, 1983), and the growth pattern and yield were studied in detail (Nelson et al., 1986). The thickness of these filament plates never reached more than a few mm.

The unusual occurrence of thick mats of *Beggiatoa*-like filaments was observed on the surface of hydrothermal sediments covering hot vents at a depth of 2,000 m in the Guaymas Basin of the Gulf of California (Nelson et al., 1989). These mats were 3-cm thick on the sediments and up to 30-cm thick between stands of vestimentiferan tube worms, the characteristic hosts of symbiotic, sulfide-oxidizing prokaryotes (see below). The unusual mass development of *Beggiatoa* at the hydrothermal vents is speculated to be due to: 1) a more efficient flux of H_2S and O_2 than by mere diffusion; 2) a continuous supply of an organic growth factor; 3) facultative chemoautotrophy providing an efficient metabolic flexibility in case of intermittent or turbulent vent emissions; and 4) an optimal temperature gradient (Jannasch et al., 1989). Furthermore, the unusually large diameter of the filaments (up to 122 μm) and an "empty" inner space (a liquid vacuole filling more than 80% of the cell, the cytoplasm being distributed only along the outer cell wall) may contribute to their growth and survival in this environment. These allow the cells to grow to an enormous size without diffusional problems, thus providing them with a structural rigidity necessary for exploiting a larger ambient space than smaller cells can without support of a substratum (e.g., sediment). Moreover, an inner low-redox reservoir may enable these organisms to survive in the absence of reduced chemical substrates during flushing by oxygenated ambient seawater for a certain period of time.

The classical filamentous and stalk-forming iron bacteria have to be considered as gradient organisms also. Bacteria such as *Leptothrix ochracea* and related bacteria, as well as *Gallionella ferruginea*, grow in drainage tubes in moist fields, preferably in the narrow zone between the anaerobic bottom layer, which is the source of ferrous iron, and the flowing water, which contains oxygen.

Horizontal Gradients

Horizontal gradients of environmental factors exist where water of unusual properties and contents is discharged into running water. Gradients are formed in the vicinity of hot springs; sulfur springs; effluents of coal, iron, and salt mines; rivers; ditches; and drainage tubes.

The water discharged from sulfur springs gives rise to a gradient in sulfide concentrations, providing sulfide-rich water and photoanaerobic conditions at the origin and photoaerobic conditions downstream. Hot sulfur springs as they occur in New Zealand, North Island (Castenholz, 1976), and Yellowstone National Park (Brock, 1978; Castenholz, 1977) provide gradients of temperature, sulfide and oxygen concentration, pH value, and secondarily, in the concentration of minerals and organic nutrients; near the spring, the effluent sulfide-rich water of 60–70°C enables the gliding green bacterium, *Chloroflexus*, to grow and form reddish orange or orange-green mats (see The Family Chloroflexaceae in Volume 7). Downstream, this green bacterium is accompanied by the cyanobacteria *Oscillatoria*, *Synechococcus*, and, eventually, *Mastigocladus*. The patterns do not vary greatly at different localities (Bauld and Brock, 1973; Castenholz, 1976, 1977, 1979).

Possible Mechanisms for Finding or Remaining in the Beneficial Layer of the Gradient

Motility and buoyancy may be involved in keeping the bacteria in horizons of favorable growth conditions. Many motile bacteria manage, by means of their chemotactic response mechanisms, to reach and stay in an area where the concentration of a nutrient or of oxygen is optimal. Bacteria that are motile by flagellation, as well as gliding bacteria, are able to move to and stay in their beneficial environment. Gas vacuoles, occurring exclusively in aquatic microorganisms (Cohen-Bazire, Kunisawa, and Pfennig, 1969), provide buoyancy and may be a means by which bacteria maintain their vertical position (Clark and Walsby, 1978a, b).

Chemotaxis Chemotaxis is widespread among motile microorganisms (Weibull, 1960) and is of ecological importance in many systems (Chet and Mitchell, 1976). Usually, the chemotactic response mechanisms result in the accumulation of microorganisms in areas of favorable metabolic conditions and depletion in areas of adverse conditions. However, there is almost no correlation between the properties of a substance to act as an attractant and to serve as an energy source (Adler, 1974; Weibull, 1960). The effect of an attractant has been shown to increase swimming periods toward the attractant after tumbling, while the swimming periods in the opposite direction are shortened (Koshland, 1974, 1976, 1980, 1981; Repaske and Adler, 1981; Adler, 1988; Hazelbauer, 1988). The role of chemotaxis in the vertical distribution is still a matter of speculation.

Aerotaxis The response of bacteria to oxygen may be a dominant mechanism enabling bacteria to find and remain in an environment favorable with respect to oxygen concentration. Vertical movements of clouds of phototrophic bacteria in Winogradsky columns observed during diurnal cycles of light and dark may serve as a model for investigating the response of bacterial populations toward oxygen and substrates.

Phototaxis Phototaxis is apparently shared by all anoxygenic phototrophic bacteria that are motile by flagellation. The phototactic response mechanism results in keeping the bacteria in an area of favorable light intensity after they accidentally enter. The response, reversal of flagellar movement, usually occurs when the cells enter an area of lower light intensity. Although phototaxis has been well studied in laboratory cultures (Hustede, Liebergesell, and Schlegel, 1989), investigations relevant to the significance of phototactic behavior in the vertical distribution of phototrophic bacteria in lakes are lacking. The function of photokinesis, the initiation or acceleration of linear velocity by light, has not yet been studied by ecologists (Nultsch, 1975).

Magnetotaxis Bacteria containing magnetite (Fe_3O_4) crystals have been discovered (Blakemore, 1975) and isolated (Blakemore et al., 1979; Frankel et al., 1979). It is hypothesized that the downward orientation of the cell within the earth's inclined magnetic field lines on the northern hemisphere provides favorable growth conditions for these motile and strongly microaerophilic organisms. However, when the studies were repeated in New Zealand and Australia, very similar organisms were found that exhibited a reversed polarity (Blakemore et al., 1980).

The anaerobic formation of magnetite by a marine magnetotactic bacterium (designated strain MV-1) was demonstrated using nitrous oxide as an electron acceptor (Bazylinski et al., 1988). This finding obliterated the notion that the biological production of magnetite can only occur in aerobic top sediments. Some nonmagnetotactic, dissimilatory iron-reducing bacteria were described by Lovley and Phillips (1987) and found also to synthesize extracellular magnetite from hydrous ferric oxide under anaerobic conditions. The formation or "biomineralization" of a ferrimagnetic iron sulfide called greigite (Fe_3S_4) has been reported in a multicellular magnetotactic bacterium that has not been isolated but is common in brackish sulfide-rich water and sediments (Mann et al., 1990). These crystals are often aligned in chains and associated with single crystals of nonmagnetic pyrite (FeS_2).

Buoyancy Assuming that gas vacuoles provide buoyancy to the cells (Walsby, 1975, 1977), there may be two ways by which distinct layers are formed. The bacteria may be able to migrate to the favorable depth, or the gas vacuoles may provide neutral buoyancy to guarantee a long residence time to the bacteria at depths supporting their growth. The second alternative has been supported by experimental evidence (Clark and Walsby, 1978b).

Gas vacuoles may also serve the distribution of cells and resting stages; this function is exemplified by the gas vacuoles of some clostridia, which are only formed during the transition phase from the vegetative cell to the spore (Duda and Makar'eva, 1977).

An interesting alteration of buoyancy and its significance in the natural habitat has been described in the case of *Metallogenium. Metallogenium* has been found in all oligotrophic lakes that contain manganese and also in mesotrophic and eutrophic lakes. Its development occurs during the periods of circulation. The vertical distribution of *Metallogenium* in these lakes is dependent on the concentration of manganese. Manganese is soluble in the reduced state. If oxidized by *Metallogenium*, manganese is precipitated at the cell surface, and the heavy manganese oxides make the cells sink down to the sediment. Under anaerobic conditions, manganese is reduced, the buoyancy is increased, and the cells return to the aerobic layers. Thus the movement of *Metallogenium* is strictly correlated to the availability of manganese and oxygen.

Microbial Associations

Under natural conditions, the various types of prokaryotes live in more or less close associations. Interactions between bacteria in a common habitat may be either weak or absent; only species with quite dissimilar nutrient requirements can be expected to show neutralism. The interactions may be strong and may be characterized as mutualism, commensalism, or parasitism. The relationships may be loose or tight, facultative or obligatory. Several examples of microbial associations studied in open systems have been reviewed by Meers (1973).

The most spectacular examples of syntrophic or of mutualistic associations have been discovered in the course of metabolic studies on bacterial cultures presumed to be pure cultures. One example concerns the partners of the culture of *Methanobacterium omelianskii* and illustrates a symbiosis with unidirectional substrate supply of mutual benefit. The other concerns symbiotic associations with bidirectional transfer of small molecules involved in energetic coupling.

Another striking discovery is the symbiotic association between chemolithotrophic sulfide- and thiosulfate-oxidizing prokaryotes and newly described marine invertebrates found to live tightly clustered around deep-sea hydrothermal vents (Cavanaugh et al., 1981; Felbeck et al., 1981; Belkin et al., 1986; Distel et al., 1988; Fisher et al., 1989). Furthermore, a new genus of marine blue mussels was found to contain methylotrophic endosymbionts in their gill cells enabling the animal to thrive near methane emissions in the vicinity of "cold seeps" on the seafloor (Childress et al., 1986). A similar association was later described in certain polychaetes collected from marine sediments at the Skagerak in the Baltic Sea (Schmaljohann and Flügel, 1987).

Interspecies Hydrogen Transfer

The concentration of many metabolites in the cell is below measurable levels. The intermediates of reactions, especially those which are catalyzed by multi-enzyme complexes, scarcely reach measurable concentrations. The intermediates are used as substrates of subsequent enzyme reactions at the same site or in the same compartment where they are produced. The absence of measurable concentrations of a compound does not mean that the compound is not involved in a metabolic reaction.

Similar situations are encountered in many ecosystems where very important intermediates of anaerobic food chains are present at scarcely detectable concentrations. In the rumen, lactate and hydrogen are present only at low concentrations, although they are major products of the predominating anaerobic fermentations; in contrast, acetate, butyrate, propionate, valerate, and formate, which are not subject to further fermentative conversion, may accumulate. In anaerobic ecosystems, only the end produces of anaerobic

food chains accumulate or are released into the environment. The concentration of some degradation products, such as hydrogen, acetate, lactate, and ethanol, is low. These compounds are consumed as soon as they are produced.

Metabolic products may be inhibitory to the cells that produce them; subsequent utilization of inhibitory products by commensals, therefore, is useful to the ecosystem. In several cases the cooperation of two or more organisms is essential, since a substrate would not be degradable at all if the concentration of the product were not kept very low. Interspecies hydrogen transfer is the most significant example of a symbiosis with unidirectional substrate supply from which mutual benefit is drawn.

The principle of interspecies hydrogen transfer became clear when the culture called *Methanobacterium omelianskii* was separated into a strain that produced hydrogen (S-organism) and a strain that oxidized hydrogen (Moh-strain) (Bryant et al., 1967; see also Virulence Strategies of Plant Pathogenic Bacteria in Volume 2). These strains carry out the following reactions:

$$\text{S-organism: } 2C_2H_5OH + 2H_2O \rightarrow 2CH_3\text{-}COOH + 4H_2$$

$$\text{Moh-strain: } 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

The S-organism grows poorly as a pure culture in media with ethanol or other utilizable alcohols because the accumulated hydrogen inhibits growth. The S-organism lacks the ability to dispose of electrons resulting from the oxidation of ethanol via electron sinks other than hydrogen. For efficient growth of the S-organism, a hydrogen-utilizing bacterium has to be included to remove the hydrogen produced (Reddy, Bryant, and Wolin, 1972a, 1972b). The methanogenic bacteria keep the partial pressure of hydrogen low; for the bovine rumen a value of about 3 times 10^{-4} atmospheres (2 times 10^{-7} M) was reported (Hungate, 1967). Therefore, both organisms benefit from this symbiotic association. The methanogenic bacterium is continuously supplied with hydrogen, and the hydrogen-producing organism can even degrade and generate energy from substrates such as lactate or ethanol, which, for thermodynamic reasons (Wolin, 1976), could not be degraded when hydrogen accumulated in the medium (Wolin and Miller, 1982).

As a result of interspecies hydrogen transfer between partners of close symbiotic associations, therefore, special ecological niches can be occupied. The principle has been exemplified by coculturing methanogenic bacteria with various hydrogen-producing bacterial species (Table 5).

For the fermentative degradation of substrates such as lactate or ethanol, the removal of hydrogen by a hydrogen-utilizing partner is obligatory; without continuous removal of hydrogen, anaerobic growth on these substrates is slight. For the degradation of other substrates, glucose included, the removal of hydrogen is not obligatory; however, it enables the cell to obtain more energy than would be otherwise possible.

In media that contain little sulfate, *Desulfovibrio vulgaris* grows only modestly on ethanol or lactate producing acetate and hydrogen. Like the S-organism, it lacks the ability to produce a sink for electrons other than protons. In coculture with *Methanobacterium,* ethanol and lactate were actively fermented with the production of acetate and methane (Bryant et al., 1977). Stable methanogenic mixed cultures, which converted glucose to methane, were also obtained by enrichment in the chemostat (Siñeriz and Pirt, 1977). Benzoate, which under anaerobic conditions can be utilized by phototrophic bacteria in the light or by chemoorganotrophs through anaerobic respiration, has been shown to be biodegradable even under fermentation conditions (Nottingham and Hungate, 1969; Boyd et al., 1983; Sleat and Robinson, 1984; Tschech, 1989); methane and carbon dioxide are the main fermentation products. Apparently, a microbial consortium of more than two bacteria is involved in methane production from benzoate (Ferry and Wolfe, 1976).

The examples discussed above of interspecies substrate transfer concerned the unidirectional transfer of substrates within food chains. In the

Table 5. Examples of interspecies hydrogen transfer by coculturing hydrogen-utilizing (methanogenic) bacteria with various H_2-producing bacterial species.

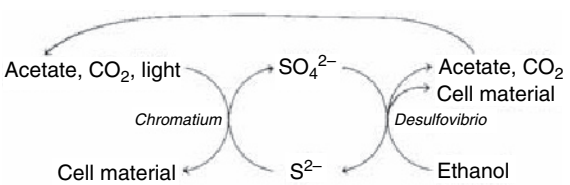
Substrate	H_2-producing species; products of pure culture	H_2-utilizing species; products of coculture	Reference
Glucose	*Ruminococcus albus*; ethanol, acetate, H_2, CO_2	*Vibrio succinogenes*; acetate, succinate	Iannotti et al., 1973
Glucose	*Selenomonas ruminantium*; lactate	*Methanobacterium ruminantium;* acetate, methane, CO_2	Chen and Wolin, 1977
Cellulose	*Clostridium thermocellum*; ethanol, acetate, H_2, CO_2	*Methanobacterium thermoautotrophicum;* acetate, methane, CO_2	Weimer and Zeikus, 1977
Cellulose	*Ruminococcus flavefaciens*; succinate, acetate, formate, H_2, CO_2	*Methanobacterium ruminantium;* acetate, methanol, CO_2	Latham and Wolin, 1977

next section, bidirectional transfer will be discussed.

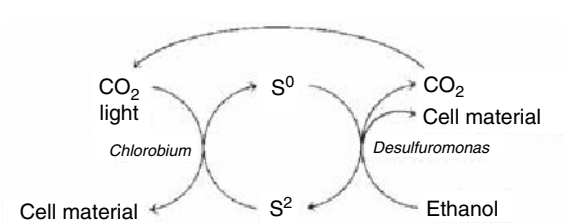
Bidirectional Transfer of Small Molecules

A bidirectional transfer of small molecules is the basis of some symbiotic associations in which sulfate (or sulfur) and hydrogen sulfide are involved in the energetic coupling of two different strains of bacteria kept in mixed culture.

Sulfate-reducing bacteria such as *Desulfovibrio vulgaris* growing with organic acids as hydrogen donors provide the hydrogen sulfide used for anoxygenic photosynthesis by *Chromatium*, which in turn reoxidizes hydrogen sulfide with the production of sulfate. The occurrence of this light-driven sulfur cycle has been repeatedly described. When sulfate is continuously added to the system, part of the sulfur intracellularly accumulated in the *Chromatium* cells may leave the cycle and be deposited. The deposition of sulfur in certain Cyrenaican (North African) lakes has been attributed mainly to this cycle (Butlin and Postgate, 1954), which is as follows:



Although the direct reduction of sulfur was already observed 80 years ago (Beijerinck, 1895), a chemoorganotrophic bacterium able to use elemental sulfur as hydrogen acceptor for growth was not known until the 1970s when a bacterium was found capable of oxidizing acetate to carbon dioxide linked to the reduction of sulfur to hydrogen sulfide (Pfennig and Biebl, 1976). This bacterium, *Desulfuromonas acetoxidans*, was isolated from a culture called *Chloropseudomonas ethylica*, which had been held as a stable mixed culture for many years. The syntrophic association of the green phototrophic bacterium, *Chlorobium*, and of *D. acetoxidans* occurs because hydrogen sulfide and sulfur permit energetic coupling of the life processes of both partners, as shown below:



In mixed cultures, low concentrations of hydrogen sulfide (7–8 mg/l) are sufficient to allow maximum growth of *Desulfuromonas* strains and of *Chlorobium* or *Prosthecochloris* strains (Biebl and Pfennig, 1978). The cell yield of the green bacterium is not limited by the hydrogen sulfide added to the culture; it depends rather on the amount of ethanol, the hydrogen donor.

To understand the function of a species in a habitat doubtless requires studies in mixed culture. Only in mixed culture under conditions of competition is the true actual niche revealed. According to van Niel (1955), Winogradsky "argued convincingly that pure culture studies may reveal characteristics that can express themselves only in the absence of potential competitors." The examples presented demonstrate the necessity for pure culture studies. Ironically enough, superb examples for the mixed culture concept were noticed when it was discovered that the "pure" cultures were not really pure.

Specific Aquatic Ecosystems: Marine Environments

Although the borderline between the aquatic and terrestrial habitat of microorganisms is diffuse, certain physical and chemical properties of water characterize the predominantly aqueous environment distinctly with respect to the indigenous microbial population. Inorganic and organic nutrients are available in dissolved and ionic form and are highly mobile and constantly dispersed by diffusion, convection, and currents. These characterizations of nutrient regime, together with the high heat capacity of water, provide the characteristic constancy of the environmental conditions upon which many of the typical aquatic microorganisms depend.

Another typical characteristic of aquatic environments is the variety of interfaces that are of special microbiological importance: the surface film (neuston); the interface between oxygenated and anoxic water or sediment; horizontal layering of water with respect to gradients of light, temperature, and salinity in stagnant waters; and finally the aqueous–solid interface of submerged surfaces. These interfaces provide specific and often highly selective environmental conditions for the accumulation and growth of microorganisms on the micro- as well as on the macroscale (see above, "Surfaces as Habitats," and Planktonic Versus Sessile Life of Prokaryotes in Volume 2).

The literature in "aquatic microbiology" largely deals with environments on the macroscale: the qualitative and quantitative assessment of populations in springs, ponds, rivers, lakes, and parts of the ocean. In addition, applied problems

led to the investigation of ground waters, acid mine waters, sewage lagoons, cooling water in pipe systems, etc. Only some of these studies focused specifically on the particular aquatic characteristics.

It was stated earlier that the lack of water-preserving mechanisms makes prokaryotes as a group appear to be typically aquatic organisms. Over and beyond this fact, certain microorganisms are specifically adapted to life at the typical conditions of aquatic environments. An example is *Thiovulum*, a highly motile and large (spherical cells of up to 25-µm diameter) chemolithotrophic organism that oxidizes hydrogen sulfide aerobically (see The Colorless Sulfur Bacteria in Volume 2). Growth of *Thiovulum* depends on its ability to locate in areas of the short-lived coexistence of hydrogen sulfide and oxygen as spontaneously reacting compounds (Wirsen and Jannasch, 1978). The biology of the typically aquatic *Caulobacter* group (see Dimorphic Prosthecate Bacteria in Volume 5) has been reviewed by Poindexter (1964) and Schmidt (1971). These prokaryotes are uniquely adjusted to metabolize at low nutrient levels (Poindexter, 1979) while largely attached to submerged solid surfaces with the aid of characteristic appendages, the prosthecae.

A large number of typically aquatic microorganisms are microaerophilic, a point of evolutionary interest, and take advantage of the fact that water provides a barrier against a quick replenishment of oxygen, as determined by the rates of dissolution and diffusion. Except for areas of high mixing, as in mountain streams, or of high photosynthesis, as in the surface layer of eutrophic lakes, concentrations of dissolved oxygen in most natural waters are well below saturation values.

The occurrence of a large number of other microorganisms, which can be easily and repeatedly isolated from natural waters, is not necessarily diagnostic for their aquatic specialization but merely for 1) their ability to survive well under these conditions or 2) a high rate of introduction from other habitats. Obvious examples are the seasonal occurrence of typical soil bacteria in streams and rivers or the common existence of enteric bacteria in waters polluted by domestic sewage.

The limited ability of enteric bacteria, as an extreme example, to compete for survival in natural waters indicates another general characteristic of aquatic habitats. The constant dispersion of dissolved nutrients by diffusion and currents has a dilution effect and leads to generally low concentrations of most of the substrates essential for microbial growth. A central theme of aquatic microbiology is the study of the physiology of growth and metabolism in the presence of low nutrient concentrations (see "Low-Nutrient Environments" in this Chapter).

Relating specific groups of prokaryotes with specific aquatic macrohabitats such as ponds, lagoons, rivers, or parts of the ocean is of limited value. A number of characteristic traits of these ecosystems, however, are of microbiological interest and are dealt with in the following sections.

Seventy-one % of the globe is covered by sea water, three-quarters of which lies below a depth of 1,000 m. Comparing the mean depth of the ocean to an arbitrarily assumed thickness of the terrestrial biosphere (depth of live soil and the zone of plant and animal life on and above the surface) at 38 m results in an aquatic/terrestrial volume ratio of 99/1. Productivity, on the other hand, is related to surface area and the availability of light and nutrients. It is limited in the sea to the phototrophic surface layer and is estimated, in spite of the above-mentioned volume ratio, in the same order of magnitude as the terrestrial photosynthetic production.

The ambient concentration of dissolved organic carbon in sea water lies in the range of 0.3–1.5 mg/liter (Menzel and Ryther, 1970). Particulate organic carbon reaches not more than one-tenth to one-fifth of that concentration. Much of this material, especially in deeper waters, is "refractory" to microbial attack ("Low-Nutrient Environments"). Except for a few nutrient-rich areas of high oxygen consumption by microorganisms, off-shore waters are aerobic down to the greatest depths. The only attempt to cover the area of marine microbiology was made by ZoBell in 1946. More recent literature reviews deal with particular problems (see below). Initial efforts in reviewing the systematics of the characteristic indigenous bacterial flora, obligately and facultatively aerobic rods, were carried out by Baumann et al. (1972). Luminescent bacteria, largely restricted to the marine environment, are described as belonging to a new genus of the Enterobacteriaceae, the *Beneckea*, and to the genus *Photobacterium* (Baumann and Baumann, 1977; Reichelt and Baumann, 1973). The former occur as free-living forms and the latter as free-living and quite distinct symbiotic forms living in specific organs of marine fishes and invertebrates (Greenberg, Hastings, and Ulitzur, 1979; Hastings and Nealson, 1977).

The Deep Sea

The deep sea comprises the largest volume of sea water characterized by the absence of light, by constant temperature around 2–3°C, by limited input of organic energy sources from the remote surface water, and by considerable hydrostatic pressure. Because of the continuous increase of

the last factor with depth, about 1 atm every 10 m, there is no particular depth at which the start of the deep sea can be defined. Oceanographers distinguish between shelf waters of up to 200 m deep, the continental slope area ranging to a depth of about 3,000 m, and the abyssal plains reaching 6,000 m. The deep trenches may reach depths of up to 11,000 m, but the total area of the world's ocean below 6,000 m covers not more than 1.2% and the total volume amounts to only 0.01% (Sverdrup, Johnson, and Fleming, 1942).

The remoteness of the deep sea from productive surface waters or from coastal input of organic energy sources causes the sparsity of life in deep waters. Yet a large number of bacteria can be isolated from sediments of almost any depth. The early work in deep-sea microbiology was reviewed by ZoBell (1970) and Morita (1976). Interest in this area was rekindled by experimental work on deep-sea in situ incubation (Jannasch and Wirsen, 1973).

The hydrostatic pressure and its possible effect on prokaryote metabolism (Marquis, 1976; Marquis and Matsumara, 1978) is of special interest. Based on physicochemical considerations, hydrostatic pressure may be assumed to affect metabolic processes only through differential volume changes. Unless dissolution and liberation of gases are involved, in principle, little or no effect can be expected at pressures below 1,000 atm.

A high variability of barotolerance in marine bacteria, mostly documented in terms of growth in various media, has long been known but is not fully understood yet. This is principally also true for "barophilism," a term introduced by ZoBell and Johnson (1949) and today defined as optimal growth response at pressures higher than normal atmospheric pressure. Pure cultures of true barophilic bacteria only first became available at the time Dietz and Yayanos (1978) introduced a technique for their isolation by using pressurized enrichments from decaying deep-sea amphipods in an organic-rich, silica-gel medium. Subsequent isolations from materials collected at depths of 5,800 and 10,500 m yielded organisms that grew optimally at pressures of about 500 and 690 atm (Yayanos et al., 1979, 1981). While some isolates grew at normal atmospheric pressure but at a rate 30-fold-lower than at optimum pressure, others did not grow at all after decompression and were called "obligate barophiles." Because of the more striking psychrophilic/barophilic behavior of isolates from greater depths, much of the earlier work concentrated on deep-sea trenches, i.e., at depths below 6,000 m, although their seawater volume is only 0.1% of the oceans' total. Thus, the results are more of physiological than of ecological significance.

Since this pioneering work appeared, large numbers of barophilic bacteria have been isolated from various depths (Deming and Colwell, 1982; Deming et al., 1981; Yayanos et al., 1982; Jannasch et al., 1982; Jannasch and Wirsen, 1982). The data generally indicate a close association between barophilism and psychrophilism, confirming earlier observations (Wirsen and Jannasch, 1975) on high barotolerance found primarily, but not exclusively, in psychrophilic isolates. The large variability of barophilism seems to merge gradually with barotolerant growth characteristics.

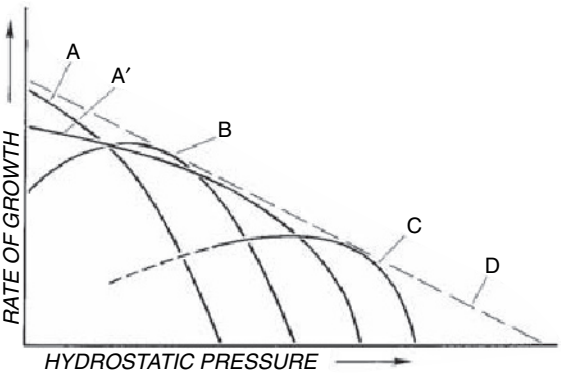


Fig. 4. Summarizing scheme of growth responses by marine bacterial isolates to hydrostatic pressure. A, low barotolerance; A′, high barotolerance; B, barophilic; C, obligate barophilic; D, slope of line indicates decrease of activity with increasing pressure. (Modified from Jannasch and Taylor, 1984.)

A direct comparison of all the published data is difficult, since they have been obtained in different growth media. However, the general situation can be schematically depicted as in Fig. 4, where A and A′ indicate a range of pressure between low and high barotolerance. Barophilic bacteria (B) also exhibit a considerable range of optimal growth pressures and are termed obligate barophiles (C) if they cannot grow at normal atmospheric pressure. The slope indicated by line D is a simplified expression of the overall decrease of microbial activity as a function of increasing pressure with depth. When considering microbial growth in the deep ocean, it should not be forgotten that both the nutrient concentration (mainly the available organic carbon) and the temperature are of predominant importance. It is now technically possible to sample enrich and isolate bacteria from the deep sea in pure culture while maintaining full pressure continuously (Jannasch et al., 1982).

Hydrothermal Vents

The decreasing cell density observed in the oceans with increasing depth has been explained as the result of the increasing remoteness from

the photosynthetically productive surface waters. A striking exception to this general rule was discovered when copious populations of large, sessile invertebrates were found at a depth of 2,700 meters clustered around warm-water vents of volcanic origin in the Galapagos Rift area (Ballard, 1977). The hydrogen sulfide content of the extruding waters (Fig. 5) was shown to provide the energy (the electron donor) for the chemosynthetically rather than photosynthetically sustained ecosystem either by free-living or by symbiotic sulfide- and thiosulfate-oxidizing prokaryotes (Jannasch and Wirsen, 1979; Grassle, 1986; Jannasch, 1989). Thus, these deep-sea animal communities are supported by terrestrial rather than solar energy (Fig. 6), the caveat to this statement being the use of photosynthetically produced oxygen in the pathway for aerobic sulfur oxidation. Although quantitatively of less importance for the production of organic carbon, anaerobic chemosynthesis has also been demonstrated by the isolation of methanogens from various sites at hydrothermal vents (Jones et al., 1989; Huber et al., 1989). Many of the aerobic chemoautotrophic and heterotrophic isolates from the warm and hot hydrothermal vent sites were extremely thermophilic (see above).

Marine Anoxic Ecosystems

Shallow estuaries are similar to rich freshwater environments, except for frequently and some-

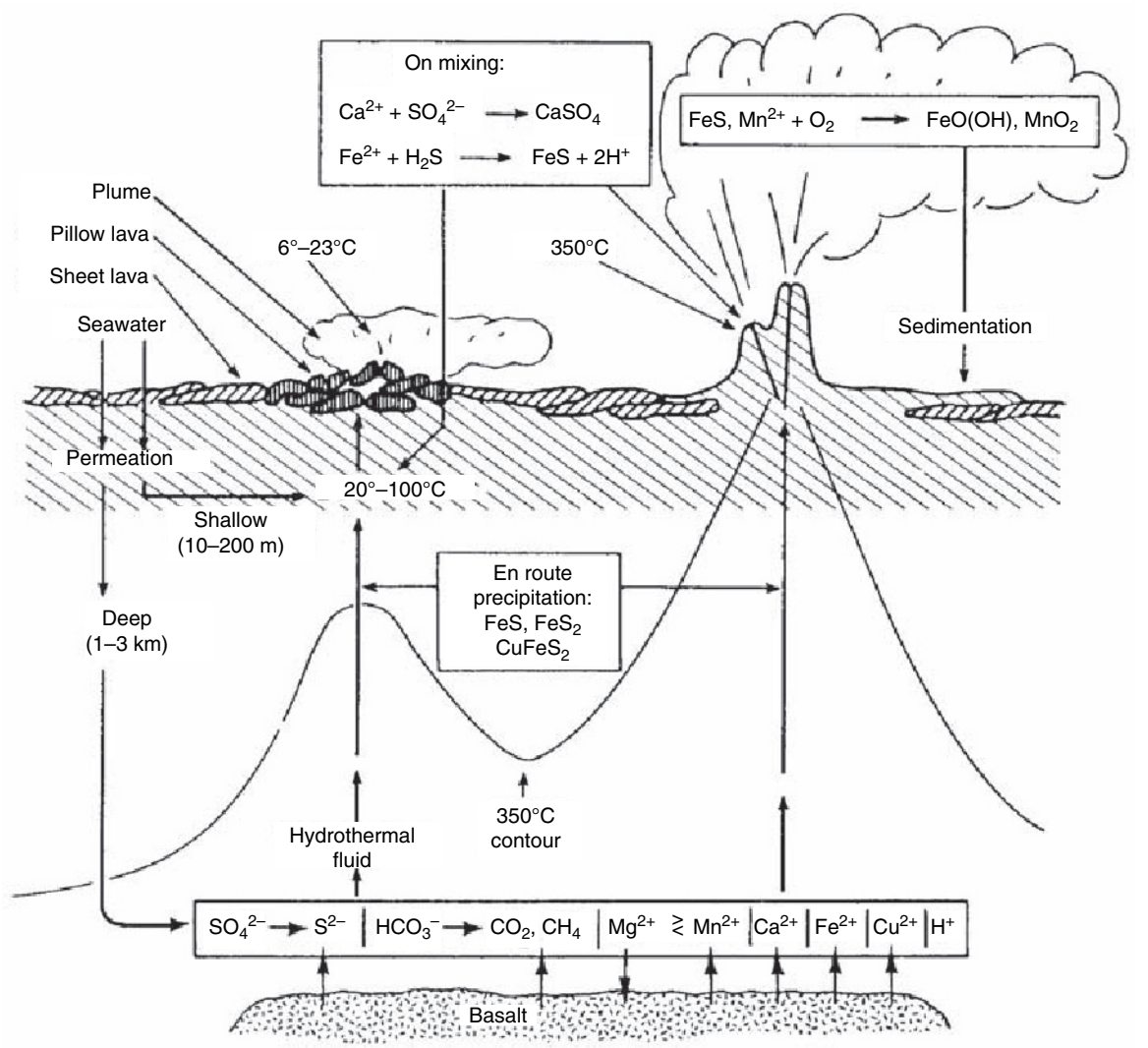


Fig. 5. Major geochemical processes occurring within the oceanic crust and on the deep-sea floor. As seawater penetrates several km into the crust, it is heated to 350–400°C, reacts with basaltic rocks, and leaches various chemical species into solution. The highly reduced "hydrothermal fluid" rises and reaches the sea floor either directly (hot vents) or after mixing with cold, oxygenated seawater before emission (warm vents). On mixing, polymetal sulfides and calcium sulfate (anhydrite) precipitate, either within subsurface lava conduits or as "chimneys" and suspended particulate matter in the "black smokers." (Modified from Jannasch and Mottl, 1985.)

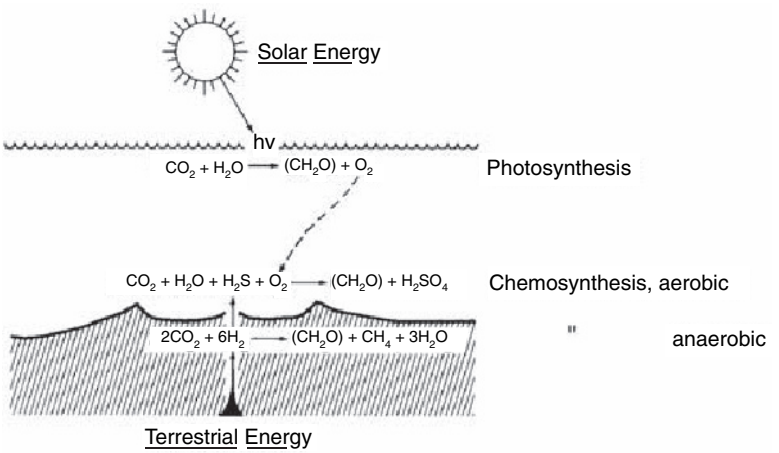


Fig. 6. Scheme of the energy supply pathway for photosynthesis and chemosynthesis, showing the role of free oxygen (O_2) at deep-sea hydrothermal vents. (From Jannasch, 1989.)

times drastically changing salinities and for the presence of certain characteristic anions and cations. The former is dealt with in the chapters on the habitats of halotolerant and halophilic prokaryotes (see The Order Halobacteriales in Volume 3; The Order Haloanaerobiales in Volume 4; and The Family Halomonadaceae in Volume 6). Next to chloride, the anion most characteristic for the marine environment is sulfate. In organically rich marine environments, the microbial reduction of sulfate to sulfide initiates the cycling of sulfur compounds with a number of concomitant environmental phenomena: the production of hydrogen sulfide, the precipitation of ferrous (and other heavy metal) sulfide often followed by the formation of a disulfide (e.g., pyrite), the deposition of elementary sulfur, and the rich populations of sulfur-oxidizing prokaryotes at the oxic/anoxic interface. Acidification rarely occurs, due to the high buffer capacity of sea water. Anaerobic marine basins, chemically stabilized by high concentrations of sulfide, range from small estuarine pockets to anoxic water masses on the scale of the Black Sea (see below). The high sodium concentration of sea water has been the key determinant for typically marine bacteria by constituting strict requirements for the cation (McLeod, 1968).

The largest anoxic ecosystems in the biosphere are marine. The Black Sea contains no free oxygen from a depth of about 150 m down to the bottom at a 2,000-m depth. In an extensive team effort, the geological, chemical, and biological characteristics have been studied (Degens and Ross, 1974). Skopintsev, Karpov, and Vershinina (1959) and Sorokin (1964) have done earlier microbiological work, finding that about 95% of the sulfide present in the deeper Black Sea water stems from sulfate reduction and that the oxidation of sulfide at the oxic/anoxic interface accounts for the large amounts of dark carbon dioxide fixation (4–6 mg C/m^3 · day). The auxiliary role of the cycling of sulfur compounds in the turnover of organic and inorganic carbon has been described (Jannasch, Trüper, and Tuttle, 1974), and the biomass and total number of microorganisms in the depth of the Black Sea have been measured (Mitskevich, 1979).

A series of research cruises on the Black Sea (April–September 1988) has resulted in some unusual oceanographic data. The oxic/anoxic interface, historically at depths between 125–175 m in the 2,000-m water column, had risen within the last decade to 80–90 m (Fig. 7). At the same time, a maximum for bacteriochlorophyll *e* was detected at the same depth where hydrogen sulfide first appears on a downward profile in the water column (Repeta et al., 1989). Such a high concentration of a prokaryotic pigment has never been observed before in the Black Sea, and it exceeded the concentration of the phytoplanktonic chlorophyll *a* in the oxic waters above (Fig. 8). This first observation of mass bacterial photosynthesis in offshore waters indicates that the rising sulfide level has reached the lower range of the photic zone. Slow-growing isolates from this zone appear to be adapted to low light intensities and are undistinguishable from *Chlorobium phaeobacteriodes* (N. Pfennig and H. Cypionka, personal communication). Other organisms isolated from the interface are aerobic, neutrophilic, obligately chemolithotrophic sulfide oxidizers, tentatively placed into the genus *Thiomicrospira*. A number of new sulfide-reducing and methanogenic bacteria were also isolated from the sediment.

The Carciaco Trench off the Venezuelan coast is another permanently anoxic basin of a depth of 1,400 m (Richards, 1975; Richards and Vaccaro, 1958). Another site is the Orca Basin in the northern gulf of Mexico, which features an anoxic and saline layer of bottom water at a depth of 2,300 m (Shokes et al., 1977). Trüper (1969) and Watson and Waterbury (1969) inves-

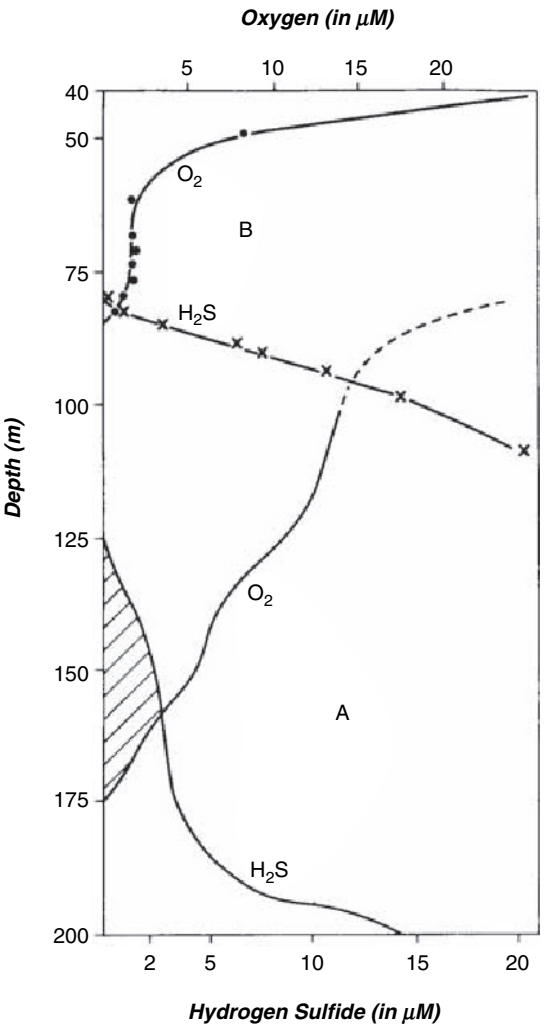


Fig. 7. Profiles of dissolved oxygen and hydrogen sulfide in the upper water column of the Black Sea, central western gyre. (A, data from Sorokin, 1972; B, data from Repeta et al., 1989.)

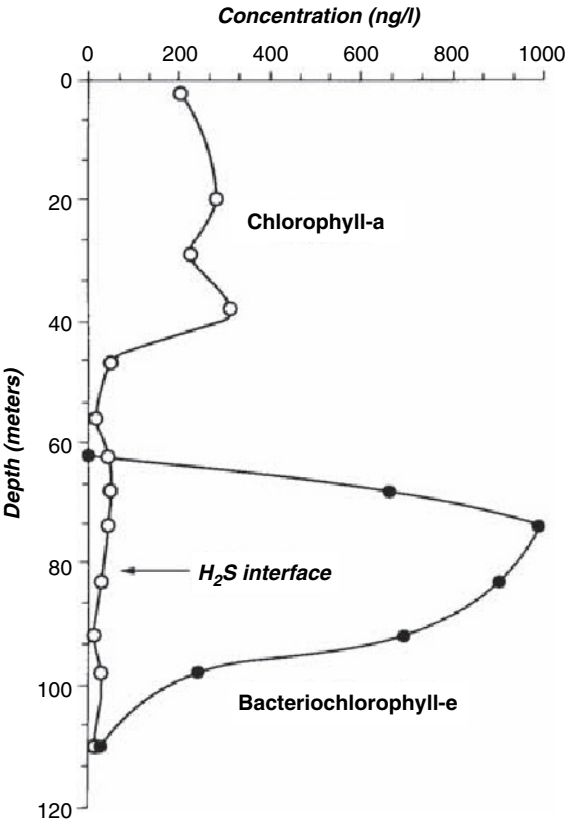


Fig. 8. Distribution of phytoplanktonic and bacterial chlorophylls in the upper water column of the Black Sea, central western gyre. (Data from Repeta et al., 1989.)

tigated anoxic hot brines (56°C) found at the bottom of the Red Sea and ascribed the absence of readily growing bacteria to toxic concentrations of heavy metals. A unique environment for the discovery of a number of new metabolic types of prokaryotes, especially the anaerobically photosynthesizing cyanobacteria, is the Solar lake near Elat in the Gulf of Aqaba, which has been described in a series of papers (see Cohen et al., 1977). The relatively low concentration of methane in most of the anoxic marine waters as compared to anoxic fresh waters is in agreement with the competitive behavior of sulfate versus carbon dioxide reduction originally suggested by Cappenberg (1974a, 1974b) and discussed in detail by Rudd and Taylor (1979).

Eukaryotes as Habitats for Bacteria

Eukaryotes present a multitude of habitats for bacteria. The surfaces, cavities, crevices, and intercellular spaces open to the air, as well as the intestinal tracts, exudates, and excretory substances, offer opportunities for the growth of many bacteria. During evolution, more or less close associations or symbioses between the eukaryotic organisms and bacteria have developed. The symbiotic relationship between the host, the larger partner, and the bacterium is either neutralistic, e.g., when bacteria feed on the waste products of the host, or mutualistic. Both of these relationships will be discussed here.

Two kinds of special abilities of the prokaryotes are exploited by the eukaryote and have opened highly specialized habitats: the ability to fix nitrogen and to hydrolyze cellulose.

Nitrogen fixation is exploited by plants. The bacterial symbionts are either held as ectosymbionts in intercellular spaces of leaves, stems, or at the root surface or as endosymbionts in root nodules and rhizothamnia. Animals do not take much advantage of nitrogen-fixing bacteria; nitrogen fixation has only been observed in the

hindgut of termites (Benemann, 1973; Breznak et al., 1973) and in the gut of humans who eat a carbohydrate-rich diet (Bergersen and Hipsley, 1970).

The ability to hydrolyze cellulose is lacking in animals that are higher on the evolutionary scale than the molluscs, with exception of the silverfish, *Lepisma lineata.* Symbiotic relationships between animals and cellulolytic protozoa and bacteria were established because of the abundance of cellulose as food and the general inability of animals to produce cellulolytic enzymes.

Associations of eukaryotic hosts with bacteria have evolutionary aspects. When the plants started to colonize the land and to shape the prerequisites for the evolution of the higher forms of life, the bacteria had already acquired a high degree of biochemical and physiological fitness—presumably the present-day status. Instead of being added to a preexisting system of higher organisms as, for example, plants and animals colonizing the recently emerged island of Surtsey, the bacteria colonized the eukaryotic host during all stages of the host's evolution. The present-day prokaryote-eukaryote relationships must be considered the result of a long selection process. Excluding parasitism, one may hypothesize that in all cases of stable associations, the relationship is of mutual advantage to the partners. The benefit may be as obvious as in the cases of nitrogen fixation in higher plants and of cellulose digestion in animals, or the benefit may just be a protective mechanism of seemingly very little importance, such as preventing harmful bacteria from entering the potential habitat.

Animal Habitats

Eliminating parasitism from this discussion, only some benign relationships will be considered. In this context the endosymbiosis of protozoa and bacteria deserves mention. Progress in this area is enormous and has been reviewed in the handbooks edited by Schwemmler and Schenk (1980) and Schenk and Schwemmler (1983). The inner and outer surfaces of animals, the intestinal tract, the skin, and several organs have to be regarded as microbial ecosystems with specific populations of indigenous and nonindigenous bacteria.

Intestinal Tract Among the associations of bacteria and animals, the intestinal tract is the ecosystem with the highest population density. The number of bacterial cells within the intestinal tract may even exceed the number of host cells. With respect to the human gut, this ratio was dramatically described by Savage (1977a): "the normal human organism can be said to be composed of over 10^{14} cells, of which only about 10% are animal cells." The ratio differs from species to species through the animal kingdom.

The intestinal tract is an open ecosystem that resembles a tube receiving food at one end and releasing waste at the other. In omnivores and carnivores, food digestion is accomplished by the animal's own intestinal digestive enzymes without involvement of microbes. There are two modifications of this "straight tube" model. Both types are found especially in herbivores, among the invertebrates as well as the vertebrates. Due to the particular nature of the diet of herbivorous animals, which is rich in celluloses, hemicelluloses, and pectins but is low in protein, the tract has been adapted to accommodate microorganisms. Extensions of the tract function as fermentation vessels and harbor protozoa and bacteria able to convert the plant polymers into microbial cells and degradation products. These fermentation vessels may be located either anteriorly or posteriorly to the areas of the intestinal tract where the gut contents are digested by the enzymes of the animal. In the ruminants, a special compartment of the stomach, the rumen-reticulum, is used as the fermentation vat. Its huge size guarantees a long residence time for the plant material to be degraded by cellulolytic and other microorganisms. Similar vessels destined for foregut fermentation are present in camels, kangaroos, hippopotamuses, and leaf-eating apes. The second modification of the straight tube is represented by extended compartments or blind sacs (ceca) near the end of the intestinal tract. Their function is also that of a fermentation vessel; examples for hindgut fermentation are found among vertebrates (horses, pigs, guinea pigs, rats, rabbits) and insects (termites, wood roaches).

Human Intestinal Tract The tract comprises six major areas: mouth, esophagus, stomach, small intestine, cecum, and large intestine. Each may be the habitat of a particular bacterial flora. And these habitats may be subdivided into further categories, such as epithelial (the bacteria grow in association with epithelial surfaces), lumenal (bacteria live free in the lumen), and crystal (bacteria live in crypts). Different areas harbor different bacteria, and each may be the primary habitat for a certain bacterium or a group of bacteria. The habitats are more or less delineated and offer rather constant conditions.

As was recognized very early (see Miller, 1890), the mouth, nasopharynx, and throat are inhabited by many aerobic and anaerobic bacteria. *Streptococcus salivarius*, *S. mutans*, *Veillonella alcalescens*, *Treponema dentium*, *Fusobacterium*, *Actinomyces*, lactobacilli, corynebacteria, and cocci belong to the normal flora of the human mouth (Hardie and Bowden, 1974). The mouth may be considered as an eco-

system of its own characterized by its anatomical and physiological development, the composition of the saliva, and the succession within the bacterial flora (Russel and Melville, 1978). The interaction of the oral streptococci and the mucosal and enamel surfaces deserves special emphasis; special methods have been developed to investigate the specificity of interactions (Rutter and Abbott, 1978), which are partially due to the kind and properties of extracellular polysaccharides (Ebisu et al., 1975; Germaine, Chludzinski, and Schachtele, 1974).

The intestinal tract contains climax populations of indigenous as well as allochthonous bacteria, yeasts, and protozoa. Hundreds of different bacteria have been isolated from the intestinal tract of many animals. In many cases, it is difficult to decide whether a certain species is autochthonous or not and which physicochemical niche it fills. Applying modern ecological theory (Alexander, 1971) to the ecosystem of the gastrointestinal tract of mammals (Savage, 1977a, 1977b), criteria for determining autochthony of microorganisms isolated from the gastrointestinal tract have been developed. "Autochthonous gastrointestinal microorganisms (i) can grow anaerobically, (ii) are always found in normal adults, (iii) colonize particular areas of the tract, (iv) colonize their habitats during succession in infant animals, (v) maintain stable population levels in climax communities in normal adults, and (vi) may associate intimately with the mucosal epithelium in the area colonized" (Savage, 1977a). These criteria are useful for distinguishing indigenous microorganisms from nonindigenous ones. For detailed information, the excellent reviews on relevant problems and on the present status of knowledge by Savage (1977a, 1977b), Clarke (1977), Bauchop (1977), Costerton et al. (1981a, 1981b, 1987), Costerton and Cheng (1981), Bitton and Marshall (1980), Marshall (1984), and Tannock (1990) and The Medically Important *Bacteroides* spp. in Health and Disease in Volume 7 should be consulted.

Because of its high acidity, the human stomach is not populated by bacteria. Behind the pylorus, fewer than 10 bacterial cells per milliliter have been counted. From the pylorus via the duodenum and jejunum to the ileum, the number of bacteria increases; up to 10^{11} bacteria per gram of feces have been counted; *Escherichia coli* represents less than 1% of the population. The predominant genus is *Bacteroides*, comprising species such as *B. fragilis* (see The Medically Important *Bacteroides* spp. in Health and Disease in Volume 7). The bacteroids are followed by *Fusobacterium*, *Eubacterium*, and *Peptostreptococcus* (Moore and Holdeman, 1974), all strictly anaerobic, Gram-negative rods. Other inhabitants belong to the streptococci, lactobacilli, and bifidobacteria (Drasar and Hill, 1974; Drasar and Barrow, 1985). The dependence of the composition of the flora on the kind of food eaten has not yet been exhaustively studied. The changes caused by the oral application of antibacterial agents like antibiotics and sulfonamides or by special foods such as garlic or cabbage have not been examined with respect to qualitative and quantitative changes of the bacterial flora.

The specific niches of the bacteria in the gastrointestinal tract are not easy to comprehend. The nutritional conditions are certainly optimal for many other bacteria which do not occur there. It is not known whether one or the other special property of intestinal bacteria as discussed by Prins (1977) is responsible for a true autochthony or whether their common occurrence in this habitat is correlated with specific chemical or structural properties.

One could imagine that for lumenal bacteria, high growth rates are required and that bacterial attachment sites and mechanisms favor the colonization of epithelia. The selection of only a few species of bacteria to grow in the gastrointestinal habitats may, among other factors, be due to the tolerance to pH, to lipolytic, peptolytic, and saccharolytic enzymes, to detergent-like bile acids, and to degradation products. Whether certain structural details of the bacterial cell envelope are common to all indigenous inhabitants of the lower intestine is an open question (Costerton et al., 1974, 1981a; Martin, 1969).

Basically, in many intestinal tracts, the presence of microorganisms is not necessary. Many animals can grow as gnotobiotes without microorganisms (Coates and Fuller, 1977). Studies with such animals have shown that the intestinal microflora confers a kind of resistance to intruding pathogens. While *Vibrio cholerae* and *Shigella dysenteriae* colonize the intestinal tract of germ-free rats easily, they do not colonize the normal gut easily. Stability of the microbial population is a major factor in the gastrointestinal ecosystem.

The use of gnotobiotic animals will help to explore neutralistic, mutualistic, and parasitic relationships of bacteria and their host. However, even simple quantitative and qualitative studies on the populations in intestinal habitats are urgently needed. It was not long ago that *Escherichia coli* was considered the dominant bacterium in the human intestinal tract. With the application to the human gut of methods originally developed for studying the ruminant bacteria, it was found that the number of strict anaerobes exceeded the oxygen-tolerant anaerobes such as *E. coli* by a thousandfold. The routine application of anaerobic techniques to other vertebrate or

invertebrate intestinal tracts will certainly result in similar unexpected relationships.

Rumen and Reticulum The best-studied ecosystem of foregut fermentation and most probably of all anaerobic ecosystems is the rumen. Rumen symbiosis is an excellent example of a highly developed ecosystem (Hobson, 1988). The principles derived from these studies have had profound influence on the study of microbial ecology (Hungate, 1966, 1975; Savage, 1977a, 1977b).

The main sources of carbohydrates for ruminants are hay, straw, and grass. About 50% of dried grass consists of fructosanes and xylanes, and the rest of the carbohydrate fraction is cellulose. The alimentary tract of ruminants is adapted to this special diet. The first two parts of the bovine stomach, the rumen and the reticulum, serve as a microbial digestive vessel; the total volume is 100–250 liters. This ruminoreticulum provides ideal conditions for the growth of many microorganisms. The temperature is constant at 37–39°C; 100–200 liters of saliva are secreted per day; it contains phosphate (10–50 mM), bicarbonate (100–140 mM), and urea (10 mM nitrogen) and is a well-buffered (pH 5.8–7.3) solution. Nutrients are periodically added in the form of well-macerated cellulose-containing fodder; the mixture is mechanically agitated by the contractions of the rumen. The rumen may be considered to be a semicontinuous culture of microorganisms (Hungate, 1975).

Between 10^{10} and 10^{11} bacteria and 10^5 and 10^6 protozoa inhabit each milliliter of rumen contents. Most of the bacteria are strict anaerobes and are especially adapted to that habitat. The oxidation-reduction potential amounts to –0.35 V. Because many rumen bacteria are instantly killed by oxygen, special techniques for manipulating nutrient media, inocula, and cultures in the absence of oxygen had to be developed (Holdeman, Cato, and Moore, 1977; Hungate, 1950). The nitrogen source of most rumen bacteria is ammonia. Many rumen bacteria require a carbon dioxide-rich (10% CO_2) atmosphere (Dehority, 1971). *Ruminococcus albus*, *R. flavefaciens*, *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens*, *Eubacterium cellulosolvens*, and *Clostridium lochheadii* are the primary species of cellulose-digesting bacteria of the rumen. The cellobiose and glucose produced from cellulose are fermented by a variety of bacteria which utilize the pectins, starch, fructosanes, proteins, and lipids as well. Fermentative degradation of these compounds leads to the accumulation of fatty acids, carbon dioxide, and hydrogen. The latter two gases are combined by *Methanobacterium ruminantium* to give methane.

Besides the bacteria mentioned above, there are many others able to ferment noncellulose carbohydrates or the products of carbohydrate fermentation. These bacteria can only be mentioned here: *Bacteroides amylophilus*, *B. ruminicola*, *Succinimonas amylolytica*, *Selenomonas ruminantium*, *Streptococcus bovis*, *Veillonella alcalescens*, *Lachnospira multiparus*, *Peptostreptococcus elsdenii*, and *Desulfotomaculum ruminis*.

In addition to these bacteria, which have been grown in pure culture, there are some that have been identified microscopically only. Quin's oval and Eadie's oval have been grown in mixed culture (Orpin, 1972, 1973). The filamentous bacterium *Oscillospira* has been found mainly in the rumen of sheep. *Lampropedia* is an obligatory aerobe; however, it is found in high numbers in the sheep rumen.

The rumen microflora is in a delicate equilibrium. If, for example, rumen methanogenesis is eliminated by the addition of chloroform to the rumen contents, a series of changes occur: gaseous hydrogen accumulates immediately, and the increased partial pressure of hydrogen results in an increase of the ratio of propionic to acetic acid. These observations indicate that interspecies hydrogen transfer plays an important role in the rumen (Iannotti et al., 1973). Hydrogen is an intermediate in substrate conversions in the rumen (Hungate, 1967, 1975).

Hindgut Fermentation Although it is not as efficient for the animal as the rumen-type fermentation, hindgut fermentation is widely distributed among herbivorous animals, vertebrates as well as insects. The fermentative organisms and their function in the blind sacs (ceca) and the large intestine of animals have been less well studied than those involved in rumen symbiosis (McBee, 1977). The majority of studies on hindgut fermentations were limited to anatomical-morphological descriptions, and preserved specimens of insects have been used to recognize or describe microorganisms. Recent investigations show that the isolation and cultivation of bacteria so far only recognized in cross-sections of cecal mucosa epithelia is possible, although with difficulties (Lee and Phillips, 1978). The comprehensive investigations of Buchner (1953) provide an excellent basis and incentive for studies on the axenic culture of protozoa and bacteria present in the insect gut and its appendices. Investigations on the protozoal and bacterial symbionts probably involved in the degradation of plant polymers or having other important functions are urgently needed (Eutick, O'Brien, and Slaytor, 1978; see Foglesong et al., 1975; McBee, 1977). Investigations on the isolation and characterization of heterotrophic bacteria from hindguts of the wood-eating termite, *Reticulotermes flavipes*, revealed the predominance of streptococci and *Bacteroides* species and indicate

the existence of a unidirectional food chain from glucose via lactate to propionate and acetate (Schultz and Breznak, 1978, 1979).

Bacteria of the Human Skin The human skin is a rather homogeneous habitat with respect to temperature. However, moisture conditions vary and create distinctive habitats for a characteristic flora. The available water is the most important environmental factor influencing the size of cutaneous populations. The occlusion of the relatively dry skin of the forearm results in a rapid increase of the bacterial population by a factor of 10^4 (from an initial colony count of 3×10^3 to 3.8×10^7 cells/cm^2) by the fourth day of occlusion. The high relative humidity of the axilla results in the survival of dense populations in this region (Marples, 1965, 1974, 1976; Noble and Somerville, 1974; Woodroffe and Shaw, 1974). Many common bacteria, such as the corynebacteria ("diphtheroids"), mycobacteria, micrococci, and streptococci, in addition to yeasts and other fungi, are inhabitants of the human skin. Nutrients are provided by sweat. Fatty acids may act as antibacterial agents and effect a counter-selection. The bacteria inhabitating the sweat glands (sebaceous glands) and hair follicles cannot be reached by normal cleaning of the skin and guarantee fast repopulation of the skin after cleaning (Noble and Pitcher, 1979; Rosebury, 1972).

Molecular Microbial Ecology

Although the sequencing and hybridization of ribosomal RNAs primarily concern the phylogenetic characteristics of organisms (see How We Do, Don't and Should Look at Bacteria and Bacteriology in Volume 1 and Woese, 1987), first results show that a search for uses of this technique in microbial ecology is well justified (Pace et al., 1986; Giovannoni et al., 1988; DeLong et al., 1988). The advantage of identifying microorganisms within complex natural populations by the use of their individual ribosomal RNA signatures is that the often-difficult procedures for isolation, cultivation, and purification becomes less critical. The disadvantage is that specifically expressed metabolic activities of the phylogenetically identified organisms or their ecological functions in situ or in vitro are not readily apparent from the data. After Torsvick and Goksoyr (1978) pioneered a method for DNA extraction from whole natural populations, hybridization probes were applied to the study of the microbial population in the rumen (Stahl et al., 1988), in soil (Holben et al. 1988), and of communities of marine planktonic microorganisms (Giovannoni et al., 1990), of microbial hot springs (Stahl et al., 1985), and of symbiotic prokaryotes (Distel et al., 1988; Unterman et al., 1989). Much of the present work continues to emphasize methodology (Somerville et al., 1989; Weller and Ward, 1989), especially the development of gene probes for individual species or genera and other methods to assess detection and abundance of microbes at specific habitats or within natural populations.

Outlook

Although the habitat is not specified in the description of a species, it is a major characteristic of a species. Microorganisms have their habitats in various ecosystems and microenvironments. Unfortunately, a discussion of the environments of microorganisms can never be complete. The purpose of the present chapter was to demonstrate the multiplicity of microbial environments with emphasis on the close relationship to the diversity of prokaryotes, their metabolic peculiarities, potential capabilities, and constraints. Obviously, more examples could have been selected, and more will be found in the literature every month. The discoveries of new morphological and metabolic types of prokaryotes, as well as of unsuspected relationships and interactions between microorganisms and their environments, over the last decades can be related, in most cases, to the study of microbial ecosystems which had received no particular attention before. The rumen and its inhabitants have been discussed as one of the most striking examples of this; the illuminated hydrogen sulfide-oxygen interfaces of lakes and lagoons are others. Highly developed symbiotic relationships between bacteria and metazoa in the luminous organs and in the hindguts of mammals and insects as well as between bacteria and protozoa have only been marginally mentioned. The ecosystems consisting of solid matter, such as the various kinds of fertile soils, virgin volcanic soils, and desert soils as well as the rhizospheres of plants, have not been dealt with at all in this chapter.

New artificial microbial ecosystems are arising in increasing numbers in, for instance, the cooling systems of nuclear power plants and the aquatic and terrestrial dumping sites of new, chemically synthesized organic compounds, including herbicides, pesticides, and widely used organic solvents. Special evolutionary pressure is being exerted on prokaryotes by the ever-changing fungicidal and bacteriocidal environments of hospitals. Microbial populations will also be the first to respond to global environmental changes such as those following the thinning of the ozone layer and the greenhouse effect. One may also be realistic enough to include here the exposure of this planet's microorganisms to extraterrestrial conditions some of which, e.g.

certain layers in the atmosphere of Venus, may indeed sustain life of some known chemolithotrophic bacteria. In any case, it can be expected that studies of prokaryotes in relation to their natural environments will remain a most exciting and productive area of microbiology.

Literature Cited

Aaronson, S. 1970. Experimental microbial ecology. New York. Academic Press.

Acher, A. J., Juven, B. J. 1977. Destruction of coliforms in water and sewage by dye-sensitized photooxidation. Applied and Environmental Microbiology 33:1019–1022.

Adler, J. 1974. Chemoreception and chemotaxis in bacteria. 107–131. Jaenicke, L. (ed.) Biochemistry of sensory functions. Berlin, Springer-Verlag.

Adler, J. 1988. Chemotaxis: Old and New. Botanica Acta 101:93–100.

Ahrens, R., Moll, G., Rheinheimer, G. 1968. Die Rolle der Fimbrien bei der eigenartigen Sternbildung von *Agrobacterium luteum*. Archiv für Mikrobiologie 63: 321–330.

Akin, D. E. 1976. Ultrastructure of rumen bacterial attachment to forage cell walls. Applied and Environmental Microbiology 31:562–568.

Akin, D. E., Amos, W. E. 1975. Rumen bacterial degradation of forage cell walls investigated by electron microscopy. Applied Microbiology 29:692–701.

Alexander, M. 1971. Microbial ecology. New York. John Wiley & Sons.

Alexander, M. 1976. Natural selection and the ecology of microbial adaption in a biosphere. 3–25. Heinrich, M. R. (ed.) Extreme environments. Mechanisms of microbial adaption. New York. Academic Press.

Alexander, M. 1977. Introduction to soil microbiology, 2nd ed. New York. John Wiley & Sons.

Anwar, M., Khan, T. H., Prebble, J., Zagalski, P. F. 1977. Membrane-bound carotenoid in *Micrococcus luteus* protects naphthoquinone from photodynamic action. Nature 270:538–540.

Aragno, M. 1978. Enrichment, isolation and preliminary characterization of a thermophilic, endospore-forming hydrogen bacterium. FEMS Microbiology Letters 3:13–15.

Atlas, R. M., Bartha, R. 1987. Microbial ecology: Fundamentals and applications, 2nd ed. Menlo-Park, CA. The Benjamin/Cummings Publishing Company, Inc.

Babenzien, H.-D. 1965. Über Vorkommen und Kultur von *Nevskia ramosa*. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1, Suppl. 1:111–116.

Babenzien, H.-D. 1967. Zur Biologie von *Nevskia ramosa*. Zeitschrift für Allgemeine Mikrobiologie 7:89–96.

Ballard, R. D. 1977. Notes on a major oceanographic find. Oceanus 20:35–44.

Barber, R. T. 1968. Dissolved organic carbon from deep waters resists microbial oxidation. Nature 220:274–275.

Baross, J. A., Deming, J. W. 1983. Growth of black smoker bacteria at temperatures of at least 250°C. Nature 303:423–426.

Baross, J. A., Morita, R. Y. 1978. Microbial life at low temperatures: Ecological aspects. 9–71. Kushner, D. J. (ed.) Microbial life in extreme environments. London, Academic Press.

Bartha, R., Atlas, R. M. 1977. The microbiology of aquatic oil spills. Advances in Applied Microbiology 22:225–266.

Bartnicki-Garcia, S., Nickerson, W. J. 1962. Nutrition, growth and morphogenesis of *Mucor rouxii*. Journal of Bacteriology 84:841–858.

Bauchop, T. 1971. Mechanism of hydrogen formation in *Trichomonas foetus*. Journal of General Microbiology 68:27–33.

Bauchop, T. 1977. Foregut fermentation. 223–310. Clarke, R. T. J., Bauchop, T. (ed.) Microbial ecology of the gut. London, Academic Press.

Bauld, J., Brock, T. D. 1973. Ecological studies of *Chloroflexus*, a gliding photosynthetic bacterium. Archiv für Mikrobiologie 92:267–284.

Baumann, L., Baumann, P., Mandel, M., Allen, R. D. 1972. Taxonomy of aerobic marine bacteria. Journal of Bacteriology 110:402–429.

Baumann, P., Baumann, L. 1977. Biology of the marine enterobacteria: Genera *Beneckea* and *Photobacterium*. Annual Review of Microbiology 31:39–61.

Bavendamm, W. 1924. Die farblosen und roten Schwefelbakterien des Süss- und Salzwassers. Pflanzenforschung 2:1–156.

Bayley, S. T., Morton, R. A. 1978. Recent developments in the molecular biology of extremely halophilic bacteria. CRC Critical Reviews in Microbiology 6:151–205.

Bayley, S. T., , Morton R. A. 1979. Biochemical evolution of halobacteria. 109–124. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Bazylinski, D. A., Frankel, R. B., Jannasch, H. W. 1988. Anaerobic magnetite production by a marine, magnetotactic bacterium. Nature 334:518–519.

Beijerinck, M. W. 1895. Über *Spirillum desulfuricans* als Ursache von Sulfatreduktion. Zentralblatt für Bakteriologie, Parasitenkunde Infektionskrankheiten und Hygiene, Abt. 2 1:1–9.

Beijerinck, M. W. 1921–1940. Verzammelde Geschriften. Nijhoff, Den Haag. 1–6.

Belkin, S., Wirsen, C. O., Jannasch, H. W. 1986. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. Appl. Environ. Microbiol. 51:1180–1185.

Belkin, S., Nelson, D. C., Jannasch, H. W. 1986. Symbiotic assimilation of CO_2 in two hydrothermal vent animals, the mussel *Bathymodiolus thermophilus* and the tube worm *Riftia pachyptila*. Biol. Bull. 170:110–121.

Benemann, J. R. 1973. Nitrogen fixation in termites. Science 181:164–165.

Bennett, A. F. 1978. Activity metabolism of the lower vertebrates. Annual Review of Physiology 40:447–469.

Berg, B., van Hofsten, B., Pettersson, G. 1972. Electron microscopic observations on the degradation of cellulose fibers by *Cellvibrio fulvus* and *Sporocytophaga myxococcoides*. Journal of Applied Bacteriology 35:215–219.

Bergensen, F. J., Hipsley, E. H. 1970. The presence of N_2-fixing bacteria in the intestine of man and animals. Journal of General Microbiology 60:61–65.

Berkeley, R. C. W., Lynch, J. M., Melling, J., Rutter, P. R., Vincent, B. 1980. Microbial adhesion to surfaces. Ellis Horwood. Chichester, UK.

Berndt, H., Ostwal, K.-P., Lalucat, J., Schumann, Ch., Mayer, F., Schlegel, H. G. 1976. Identification and physiological

characterization of the nitrogen fixing bacterium *Corynebacterium autotrophicum* GZ 29. Archives of Microbiology 108:17–26.

Bezdek, H. G., Carlucci, A. F. 1972. Surface concentration of marine bacteria. Limnology and Oceanography 17:566–569.

Bhuiya, Z. H., Walker, N. 1977. Autotrophic nitrifying bacteria in acid tea soils from Bangladesh and Sri Lanka. Journal of Applied Bacteriology 42:253–257.

Biebl, H., Pfennig, N. 1978. Growth yield of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Archives of Microbiology 117:9–16.

Bitton, G., Marshall, K. C. 1980. Adsorption of microorganisms to surfaces. New York. John Wiley.

Blakemore, R. P. 1975. Magnetotactic bacteria. Science 190:377–379.

Blakemore, R. P., Frankel, R. B., Kalmijn, A. J. 1980. South-seeking magnetotactic bacteria in the Southern Hemisphere. Nature 286:384–385.

Blakemore, R. P., Maratea, D., Wolfe, R. S. 1979. Isolation and pure culture of a freshwater magnetic spirillum in chemically defined medium. Journal of Bacteriology 140:720–729.

Bland, J. A., Staley, J. T. 1978. Observations on the biology of *Thiothrix*. Archives of Microbiology 117:79–87.

Blumershine, R. V., Savage, D. C. 1978. Filamentous microbes indigenous to the murine small bowel: A scanning electron microscopic study of their morphology and attachment to the epithelium. Microbial Ecology 4:95–103.

Bokor, R. 1933. Die Mikrobiologie der Szik- (Salz- oder Alkali-) Böden mit besonderer Berücksichtigung ihrer Fruchtbarmachung. 221–258. Fehäer, D. (ed.) Untersuchungen über die Mikrobiologie des Waldbodens. Berlin, Springer-Verlag.

Bothe, H., De Bruijn, F. J., Newton, W. E. (ed.). 1988. Nitrogen fixation: Hundred years after. Gustav-Fischer-Verlag. Stuttgart, Germany.

Bousfield, I. J., MacKenzie, A. R. 1976. Inactivation of bacteria by freeze-drying. Society for Applied Bacteriology Symposium Series 5:329–344.

Boyd, S. A., Shelton, D. R., Berry, D., Tiedje, J. M. 1983. Anaerobic biodegradation of phenolic compounds in digested sludge. Applied and Environmental Microbiology 46:50–54.

Boyer, E. V., Ingle, M. B., Merver, G. D. 1973. *Bacillus alcalophilus* subsp. *halodurans* subsp. nov.: An alkaline-amylase-producing, alkalophilic organism. International Journal of Systematic Bacteriology 23:238–242.

Boylen, C. W. 1973. Survival of *Arthrobacter crystallopoietes* during prolonged periods of extreme desiccation. Journal of Bacteriology 113:33–37.

Breznak, J. A., Brill, W. J., Mertins, J. W., Coppel, H. C. 1973. Nitrogen fixation in termites. Nature 244:577–579.

Brierley, C. L. 1977. Thermophilic microorganisms in extraction of metals from ores. 273–284. Underkofler L. A. (ed.) Developments in industrial microbiology. Proceedings of the 33rd General Meeting of the Society for Industrial Microbiology, vol 18. Washington. American Institute of Biological Sciences.

Brierley, C. L. 1978. Bacterial leaching. CRC Critical Reviews in Microbiology 6:207–262.

Brierley, C. L., Brierley, J. A. 1973. A chemoautotrophic and thermophilic microorganism isolated from an acid hot spring. Canadian Journal of Microbiology 19:183–188.

Brierley, J. A. 1978. Thermophilic iron-oxidizing bacteria found in copper leaching dumps. Applied and Environmental Microbiology 36:523–525.

Brierley, J. A., Lockwood, S. J. 1977. The occurrence of thermophilic iron-oxidizing bacteria in a copper leaching system. FEMS Microbiology Letters 2:163–165.

Briston, J., Courtois, D., Denis, F. 1974. Microbiological study of a hypersaline lake in French Somaliland. Applied Microbiology 27:819–822.

Brock, T. D. 1967. Life at high temperatures. Science 158:1012–1019.

Brock, T. D. 1969. Microbial growth under extreme conditions. Society for General Microbiology Symposium. 19:15–41.

Brock, T. D. 1970. High temperature systems. Annual Review of Ecology and Systematics 1:191–220.

Brock, T. D. 1978. Thermophilic microorganisms and life at high temperatures. New York. Springer-Verlag.

Brock, T. D. 1979. Ecology of saline lakes. 29–47. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Brock, T. D. (ed.). 1986. Thermophiles: General, molecular and applied microbiology. J. Wiley & Sons, Inc.. New York.

Brock, T. D. 1987. The study of microorganisms in situ: Progress and problems. 1–17. Fletscher, M., Gray, T. R. G., Jones, J. G. (ed.) Ecology of microbial communities. Cambridge, Cambridge University Press.

Brock, T. D., Boylen, K. L. 1973. Presence of thermophilic bacteria in laundry and domestic hot-water heaters. Applied Microbiology 25:72–76.

Brock, T. D., Freeze, H. 1969. *Thermus aquaticus* gen. n. and sp. n., a non-sporulating extreme thermophile. Journal of Bacteriology 98:289–297.

Brock, T. D., Brock, M. L., Bott, T. L., Edwards, M. R. 1971. Microbial life at 90°C: The sulfur bacteria of Boulder Spring. Journal of Bacteriology 107:303–314.

Brock, T. D., Brock, K. M., Belly, R. T., Weiss, R. L. 1972. *Sulfolobus:* A new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Archiv für Mikrobiologie 84:54–68.

Brown, A. D. 1978. Microbial water stress. Bacteriological Reviews 40:803–846.

Bryant, M. P., Wolin, E. A., Wolin, M. J., Wolfe, R. S. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Archiv für Mikrobiologie 59:20–31.

Bryant, M. P., Campbell, L. L., Reddy, C. A., Crabill, M. R. 1977. Growth of *Desulfovibrio* in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Applied and Environmental Microbiology 33:1162–1169.

Buchner, P. 1953. Endosymbiose der Tiere mit pflanzlichen Mikroorganismen. Basel, Birkhäuser Verlag.

Buder, J. 1919. Zur Biologie des Bacteriopurpurins und der Purpurbakterien. Jahrbücher der Wissenschaftlichen Botanik 58:525–628.

Burggraf, S., Jannasch, H. W., Nicolaus, B., Stetter, K. O. 1990. *Archaeoglobus profundus* sp. nov., represents a new species of within the sulfate-reducing archaebacteria. Syst. Appl. Microbiol. 10:24–28.

Butlin, K. R., Postgate, J. R. 1954. The microbiological formation of sulphur in Cyrenaican lakes. 112–122. Cloudsley-Thompson, J. L. (ed.) Biology of deserts. London, Institute of Biology.

Cagle, G. D. 1975. Fine structure and distribution of extracellular polymer surrounding selected aerobic bacteria. Canadian Journal of Microbiology 21:395–408.

Cappenberg, Th. E. 1974a. Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh-water lake. I. Field of observations. Antonie van Leeuwenhoek Journal of Microbiology and Serology 40:285–295.

Cappenberg, Th. E. 1974b. Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh-water lake. II. Inhibition experiments. Antonie van Leeuwenhoek Journal of Microbiology and Serology 40:297–306.

Castenholz, R. W. 1969. Thermophilic blue-green algae and the thermal environment. Bacteriological Reviews 33:476–504.

Castenholz, R. W. 1976. The effect of sulfide on the blue-green algae of hot springs. I. New Zealand and Iceland. Journal of Phycology 12:54–68.

Castenholz, R. W. 1977. The effect of sulfide on the blue-green algae of hot springs. II. Yellowstone National Park. Microbial Ecology 3:79–105.

Castenholz, R. W. 1979. Evolution and ecology of thermophilic microorganisms. 373–392. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Cavanaugh, C. M., Gardiner, S. L., Jones, M. L. S., Jannasch, H. W., Waterbury, J. B. 1981. Prokaryotic cells in the hydrothermal vent tube worm, *Riftia pachyptila:* Possible chemoautotrophic symbionts. Science 213:340–342.

Characklis, W. G., Marshall, K. C. 1990. Biofilms. Wiley Interscience. New York.

Chen, M., Wolin, M. J. 1977. Influence of CH_4 production by *Methanobacterium ruminantium* on the fermentation of glucose and lactate by *Selenomonas ruminantium*. Applied and Environmental Microbiology 34:756–759.

Chet, I., Mitchell, R. 1976. Ecological aspects of microbial chemotactic behavior. Annual Review of Microbiology 30:221–239.

Childress, J. J., Fisher, C. R., Brook, J. M., Kennicutt, M. C., II., Bridigare, R., Anderson, A. E. 1986. A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: Mussels fueled by gas. Science 233:1306–1308.

Chislett, M. E., Kushner, D. J. 1961. A strain of *Bacillus circulans* capable of growing under highly alkaline conditions. Journal of General Microbiology 24:187–190.

Clark, A. E., Walsby, A. E. 1978a. The occurrence of gas-vacuolate bacteria in lakes. Archives of Microbiology 118:223–228.

Clark, A. E., Walsby, A. E. 1978a. The development and vertical distribution of populations of gas-vacuolate bacteria in a eutrophic, monomictic lake. Archives of Microbiology 118:229–233.

Clark, F. E. 1967. Bacteria in soil. 15–49. Burges, A., Raw, F. (ed.) Soil biology. London, Academic Press.

Clarke, R. T. J. 1977. The gut and its micro-organisms. 35–71. Clarke, R. T. J., Bauchop, T. (ed.) Microbial ecology of the gut. London, Academic Press.

Coates, M. E., Fuller, R. 1977. The gnotobiotic animal in the study of gut microbiology. 311–346. Clark, R. T. J., Bauchop, T. (ed.) Microbial ecology of the gut. London, Academic Press.

Cohen, Y., Padan, E., Shilo, M. 1975. Facultative anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica*. Journal of Bacteriology 123:855–861.

Cohen, Y., Krumbein, W. E., Goldberg, M., Shilo, M. 1977. Solar lake (Sinai). I. Physical and chemical limnology. Limnology and Oceanography 22:597–608.

Cohen, Y., Rosenberg, E. 1989. Microbial Mats—Physiological ecology of benthic microbial communities. American Society for Microbiology. Washington, DC.

Cohen-Bazire, G., Stainer, R. Y. 1958. Inhibition of carotenoid synthesis in photosynthetic bacteria. Nature 181:250–252.

Cohen-Bazire, G., Kunisawa, R., Pfennig, N. 1969. Comparative study of the structure of gas vacuoles. Journal of Bacteriology 100:1049–1061.

Cohn, F. 1881. Gutachten über die Abwässer verschiedener Zuckerfabriken im Winter 1881. Quoted from Kolkwitz, 1906.

Colmer, A. R., Temple, K. L., Hinkle, M. E. 1950. An iron-oxidizing bacterium from the drainage of some bituminous coal mines. Journal of Bacteriology 59:317–328.

Cornax, R., Morinigo, M. A., Romero, P., Borrego, J. J. 1990. Survival of pathogenic microorganisms in seawater. Curr. Microbiology 220:293–298.

Costerton, J. W., Cheng, K. J. 1981. The Bacterial glycocalyx in nature and disease. Annual Review of Microbiology 35:299–324.

Costerton, J. W., Cheng, K.-J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., Marrie, T. J. 1987. Bacterial biofilms in nature and disease. Annual Review of Microbiology 41:435–464.

Costerton, J. W., Geesey, G. G., Cheng, K.-J. 1978. How bacteria stick. Scientific American 238:86–95.

Costerton, J. W., Ingram, J. M., Cheng, K.-J. 1974. Structure and function of the cell envelope of Gram-negative bacteria. Bacteriological Reviews 38:87–110.

Costerton, J. W., Irvin, R. T., Cheng, K. J. 1981a. The role of bacterial surface structures in pathogenesis. Critical Reviews in Microbiology 8:303–338.

Costerton, J. W., Marrie, T. J., Cheng, K. J. 1985. Phenomena of bacterial adhesion. 3–43. Savage, D. C., and Fletcher, M. (ed.) Bacterium adhesion. Plenum Press. New York.

Cross, T. 1968. Thermophilic actinomycetes. Journal of Applied Bacteriology 31:36–53.

Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiological Review 53:121–147.

Cundell, A. M., Sleeter, T. D., Mitchell, R. 1977. Microbial populations associated with the surface of the brown alga *Ascophyllum nodosum*. Microbial Ecology 4:81–91.

Dazzo, F. B., Yanke, W. E., Brill, W. J. 1978. Trifoliin: A *Rhizobium* recognition protein from white clover. Biochimica et Biophysica Acta 539:276–286.

De Bont, J. A. M., Mulder, E. G. 1974. Nitrogen fixation and co-oxidization of ethylene by a methane-utilizing bacterium. Journal of General Microbiology 83:113–121.

Degens, E. T., Ross, D. A. 1974. The Black Sea: Geology, chemistry and biology. Memoir 20. Tulsa, American Association of Petroleum.

Dehority, B. A. 1971. Carbon dioxide requirement of various species of rumen bacteria. Journal of Bacteriology 105:70–76.

DeLong, E. F., Wickham, G. S., Pace, N. R. 1988. Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363.

Deming, J. W., Colwell, R. R. 1982. Barophilic bacteria associated with digestive tracts of abyssal holothurians. Appl. Environ. Microbiol. 44:1222–1230.

Deming, J. W., Tabor, P. S., Colwell, R. R. 1981. Barophilic growth from intestinal tracts of deep-sea invertebrates. Microb. Ecol. 7:85–94.

Demoll, R., Liebmann, H. 1952. Über die Verteilung von *Sphaerotilus natans* im Fluss. Schweizerische Zeitschrift für Hydrologie 14:289–297.

Dietz, A. S., Yayanos, A. A. 1978. Silica gel for isolating and studying bacteria under hydrostatic pressure. Appl. Environ. Microbiol. 36:966–968.

Distel, D. L., Lane, D. J., Olsen, G. J., Giovannoni, S. J., Pace, B., Pace, N. R., Stahl, D. A., Felbeck, H. 1988. Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences. J. Bacteriol. 170:2506–2510.

Dondero, N. C. 1961. *Sphaerotilus*, its nature and economic significance. Advances in Applied Microbiology 3:77–107.

Dondero, N. C. 1975. The *Sphaerotilus-Leptothrix* group. Annual Review of Microbiology 29:407–465.

Drasar, B. S., Barrow, P. A. 1985. Intestinal microbiology (Aspects of Microbiology 10). England. van Nostrand Reinholf.

Drasar, B. S., Hill, M. J. 1974. Human intestinal flora. London, Academic Press.

Duckworth, R. B. 1975. Water relations in foods. Proceedings of an International Symposium in Glasgow, September 1974. London, Academic Press.

Duda, V. I., Makaer'eva, D. E. 1977. Morphogenesis and function of gas caps on spores of anaerobic bacteria of the genus *Clostridium*. In Russian, with English summary. Mikrobiologiya 46:689–694.

Dugan, P. R., MacMillan, C. B., Pfister, R. M. 1970. Aerobic heterotrophic bacteria indigenous to pH 2.8 acid mine water: Microscopic examination of acid streamers. Journal of Bacteriology 101:973–981.

Dundas, I. D., Larsen, H. 1962. The physiological role of the carotenoid pigments of *Halobacterium salinarium*. Archiv für Mikrobiologie 44:233–239.

Dundas, I. E. D. 1977. Physiology of Halobacteriaceae. Advances in Microbial Physiology 15:85–120.

Ebisu, S., Kato, K., Kotani, S., Misaki, A. 1975. Structural differences in fructans elaborated by *Streptococcus mutans* and *S. salivarius*. Journal of Biochemistry 78:879–887.

Ellwood, D. C., Hedger, J. N., Latham, M. H., Lynch, J. M., Slater, J. H. 1980. Contemporary microbial ecology. London, Academic Press.

Ensign, J. C., Wolfe, R. S. 1964. Nutritional control of morphogenesis in *Arthrobacter crystallopoietes*. Journal of Bacteriology 87:924–932.

Eutick, M. L., O'Brien, R. W., Slaytor, M. 1978. Bacteria from the gut of Australian termites. Applied and Environmental Microbiology 35:823–828.

Fairbairn, D. 1970. Biochemical adaptation and loss of genetic capacity in helminth parasites. Biological Reviews 45:29–72.

Felbeck, H., Childress, J. J., Somero, G. N. 1981. Calvin-Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. Nature 293:291–293.

Fenchel, T. M. 1969. The ecology of marine microbenthos. IV. Structure and function of the benthic ecosystem. Ophelia 6:1–182.

Fenchel, T. M., Jørgensen, B. B. 1977. Detritus food chains in aquatic ecosystems: The role of bacteria. Advances in Microbiol Ecology 1:1–58.

Fenchel, T. M., Riedl, R. J. 1970. The sulfide system: A new biotic community underneath the oxidized layer of marine sand bottoms. Marine Biology 7:255–268.

Fenchel, T. M., Staarup, B. J. 1971. Vertical distribution of photosynthetic pigments and the penetration of light in marine sediments. Oikos 22:172–182.

Ferry, J. G., Wolfe, R. S. 1976. Anaerobic degradation of benzoate to methane by a microbial consortium. Archives of Microbiology 107:33–40.

Fiala, G., Stetter, K. O., Jannasch, H. W., Langworthy, T. A., Madon, J. 1986. *Staphylothermus marinus* sp. nov. represents a novel genus of extremely thermophilic submarine heterotrophic archaebacteria growing up to 98°C. System. Appl. Microbiol. 8:106–113.

Fisher, C. R., Childress, J. J., Minnich, E. 1989. Autotrophic carbon fixation by the chemoautotrophic symbionts of *Riftia pachyptila*. Biol. Bull. 177:372–385.

Fletcher, M., Loeb, G. I. 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Applied and Environmental Microbiology 37:67–72.

Fletcher, M., Marshall, K. C. 1982. Are solid surfaces of ecological significance to aquatic bacteria? Marshall, K. C. (ed.) Advances in microbial ecology. New York. Plenum Press.

Fliermans, C. B., Brock, T. D. 1972. Ecology of sulfur-oxidizing bacteria in hot acid soils. Journal of Bacteriology 111:343–350.

Focht, D. D., Verstraete, W. 1977. Biochemical ecology of nitrification and denitrification. Advances in Microbial Ecology 1:135–214.

Foglesong, M. A., Walker, D. H., Jr., Puffer, J. S., Markovetz, A. J. 1975. Ultrastructural morphology of some prokaryotic microorganisms associated with the hindgut of cockroaches. Journal of Bacteriology 123:336–345.

Foster, J. W. 1949. Chemical activities of fungi. New York. Academic Press.

Frankel, R. B., Blakemore, R. P., Wolfe, R. S. 1979. Magnetite in freshwater magnetotactic bacteria. Science 203:1355–1356.

Fridovich, I. 1974. Superoxide dismutases. Advances in Enzymology 41:35–97.

Fridovich, I. 1975. Oxygen: Boon and bane. American Scientist 63:54–59.

Fridovich, I. 1976. Oxygen radicals, hydrogen peroxide, and oxygen toxicity. 239–277. Pryor, W. A. (ed.) Free radicals in biology, vol. 1. New York. Academic Press.

Gerber, N. N. 1975. Prodigiosin-like pigments. CRC Critical Reviews in Microbiology 3:469–485.

Germaine, G. R., Chludzinski, A. M., Schachtele, C. F. 1974. *Streptococcus mutans* dextransucrase: Requirement for primer dextran. Journal of Bacteriology 120:287–294.

Gillespy, T. G., Thorpe, R. H. 1968. Occurrence and significance of thermophiles in canned foods. Journal of Applied Bacteriology 31:59–65.

Giovannoni, S. J., Britschgi, T. B., Moyer, C. L., Field, K. G. 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature 345(6270):60–63.

Giovannoni, S. J., DeLong, E. F., Olsen, G. J., Pace, N. R. 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. 170:720–726.

Golovacheva, R. S. 1976. Thermophilic nitrifying bacteria from hot springs. [In Russian, with English summary.] Mikrobiologiya 45:377–379.

Golovacheva, R. S. 1979. Attachment of *Sulfobacillus thermosulfidooxidans* cells to the surface of sulfide minerals. [In Russian, with English summary.] Mikrobiologiya 48:528–533.

Golovacheva, R. S., Karavaiko, G. I. 1978. *Sulfobacillus,* a new genus of thermophilic sporeforming bacteria. [In Russian, with English summary.] Mikrobiologiya 47:815–822.

Gorini, L. 1960. Antagonism between substrate and repressor in controlling the formation of a biosynthetic enzyme. Proceedings of the National Academy of Sciences of the United States of America 46:682–690.

Gorlenko, W. M., Dubinina, G. A., Kuznezow, S. J. 1977. Ecology of aquatic microorganisms. [In Russian.] Moscow, Nauka.

Goto, E., Kodama, T., Minoda, Y. 1977. Isolation and culture conditions of thermophilic hydrogen bacteria. Agricultural and Biological Chemistry 41:685–690.

Gottlieb, S. F. 1971. Effect of hyperbaric oxygen on microorganisms. Annual Review of Microbiology 25:111–152.

Grant, W. D., Mills, A. A., Schofield, A. K. 1979. An alkalophilic species of *Ectothiorhodospira* from a Kenyan soda lake. Journal of General Microbiology 110:137–142.

Grassle, J. F. 1986. The ecology of deep-sea hydrothermal vent communities. Adv. Mar. Biol. Ecol. 23:301–362.

Greenberg, E. P., Hastings, J. W., Ulitzur, S. 1979. Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. Archives of Microbiology 120:87–91.

Griffin, D. M. 1981. Water and microbial stress. Advances in Microbial Ecology 5:91–136.

Griffin, D. M., Luard, E. J. 1979. Water stress and microbial ecology. 49–63. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Gromet-Elhanan, Z. 1977. Electron transport and photophosphorylation in photosynthetic bacteria. 637–662. Trebst, A., and Avron, M. (ed.) Encyclopaedia of plant physiology, vol. 5. Berlin, Springer-Verlag.

Gunner, H. B., Alexander, M. 1964. Anaerobic growth of *Fusarium oxysporum*. Journal of Bacteriology 87:1309–1316.

Hansen, M. H., Ingvorsen, K., Jørgensen, B. B. 1978. Mechanisms of hydrogen sulfide release from coastal marine sediments to the atmosphere. Limnology and Oceanography 23:68–76.

Harder, W., Veldkamp, H. 1968. Physiology of an obligate psychrophilic marine *Pseudomonas* species. Journal of Applied Bacteriology 31:12–33.

Harder, W., Veldkamp, H. 1971. Competition of marine psychrophilic bacteria at low temperatures. Antonie van Leeuwenhoek Journal of Microbiology and Serology 37:51–63.

Hardie, J. M., Bowden, G. H. 1974. The normal microbial flora of the mouth. 47–83. Skinner, F. A., Carr, J. G. (ed.) The normal microbial flora of man. London, Academic Press.

Harold, R., Stanier, R. Y. 1955. The genera *Leucothrix* and *Thiothrix*. Bacteriological Reviews 19:49–58.

Harris, R. H., Mitchell, R. 1973. The role of polymers in microbial aggregation. Annual Review of Microbiology 27:27–50.

Hassan, H. M., Fridovich, I. 1979. Superoxide dismutase and its role for survival in the presence of oxygen. 179–193. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Hastings, J. W., Nealson, K. H. 1977. Bacterial bioluminescence. Annual Review of Microbiology 31:549–595.

Hattori, T., Ishida, Y., Maruyama, Y., Morita, R. Y., Uchida, A. 1989. Recent advances in microbial ecology. Proceedings of the 5th International Symposium on Microbial Ecology, Jap. Sci. Soc. Press. Tokyo.

Hazelbauer, G. L. 1988. The bacterial chemosensory system. Canadian Journal of Microbiology 34:466–474.

Heinen, W. 1974. Proceedings of the first European workshop on microbial adaptation to extreme environments. Biosystems 6:57–80.

Held, A. A. 1970. Nutrition and fermentative energy metabolism of the water mold *Aqualinderella fermentans*. Mycologia 62:339–358.

Held, A. A., Emerson, R., Fuller, M. S., Gleason, F. H. 1969. *Blastocladia* and *Aqualinderella*: Fermentative water molds with high carbon dioxide optima. Science 165:706–708.

Henrici, A. T., Johnson, D. E. 1935. Studies of freshwater bacteria. II. Stalked bacteria, a new order of Schizomycetes. Journal of Bacteriology 30:61–86.

Herdman, M., Janvier, M., Waterbury, J. B., Rippka, R., Stanier, R. Y. 1979. Deoxyribonucleic acid base composition of cyanobacteria. Journal of General Microbiology 111:63–71.

Heukelekian, H., Heller, A. 1940. Relation between food concentration and surface for bacterial growth. Journal of Bacteriology 40:547–558.

Heumann, W., Marx, R. 1964. Feinstruktur und Funktion der Fimbrien bei dem sternbildenden Bakterium *Pseudomonas echinoides*. Archiv für Mikrobiologie 47:325–337.

Hirsch, P. 1974. Budding bacteria. Annual Review of Microbiology 28:392–444.

Hirsch, P. 1979. Life under conditions of low nutrient concentrations. 357–372. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Hirsch, P., Pankratz, St. H. 1970. Study of bacterial populations in natural environments by use of submerged electron microscope grids. Zeitschrift für Allgemeine Mikrobiologie 10:589–605.

Hobson, P. N. 1988. The rumen microbial ecosystem. Elsevier Science Publishers, London.

Hochachka, P. W., Mustafa, T. 1972. Invertebrate facultative anaerobiosis. Science 178:1056–1060.

Hochachka, P. W., Somero, G. N. 1973. Strategies of biochemical adaptation. London, W.B. Saunders.

Hoffmann, C. 1942. Beiträge zur Vegetation des FarbstreifenSandwattes. Kieler Meeresforschungen 4:85–108.

Holben, W. E., Jansson, J., Chelm, B., Tiedje, T. 1988. DNA probe method for detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. 54:703–711.

Holdemann, L. V., Cato, E. P., Moore, W. E. C. 1977. Anaerobe laboratory manual, 4th ed. Blacksburg, Virginia Polytechnic Institute and State University.

Horikoshi, K., Akiba, T. 1982. Alkalophilic microorganisms. A new microbial world. Japan Scientific Societies Press, Berlin. Springer, New York.

Huber, R., Huber, G., Segerer, A., Seger, J., Stetter, K. O. 1987a. Aerobic and anaerobic extremely thermophilic autotrophs. 44–51. van Verseveld, H. W., and Duine, J. A. (ed.) Proceedings of the 5th International Symposium. Martinus Nijhoff Publ., Dordrecht.

Huber, R., Kristjansson, J., Stetter, K. O. 1987b. *Pyrobaculum* gen. nov., a new genus of neutrophilic, rod-shaped

archaebacteria from continental solfataras growing optimally at 100°C. Arch. Microbiol. 149:95–101.

Huber, R., Kurr, M., Jannasch, H. W., Stetter, K. O. 1989. A novel group of abyssal methanogenic archaebacteria (*Methanopyrus*) growing at 110°C. Nature 342:833–834.

Huber, R., Langworthy, T. A., König, H., Thomm, M., Woese, C. R., Sleytr, U. B., Stetter, K. O. 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. 144:324–333.

Huber, R., Stoffers, P., Cheminee, J. L., Richnow, H. H., Stetter, K. O. 1990. Hyperthermophilic archaebacteria within th crater and open-sea plume of erupting Macdonald Seamount. Nature 345:179–181.

Hughes, M. N., Poole, R. K. 1989. Metals and microorganisms. London, Chapman and Hall.

Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriological Reviews 14:1–49.

Hungate, R. E. 1962. Ecology of bacteria. 95–119. Gunsalus, J. C., and Stanier, R. Y. (ed.) The bacteria, vol. IV: The physiology of growth. New York. Academic Press.

Hungate, R. E. 1966. The rumen and its microbes. New York. Academic Press.

Hungate, R. E. 1967. Hydrogen as an intermediate in the rumen fermentation. Archiv für Mikrobiologie 59:158–164.

Hungate, R. E. 1975. The rumen microbial ecosystem. Annual Review of Ecology and Systematics 6:39–66.

Hussain, H. M. 1973. Ökologische Untersuchungen über die Bedeutung thermophiler Mikroorganismen für die Selbsterhitzung von Heu. Zeitschrift für Allgemeine Mikrobiologie 13:323–334.

Hustede, E., Liebergesell, M., Schlegel, H. G. 1989. The photophobic response of various sulfur and nonsulfur purple bacteria. Photochemistry and Photobiology 50:809–815.

Iannotti, E. L., Kafkewit, D., Wolin, M. J., Bryant, M. P. 1973. Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: Changes caused by interspecies transfer of H_2. Journal of Bacteriology 114:1231–1240.

Imhoff, J. F., Trüper, H. G. 1977. *Ectothiorhodospira halochloris* sp. nov., a new extremely halophilic bacterium containing bacteriochlorophyll b. Archives of Microbiology 114:115–121.

Inniss, W. E. 1975. Interaction of temperature and psychrophilic microorganisms. Annual Review of Microbiology 29:445–465.

Inniss, W. E., Ingraham, J. L. 1978. Microbial life at low temperatures: Mechanisms and molecular aspects. 73–104. Kushner, D. J. (ed.) Microbial life in extreme environments. London, Academic Press.

Jaggar, J. 1983. Physiological effects of near-ultraviolet radiation on bacteria. Photochemical and Photobiological Reviews 7:1–75.

Jannasch, H. W. 1955. Zur Ökologie der zymogenen planktischen Bakterienflora natürlicher Gewässer. Archiv für Mikrobiologie 23:146–180.

Jannasch, H. W. 1957. Die bakterielle Rotfärbung der Salzseen des Wadi Natrun (Ägypten). Archiv für Hydrobiologie 53:425–433.

Jannasch, H. W. 1958. Studies of planktonic bacteria by means of a direct membrane filter method. Journal of General Microbiology 18:609–620.

Jannasch, H. W. 1960. Versuche über Denitrifikation und die Verfügbarketi des Sauerstoffes in Wasser und Schlamm. Archiv für Hydrobiologie 56:335–369.

Jannasch, H. W. 1967. Enrichment of aquatic bacteria in continuous culture. Archiv für Mikrobiologie 59:165–173.

Jannasch, H. W. 1977. Growth kinetics of aquatic bacteria. Society for Applied Bacteriology Symposium Series 6:55–68.

Jannasch, H. W. 1978. Microorganisms and their aquatic habitat. 17–24. Krumbein, W. E. (ed.) Environmental biogeochemistry and geomicrobiology, vol. 1. Ann Arbor, MI. Ann Arbor Scientific Publications.

Jannasch, H. W. 1979. Microbial ecology of aquatic low-nutrient habitats. 243–260. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Jannasch, H. W. 1984. Microbial processes at deep sea hydrothermal vents. 677–709. Rona, P. A., Bostrom, K., Laubier, L., and Smith, K. L. (ed.) Hydrothermal processes at seafloor spreading centers. Plenum Publ. New York.

Jannasch, H. W. 1989. Chemosynthetically sustained ecosystems in the deep sea. 147–166. Schlegel, H. G., and Bowien, B. (ed.) Autotrophic bacteria. Springer-Verlag. Berlin.

Jannasch, H. W., Huber, R., Belkin, S., Stetter, K. O. 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch. Microbiol. 150:103–104.

Jannasch, H. W., Mateles, R. I. 1974. Experimental bacterial ecology studies in continuous culture. Advances in Microbial Physiology 11:165–212.

Jannasch, H. W., Nelson, D. C., Wirsen, C. O. 1989. Massive natural occurrence of unusually large bacteria (*Beggiatoa* sp.) at a hydrothermal deep-sea vent site. Nature 342:834–836.

Jannasch, H. W., Pritchard, P. H. 1972. The role of inert particulate matter in the activity of aquatic microorganisms. Melchiorri-Santolinie, U., and Hopton, J. W. (ed.) Detritus and its role in aquatic ecosystems. Memorie dell'Istituto Italiano di Idrobiologia Dott Marco de Marchi Pallanza Italy Suppl. 29:289–308.

Jannasch, H. W., Taylor, C. D. 1984. Deep-sea microbiology. Ann. Rev. Microbiol. 37:487–514.

Jannasch, H. W., Trüper, H. G., Tuttle, J. H. 1974. The microbial sulfur cycle in the Black Sea. 419–425. Dergens, E. T., Ross, D. A. (ed.) The Black Sea: Its geology, chemistry and biology, Memoir 20. Tulsa, American Association of Petroleum.

Jannasch, H. W., Wirsen, C. O. 1973. Deep-sea microorganisms: In situ response to nutrient enrichment. Science 180:641–643.

Jannasch, H. W., Wirsen, C. O. 1977. Retrieval of concentrated and undecompressed microbial populations from the deep sea. Applied and Environmental Microbiology 33:642–646.

Jannasch, H. W., Wirsen, C. O. 1979. Chemosynthetic primary production at East Pacific sea floor spreading centers. Bioscience 29:592–598.

Jannasch, H. W., Wirsen, C. O. 1981. Morphological survey of microbial mats near deep sea thermal vents. Appl. Environ. Microbiol. 41:528–538.

Jannasch, H. W., Wirsen, C. O. 1982. Microbial activities in undecompressed and decompressed deep-seawater samples. Appl. Environ. Microbiol. 43:1116–1124.

Jannasch, H. W., Wirsen, C. O., Taylor, C. D. 1976. Undecompressed microbial populations from the deep sea. Applied and Environmental Microbiology 32:360–367.

Jannasch, H. W., Wirsen, C. O., Taylor, C. D. 1982. Deep-sea bacteria: isolation in the absence of decompression. Science 216:1315–1317.

Jones, O. T. G. 1977. Electron transport and ATP synthesis in the photosynthetic bacteria. 151–183. Haddock, B. A., and Hamilton, W. A. (ed.) Microbial energetics. Cambridge, Cambridge University Press.

Jones, W. J., Leigh, J. A., Mayer, F., Woese, C. R., Wolfe, R. S. 1983. *Methanococcus jannaschii,* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. 163:154–261.

Jones, W. J., Stugard, C. E., Jannasch, H. W. 1989. Comparison of thermophilic methanogens from submarine hydrothermal vents. Arch. Microbiol. 151: 314–318.

Jørgensen, B. B. 1982. Ecology of the bacteria of the sulfur cycle with special reference to anoxic-oxic interface environments. Phil. Tran. R. Soc. Lond., Biol. Sci. 298:543–561.

Jørgensen, B. B., Zawacki, L. X., Jannasch, H. W. 1990. Thermophilic bacterial sulfate reduction in deep-sea sediments at the Guaymas Basin hydrothermal vent site (Gulf of California). Deep-Sea Res. 37:695–710.

Jørgensen, B. B., Revsbech, N. P. 1983. Colorless sulfur bacteria, *Beggiatoa* spp. and *Thiovulum* spp., in O_2 and H_2S microgradients. Appl. Environ. Microbiol. 45:1261–1270.

Kato, G., Maruyama, Y., Nakamura, M. 1979. Role of lectins and lipopolysaccharides in the recognition process of specific legume-*Rhizobium* symbiosis. Agricultural and Biological Chemistry 43:1085–1092.

Kefford, B., Kjelleberg, S., Marshall, K. C. 1982. Bacterial scavenging: utilization of fatty acids localized at a solid-liquid interface. Arch. Microbiol. 133:257–260.

Kelly, M. T., Brock, T. D. 1969. Physiological ecology of *Leucothrix mucor*. Journal of General Microbiology 59:153–162.

Kjelleberg, S., Hermansson, M., Mardén, P., Jones, G. W. 1987. The transient phase between growth and non growth of heterotrophic bacteria, with emphasis on the marine environment. Annual Review of Microbiology 41:25–49.

Kluyver, A. J., Donker, H. J. L. 1925. The unity of the chemistry of the fermentative sugar dissimilation processes of microbes. Proceedings of the Royal Academy of Amsterdam 28:297–313.

Kluyver, A. J., Donker, H. J. L. 1926. Die Einheit in der Biochemie. Chemie der Zelle und Gewebe 13:134–190.

Koch, A. L. 1979. Microbial growth in low concentrations of nutrients. 261–279. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Kolkwitz, R. 1904–1906. Mykologie und Reinigung der städtischen und der Zuckerfabriksabwässer. 391. Lafar, F. (ed.) Handbuch der technischen Mykologie, vol. 3. Jena, Gustav Fischer Verlag.

Korhonen, T. K., Nurmiaho, E.-L., Tuovinen, O. H. 1978. Fimbriation in *Thiobacillus* A2. FEMS Microbiology Letters 3:195–198.

Koshland, D. E., Jr. 1974. The chemotactic response in bacteria. 133–160. Jaenicke, L. (ed.) Biochemistry of sensory functions. Berlin, Springer-Verlag.

Koshland, D. E., Jr. 1976. Bacterial chemotaxis as a simple model for a sensory system. Trends in Biochemical Sciences 1:1–3.

Koshland, D. E. 1980. Bacterial chemotaxis as a model behavioral system. New York. Raven Press.

Koshland, D. E. 1981. Biochemistry of sensing and adaptation in a simple bacterial system. Annual Review of Biochemistry 50:765–782.

Krinsky, N. I. 1979. Carotenoid pigments: Multiple mechanisms for coping with the stress of photosensitized oxidations. 163–177. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Krul, J. M., Hirsch, P., Staley, J. T. 1970. *Toxothrix trichogenes* (Chol.) Beger et Bringmann: The organism and its biology. Antonie van Leeuwenhoek Journal of Microbiology and Serology 36:409–420.

Krulwich, T. A., Guffanti, A. A. 1983. Physiology of acidophilic and alkalophilic bacteria. Advances in Microbial Physiology 24:173–214.

Kuenen, J. G., Boonstra, H. G., Schröder, J., Veldkamp, H. 1977. Competition for inorganic substrates among chemoorganotrophic and chemolithotrophic bacteria. Microbial Ecology 3:119–130.

Kushner, D. J. 1971. Life in extreme environments. 485–491. Buvet, R., and Ponnamperuma, C. (ed.) Chemical evolution and origin of life. Amsterdam, North-Holland.

Kushner, D. J. 1978. Life in high salt and solute concentrations: Halophilic bacteria. 317–368. Kushner, D. J. (ed.) Microbial life in extreme environments. London, Academic Press.

Kuznezow, S. I. 1959. Die Rolle der Mikroorganismen im Stoffkreislauf der Seen. Berlin, VEB Deutscher Verlag der Wissenschaften.

Kuznezow, S. I. 1977. Trends in the development of ecological microbiology. 1–48. Droop, M. R., and Jannasch, H. W. (ed.) Advances in aquatic microbiology. London, Academic Press.

Langworthy, T. A. 1978. Microbial life in extreme pH values. 279–315. Kushner, D. J. (ed.) Microbial life in extreme environments. London, Academic Press.

Lanyi, J. K. 1979. Physical-chemical aspects of salt-dependence in Halobacteria. 93–107. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Lapage, S. P., Shelton, J. E., Mitchell, T. G., MacKenzie, A. R. 1970. Culture collections and the preservation of bacteria. 135–228. Norris, J. R., and Ribbons, D. W. (ed.) Methods in microbiology, vol. 3A. London, Academic Press.

la Riviére, J. W. M. 1963. Cultivation and properties of *Thiovulum majus* Hinze. 61–72. Oppenheimer, C. H. (ed.) Marine microbiology. Springfield, Illinois. Charles C. Thomas.

la Riviére, J. W. M. 1965. Enrichment of colorless sulfur bacteria. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1, Suppl. 1:17–27.

Larsen, H. 1967. Biochemical aspects of extreme halophilism. Advances in Microbial Physiology 1:97–132.

Larsen, H. 1971. Halophilism, microbial. McGraw-Hill encyclopedia of science and technology, 3rd ed. New York. McGraw-Hill.

Larsen, H. 1973. The halobacteria's confusion to biology. The fourth A.J. Kluyver memorial lecture delivered before the Netherlands Society for Microbiology, April 1972 at

the Delft University of Technology. Antonie van Leeuwenhoek Journal of Microbiology and Serology 39:383–396.

Larsen, H. 1986. Halophilic and holotolerant microorganisms—an overview and historical perspective. FEMS Microbiology Reviews 39:3–7.

Latham, M. J., Wolin, M. J. 1977. Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*. Applied and Environmental Microbiology 34:297–301.

Latham, M. J., Brooker, B. E., Pettipher, G. L., Harris, P. J. 1978. *Ruminococcus flavefaciens* cell coat and adhesion to cotton cellulose and to cell walls in leaves of perennial ryegrass (*Lolium perenne*). Applied and Environmental Microbiology 35:156–165.

Leathen, W. W., Braley, S. A., Sr., McIntyre, L. D. 1953. The role of bacteria in the formation of acid from certain sulfuritic constituents associated with bituminous coal. II. Ferrous iron oxidizing bacteria. Applied Microbiology 1:65–68.

Lee, A., Phillips, M. 1978. Isolation and cultivation of spirochetes and other spiral-shaped bacteria associated with the cecal mucosa of rats and mice. Applied and Environmental Microbiology 35:610–613.

Leifson, E. 1962. The bacterial flora of distilled and stored water. I. General observations, techniques and ecology. International Bulletin of Bacteriological Nomenclature and Taxonomy 12:133–153.

Le Roux, N. W., Wakerley, D. S., Hunt, S. D. 1977. Thermophilic *Thiobacillus*-type bacteria from Icelandic thermal areas. Journal of General Microbiology 100:197–201.

Levi, P., Linkletter, A. 1989. Metals, microorganisms and biotechnology. Hughes, M. N., and Poole, R. K. (ed.) Metals and microorganisms. London, Chapman and Hall.

Liener, I. E. 1976. Phytohemagglutinins (phytolectins). Annual Review of Plant Physiology 27:291–319.

Loesche, W. J. 1969. Oxygen sensitivity of various anaerobic bacteria. Applied Microbiology 18:723–727.

Lovley, D. R., Phillips, E. J. P. 1987. Novel mode of microbial energy metabolism: Organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl. Environ. Microbiol. 54:1472–1480.

Madigan, M. T., Brock, T. D. 1977. Adaptation by hot springs phototrophs to reduced light intensities. Archives of Microbiology 113:111–120.

Mandel, M., Leadbetter, E. R., Pfennig, N., Trüper, H. G. 1971. Deoxyribonucleic acid base compositions of phototrophic bacteria. International Journal of Systematic Bacteriology 21:222–230.

Mann, S., Sparks, N. H., Frankel, R. B., Bazylinski, D. A., Jannasch, H. W. 1990. Biomineralization of ferrimagnetic greigite (FeS) and iron pyrite (FeS) in a magnetotactic bacterium. Nature 343:258–261.

Marchlewitz, B., Schwartz, W. 1961. Untersuchungen über die Mikroben-Assoziation saurer Grubenwässer. Zeitschrift für Allgemeine Mikrobiologie 1:100–114.

Marples, M. J. 1965. The ecology of the human skin. Springfield, Illinois. Charles C. Thomas.

Marples, M. J. 1974. The normal microbial flora of the skin. 7–12. Skinner, F. A., and Carr, J. G. (ed.) The normal microbial flora of man. London, Academic Press.

Marples, M. J. 1976. Life on the human skin. Scientific American 220:108–115.

Marquis, R. E. 1976. High-pressure microbial physiology. 159–241. Rose, A. H., and Tempest, D. W. (ed.) Advances in microbial physiology, vol. 14. London, Academic Press.

Marquis, R. E., Matsumara, P. 1978. Microbial life under pressure. 105–158. Kushner, D. J. (ed.) Microbial life in extreme environments. London, Academic Press.

Marshall, K. C. 1976. Interfaces in microbial ecology. Cambridge, London, Harvard University Press.

Marshall, K. C. 1979. Growth at interfaces. 281–290. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Marshall, K. C. (ed.). 1984. Microbial adhesion and aggregation. Dahlem workshop reports, Life Science, vol. 31. Springer, New York.

Martin, H. H. 1969. Die Struktur der Zellwand bei Gramnegativen Bakterien. Arzneimittel-Forschung 19:266–272.

Martin, S. M. 1964. Conservation of microorganisms. Annual Review of Microbiology 18:1–16.

Marx, J. L. 1977. Looking at lectins: Do they function in recognition processes?. Science 196:1429–1430.

Marx, R., Heumann, W. 1962. Uber Geisselfeinstrukturen und Fimbrien bei zwei *Psuedomonas*-Stämmen. Archiv für Mikrobiologie 43:245–254.

Matin, A. 1979. Microbial regulatory mechanisms at low nutrient concentrations as studies in chemostat. 323–339. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Matin, A., Auger, E. A., Blum, P. H., Schultz, J. E. 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Annual Review of Microbiology 43:293–316.

Matin, A., Veldkamp, H. 1978. Physiological basis of the selective advantage of a *Spirillum* sp. in a carbon-limited environment. Journal of General Microbiology 105:187–197.

Matin, A., Veldhuis, C., Stegemann, V., Veenhuis, M. 1979. Selective advantage of a *Spirillum* sp. in a carbon-limited environment. Accumulation of poly-β-hydroxybutyric acid and its role in starvation. Journal of General Microbiology 112:349–355.

Matthews, M. M., Sistrom, W. R. 1959. Function of carotenoid pigments in non-photosynthetic bacteria. Nature 184:1892–1893.

Mayer, F. 1971. Elektronenmikroskopische Untersuchung der Fimbrienkontraktion bei dem sternbildenden Bodenbakterium *Pseudomonas echinoides*. Archiv für Mikrobiologie 76:166–173.

Mayer, F., Schmitt, R. 1971. Elektronenmikroskopische, diffraktometrische und disc-elektrophoretische Untersuchungen an Fimbrien des sternbildenden Bodenbakteriums *Pseudomonas echinoides* und einer nicht-sternbildenden Mutante. Archiv für Mikrobiologie 79:311–326.

Mazanec, K., Kocur, M., Martinec, T. 1965. Electron microscopy of ultrathin sections of *Sporosarcina ureae*. Journal of Bacteriology 90:808–816.

McBee, R. H. 1977. Fermentation in the hindgut. 185–222. Clarke, R. T. J., and Bauchop, T. (ed.) Microbial ecology of the gut. London, Academic Press.

McLeod, R. A. 1968. On the role of inorganic ions in the physiology of marine bacteria. Advances in the Microbiology of the Sea 1:95.

Meers, J. L. 1973. Growth of bacteria in mixed cultures. CRC Critical Reviews in Microbiology 2:139–184.

Megusar, F., Gantar, M. (ed.). 1986. Perspectives in microbial ecology. Slovene Society for Microbiology. Ljubljana, Yugoslavia.

Menzel, D. W., Ryther, J. H. 1970. Distribution and cycling of organic matter in the oceans. Hood, D. W. (ed.) Organic matter in natural waters. Alaska. Institute of Marine-Sciences.

Millar, W. N. 1973. Heterotrophic bacterial population in acid coal mine water: *Flavobacterium acidurans*, sp. n. International Journal of Systematic Bacteriology 23:142–150.

Miller, R. E., Simons, L. A. 1962. Survival of bacteria after twenty-one years in the dried state. Journal of Bacteriology 84:1111–1114.

Miller, W. D. 1890. The micro-organisms of the human mouth, Philadelphia 1890 [unaltered reprint from original work]. Basel, Karger.

Minato, H., Suto, T. 1978. Technique for fractionation of bacteria in rumen microbial ecosystem. II. Attachment of bacteria isolated from bovine rumen to cellulose powder in vitro and elution of bacteria attached therefrom. Journal of General and Applied Microbiology 24:1–16.

Mitskevich, I. N. 1979. The total number of biomass of microorganisms in deep waters of the Black Sea. [In Russian, with English summary.] Mikrobiologiya 48:552–557.

Moore, W. E. C., Holdeman, L. V. 1974. Human fecal flora: The normal flora of 20 Japanese-Hawaiians. Applied Microbiology 27:961–979.

Morita, R. Y. 1975. Psychrophilic bacteria. Bacteriological Reviews 39:144–167.

Morita, R. Y. 1976. Survival of bacteria in cold and moderate hydrostatic pressure environments with special reference to psychrophilic and barophilic bacteria. 279–298. Gray, T. G. R., and Postgate, J. R. (ed.) The survival of vegetative microbes. Cambridge, Cambridge University Press.

Morris, J. G. 1975. The physiology of obligate anaerobiosis. Advances in Microbial Physiology 12:169–246.

Morris, J. G. 1976. Fifth Stenhouse-Williams Memorial Lecture—oxygen and the obligate anaerobe. Journal of Applied Bacteriology 40:229–244.

Morris, J. G. 1978. The biochemistry of anaerobiosis. Biochemical Society Transactions 6:353–356.

Morris, J. G. 1979. Nature of oxygen toxicity in anaerobic microorganisms. 149–162. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Morris, J. G., O'Brien, R. W. 1971. Oxygen and clostridia: A review. 1–37. Barker, A. N., Gould, G. W., and Wolf, J. (ed.) Spore research 1971. London, Academic Press.

Mossel, D. A. A. 1975. Water and micro-organisms in foods—a synthesis. 347–361. Duckworth, R. B. (ed.) Water relations of foods. London, Academic Press.

Mossel, D. A. A., Ingram, M. 1955. The physiology of the microbial spoilage of foods. Journal of Applied Bacteriology 18:232–268.

Mountfort, D. O., Asher, R. A. 1983. Role of catabolic regulatory mechanism in control of carbohydrate utilization by the rumen anaerobic fungus *Neocallimastix frontalis*. Applied and Environmental Microbiology 46:1331–1338.

Müller, M. 1975. Biochemistry of protozoan microbodies: Peroxisomes, glycerophosphate oxidase bodies, hydrogenosomes. Annual Review of Microbiology 29:467–483.

Müller-Neuglück, M., Engel, H. 1961. Photoinaktivierung von *Nitrobacter winogradskyi* Buch. Archiv für Mikrobiologie 39:130–138.

Mulder, E. G., Brotonegoro, S. 1974. Free-living heterotrophic nitrogen-fixing bacteria. 37–85. Quispel, A. (ed.) The biology of nitrogen fixation. Amsterdam, North-Holland.

Nasim, A., James, A. P. 1978. Life under conditions of high irradiation. 409–439. Kushner, D. J. (ed.) Microbial life in extreme environments. London, Academic Press.

Neijssel, O. M., Hueting, S., Crabbendam, K. J., Tempest, D. W. 1975. Dual pathways of glycerol assimilation in *Klebsiella aerogenes* NCIB 418. Their regulation and possible functional significance. Archives of Microbiology 104:83–87.

Nelson, D. C., Jannasch, H. W. 1983. Chemoautotrophic growth of a marine *Beggiatoa* in sulfide-gradient cultures. Arch. Microbiol. 136:262–269.

Nelson, D. C., Jørgensen, B. B., Revsbech, N. P. 1986. Growth pattern and yield of a chemoautotrophic *Beggiatoa* sp. in oxygen-sulfide microgradients. Appl. Environ. Microbiol. 53:225–233.

Nelson, D. C., Wirsen, C. O., Jannasch, H. W. 1989. Characterization of large, autotrophic *Beggiatoa* spp. abundant at hydrothermal vents of the Guaymas Basin. Appl. Environ. Microbiol. 55:2909–2917.

Noble, W. C., Pitcher, D. G. 1979. Microbial ecology of the human skin. Advances in Microbial Ecology 2:245–289.

Noble, W. C., Somerville, D. A. 1974. Microbiology of human skin. London, W.B. Saunders.

Nottingham, P. M., Hungate, R. E. 1969. Methanogenic fermentation of benzoate. Journal of Bacteriology 98:1170–1172.

Nultsch, W. 1975. Phototaxis and photokinesis. 29–90. Carlile, M. J. (ed.) Primitive sensory and communication systems: The taxes and tropisms of microorganisms and cells. London, Academic Press.

O'Brien, R. W., Morris, J. G. 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. Journal of General Microbiology 68:307–318.

Odum, E. P. 1977. Ecology: The link between the natural and the social sciences, 2nd ed. London, UK. Holt, Rinehart & Winston.

Ohta, K., Kiyomiya, A., Koyama, N., Nosoh, Y. 1975. The basis of the alkalophilic property of a species of bacillus. Journal of General Microbiology 86:259–266.

Okon, Y., Albrecht, S. L., Burris, R. H. 1976. Factors affecting growth and nitrogen fixation of *Spirillum lipoferum*. Journal of Bacteriology 127:1248–1254.

Oren, A., Padan, E. 1978. Induction of anaerobic, photoautotrophic growth in the cyanobacterium *Oscillatoria limnetica*. Journal of Bacteriology 133:558–563.

Oren, A., Shilo, M. 1979. Anaerobic heterotrophic dark metabolism in the cyanobacterium *Oscillatoria limnetica:* Sulfur respiration and lactate fermentation. Archives of Microbiology 122:77–84.

Orpin, C. G. 1972. The culture in vitro of the rumen bacterium Quin's oval. Journal of General Microbiology 73:523–530.

Orpin, C. G. 1973. The intracellular polysaccharide of the rumen bacterium Eadie's oval. Archiv für Mikrobiologie 90:247–254.

Orpin, C. G., Joblin, K. N. 1988. The rumen anaerobic fungi. Hobson, P. N. (ed.) The rumen microbial ecosystem. London, Elsevier Science Publishers.

Ottow, J. C. G. 1975. Ecology, physiology, and genetics of fimbriae and pili. Annual Review of Microbiology 29:79–108.

Overbeck, J. 1972. Zur Struktur und Funktion des aquatischen Ökosystems. Berichte der Deutschen Botanischen Gesellschaft 85:553–579.

Pace, N. R., Stahl, D., Lane, D. J., Olsen, G. J. 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microbiol. Ecol. 9:1–55.

Padan, E. 1979a. Facultative anoxygenic photosynthesis in cyanobacteria. Annual Review of Plant Physiology 30:27–40.

Padan, E. 1979b. Impact of facultatively anaerobic photoautotrophic metabolism on ecology of cyanobacteria (blue-green algae). Advances in Microbiol Ecology 3:1–48.

Pask-Hughes, R. A., Williams, R. A. D. 1975. Extremely thermophilic Gram-negative bacteria from hot tap water. Journal of General Microbiology 88:321–328.

Pask-Hughes, R. A., Williams, R. A. D. 1977. Yellow-pigmented strains of *Thermus* spp. from Icelandic hot springs. Journal of General Microbiology 102:375–383.

Patterson, H., Irvin, R., Costerton, J. W., Cheng, K.-J. 1975. Ultrastructure and adhesion properties of *Ruminococcus albus*. Journal of Bacteriology 122:278–287.

Pfennig, N. 1961. Eine vollsynthetische Nährlösung zur selektiven Anreicherung einiger Schwefelpurpurbakterien. Naturwissenschaften 48:136.

Pfennig, N. 1965. Anreicherungskulturen für rote und grüne Schwefelbakterien. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1, Suppl. 1:179–189, 503–504.

Pfennig, N. 1967. Photosynthetic bacteria. Annual Review of Microbiology 21:286–324.

Pfennig, N. 1979. General physiology and ecology of photosynthetic bacteria. 3–18. Sistrom, W. R., and Clayton, R. (ed.) Photosynthetic bacteria. New York. Plenum.

Pfennig, N., Biebl, H. 1976. *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Archives of Microbiology 110:3–12.

Pfennig, N., Cohen-Bazire, G. 1967. Some properties of the green bacterium *Pelodictyon clathratiforme*. Archiv für Mikrobiologie 59:226–236.

Pierson, B. K., Castenholz, R. W. 1974. A phototrophic gliding filamentous bacterium of hot springs. Chloroflexus aurantiacus, gen. and sp. nov. Archives of Microbiology 100:5–24.

Poindexter, J. S. 1964. Biological properties and classification of the *Caulobacter* group. Bacteriological Reviews 28:231–295.

Poindexter, J. S. 1979. Morphological adaptation to low nutrient concentrations. 341–356. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Prebble, J., Huda, S. 1977. The photosensitivity of the malate oxidase system of a pigmented strain and a carotenoidless mutant of *Sarcina lutea* (*Micrococcus luteus*). Archives of Microbiology 113:39–42.

Pringsheim, E. G. 1957. Observations on *Leucothrix mucor* and *Leucothrix cohaercus* nov. sp. with a survey of colorless filamentous organisms. Bacteriological Reviews 21:69–81.

Prins, R. A. 1977. Biochemical activities of gut microorganisms. 73–183. Clarke, R. T. J., and Bauchop, T. (ed.) Microbial ecology of the gut. London, Academic Press.

Raj, H. D. 1977. *Leucothrix*. CRC Critical Reviews in Microbiology 5:271–301.

Ramaley, R. F., Hixson, J. 1970. Isolation of a nonpigmented, thermophilic bacterium similar to *Thermus aquaticus*. Journal of Bacteriology 103:527–528.

Reddy, C. A., Bryant, M. P., Wolin, M. J. 1972a. Characteristics of S organism isolated from *Methanobacillus omelianskii*. Journal of Bacteriology 109:539–545.

Reddy, C. A., Bryant, M. P., Wolin, M. J. 1972b. Ferredoxin-independent conversion of acetaldehyde to acetate and H_2 in extracts of S organism. Journal of Bacteriology 110:133–138.

Reichelt, J. L., Baumann, P. 1973. Taxonomy of the marine, luminous bacteria. Archiv für Mikrobiologie 94:283–330.

Repaske, D. R., Adler, J. 1981. Change in intracellular pH of *escherichia coli* mediates the chemotactic response to certain attractants and repellents. Journal of Bacteriology 145:1196–1208.

Repeta, D. J., Simpson, D. J., Jørgensen, B. B., Jannasch, H. W. 1989. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature 342:69–72.

Richards, F. A., Vaccaro, R. F. 1958. The Cariaco Trench, an anaerobic basin in the Caribbean Sea. Deep-Sea Research 3:214–228.

Richards, F. R. 1975. The Cariaco basin (Trench). Oceanography and Marine Biology Annual Review 13:11–67.

Rittenberg, S. C. 1979. *Bdellovibrio*: A model of biological interactions in nutrient impoverished environments? 305–322. Shilo, M. (ed.) Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Robinson, J. B., Salonius, P. O., Chase, F. E. 1965. A note on the differential response of *Arthrobacter* spp. and *Pseudomonas* spp. to drying in soil. Canadian Journal of Microbiology 11:746–748.

Rose, A. H. 1968. Physiology of microorganisms at low temperatures. Journal of Applied Bacteriology 31:1–11.

Rosebury, T. 1972. Der Reinlichkeitstick. Hamburg, Hoffmann & Campe Verlag.

Rudd, J. W. M., Taylor, C. D. 1980. Methane cycling in aquatic environments. Advances in Aquatic Microbiology 2:77–150.

Rupela, O. P., Tauro, P. 1973. Isolation and characterization of *Thiobacillus* from alkali soils. Soil Biology and Biochemistry 5:891–897.

Russell, C., Melville, T. H. 1978. A review: Bacteria in the human mouth. Journal of Applied Bacteriology 44:163–181.

Rutter, P. R., Abbott, A. 1978. A study of the interaction between oral streptococci and hard surfaces. Journal of General Microbiology 105:219–226.

Sadoff, H. L. 1973. Comparative aspects of morphogenesis in three prokaryotic genera. Annual Review of Microbiology 27:133–153.

Sadoff, H. L. 1975. Encystment and germination in *Azotobacter vinelandii*. Bacteriological Reviews 39:516–539.

Savage, D. C. 1977a. Microbial ecology of the gastrointestinal tract. Annual Review of Microbiology 31:107–133.

Savage, D. C. 1977b. Interactions between the host and its microbes. 277–310. Clarke, R. T. J., and Bauchop, T. (ed.) Microbial ecology of the gut. London, Academic Press.

Savage, D. C., Fletcher, M. 1985. Bacterial adhesion. New York. Plenum Press.

Scarr, M. P. 1968. Thermophiles in sugar. Journal of Applied Bacteriology 31:66–74.

Schenk, A., Aragno, M. 1979. *Bacillus schlegelii*, a new species of thermophilic, facultatively chemolithoautotrophic bacterium oxidizing molecular hydrogen. Journal of General Microbiology 115:333–341.

Schenk, E. A., Schwemmler, W. 1983. Endocytobiology II. Walter de Gruyter. Berlin,

Schlegel, H. G. (ed.) 1965. Anreicherungskultur und Mutantenauslese. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1 Orig., Suppl. 1, 179–189.

Schlegel, H. G., Bowien, B. 1989. Autotrophic bacteria. Madison, WI. Science Tech Publishers. New York. NY. Springer-Verlag.

Schlegel, H. G., Jannasch, H. W. 1967. Enrichment cultures. Annual Review of Microbiology 21:49–70.

Schlegel, H. G., Pfennig, N. 1961. Die Anreicherungskultur einiger Schwefelpurpurbakterien. Archiv für Mikrobiologie 38:1–39.

Schmaljohann, R., Flügel, H. J. 1987. Methane-oxidizing bacteria in *Pogonophora*. Sarsia 72:91–98.

Schmidt, J. M. 1971. Prosthecate bacteria. Annual Review of Microbiology 25:93–110.

Schmidt-Lorenz, W. 1967. Behavior of microorganisms at low temperatures. Bulletin de l'Institut International du Froid 1–59.

Schnaitman, C., Lundgren, D. G. 1965. Organic compounds in the spent medium of *Ferrobacillus ferrooxidans*. Canadian Journal of Microbiology 11:23–27.

Schön, G. H., Engel, H. 1962. Der Einfluss des Lichtes auf *Nitrosomonas europaea* Win. Archiv für Mikrobiologie 42:415–428.

Schroff, G., Schöttler, U. 1977. Anaerobic reduction of fumarate in the body wall musculature of *Arenicola marina* (Polychaeta). Journal of Comparative Physiology 116:325–336.

Schultz, J. E., Breznak, J. A. 1978. Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. Applied and Environmental Microbiology 35:930–936.

Schultz, J. E., Breznak, J. A. 1979. Cross-feeding of lactate between *Streptococcus lactis* and *Bacteroides* sp. isolated from termite hindguts. Applied and Environmental Microbiology 37:1206–1210.

Schweinfurth, G., Lewin, L. 1898. Beiträge zur Topographie und Geochemie des ägyptischen Natron-thals. Zeitschrift für die Gesamte Erdkunde 33:1–25.

Schwemmler, W., Schenk, E. A. 1980. Endocytobiology. Berlin, Walter de Gruyter.

Shilo, M. (ed.). 1979. Strategies of microbial life in extreme environments. Weinheim, Verlag Chemie.

Shokes, R. F., Trabant, P. K., Presley, B. J., Reid, D. F. 1977. Anoxic, hypersaline basin in the northern Gulf of Mexico. Science 196:1443–1446.

Siñeriz, F., Pirt, S. J. 1977. Methane production from glucose by a mixed culture of bacteria in the chemostat: The role of *Citrobacter*. Journal of General Microbiology 101:57–64.

Singer, C. E., Ames, B. N. 1970. Sunlight ultraviolet and bacterial DNA base ratios. Science 170:822–826.

Skopintsev, B. A., Karpov, A. V. Vershinina, O. A. 1959. Study of the dynamics of some sulfur compounds in the Black Sea under experimental conditions. Soviet Oceanography Series [English translation] 4:55–72.

Sleat, R., Robinson, J. P. 1984. The bacteriology of anaerobic degradation of aromatic compounds. Journal of Applied Bacteriology 57:381–394.

Smith, D. W. 1978. Water relations of microorganisms in nature. 369–380. Kushner, D. J. (ed.) Microbial life in extreme environments. London, Academic Press.

Sneath, P. H. A. 1962. Longevity of micro-organisms. Nature 195:643–646.

Somerville, C. C., Knight, I. T. Straube, W. L., Colwell, R. R. 1989. Simple, rapid method for direct isolation of nucleic acids from aquatic environments. Appl. Environ. Microbiol. 55:548–554.

Sorokin, Y. I. 1964. On the primary production and bacterial activities in the Black Sea. Journal du Conseil, Conseil International pour l'Exploration de la Mer 29:41–60.

Sorokin, Y. I. 1970. Interrelations between sulphur and carbon turnover in meromictic lakes. Archiv für Hydrobiologie 66:391–446.

Sorokin, Y. I. 1972. The bacterial population and process of sulfide oxidation in the Black Sea. J. Cons. Int. Explor. Mer 34:423–454.

Souza, K. A., Deal, P. H. 1977. Characterization of a novel extremely alkaline bacterium. Journal of General Microbiology 101:103–109.

Souza, K. A., Deal, P. H., Mack, H. M., Turnbill, C. E. 1974. Growth and reproduction of microorganisms under extremely alkaline conditions. Applied Microbiology 28:1066–1068.

Stahl, D. A., Flesher, B., Mansfield, H. R., Montgomery, L. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. 54:1079–1084.

Stahl, D. A., Lane, D. J., Olsen, G. J., Pace, N. R. 1985. Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl. Environ. Microbiol. 49:1379–1384.

Stanier, R. Y. 1942. The cytophaga group: A contribution to the biology of Mycobacteria. Bacteriological Reviews 6:143–196.

Stapp, C., Bortels, H. 1931. Der Pflanzenkrebs und sein Erreger *Pseudomonas tumefaciens*. II. Mitteilung: Über den Lebenskreislauf von *Pseudomonas tumefaciens*. Zeitschrift für Parasitenkunde 4:101–125.

Stapp, C., Knösel, D. 1954. Zur Genetik sternbildender Bakterien. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 2. 108:244–259.

Steinbüchel, A. 1986. Anaerobic pyruvate metabolism of *Trichomonas foetus* and *Trichomonas vaginalis* hydrogenosomes. Molecular Biochemistry Parasitology 20:57–65.

Stetter, K. O. 1986. Diversity of extremely thermophilic archaebacteria. 39–74. Brock, T. D. (ed.) Thermophiles, general, molecular and applied microbiology. New York. J. Wiley & Sons.

Stetter, K. O. 1989. Extremely thermophilic chemolithoautotrophic archaebacteria. 167–176. Schlegel, H. G., Bowien, B. (ed.) Autotrophic bacteria. Madison, WI. Science Tech Publishers.

Stetter, K. O., Fiala, G., Huber, G., Huber, R., Segerer, A. 1990. Hyperthermophilic microorganisms. FEMS Microbiology Reviews 75:117–124.

Stetter, K. O., König, H., Stackebrandt, E. 1983. *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulphur reducing archaebacteria growing optimally at 105°C. Syst. Appl. Microbiol. 4:535–551.

Stetter, K. O., Zillig, W. 1985. *Thermoplasma* and the thermophilic sulfur-dependent archaebacteria. 85–170. Wolfe, R. S., and Woese, C. R. (ed.) The bacteria, vol. 8. New York. Academic Press.

Stockhausen, F. 1907. Okologie, "Anhäufungen" nach Beijerinck. Berlin, Institut für Gärungsgewerbe.

Strange, R. E. 1976. Microbiol response to mild stress. Durham, England. Meadowfield Press.

Strength, W. J., Isani, B., Linn, D. M., Williams, F. D., Vandermolen, G. E., Laughon, B. E., Krieg, N. R. 1976. Isolation and characterization of *Aquaspirillum fascilus* sp. nov., a rod-shaped, nitrogen-fixing bacterium having unusual flagella. International Journal of Systematic Bacteriology 26:253–268.

Strohl, W. R., Larkin, J. M. 1979. Enumeration, isolation, and characterization of *Beggiatoa* from freshwater sediments. Applied and Environmental Microbiology 36:755–770.

Stumm, C. K., Zwart, K. B. 1986. Symbiosis of protozoa with hydrogen-utilizing methanogens. Microbiological Sciences 3:100–105.

Sverdrup, H. W., Johnson, M. W., Fleming, R. H. 1942. The oceans. London, Prentice-Hall.

Swart-Füchtbauer, H., Rippel-Baldes, A. 1951. Die baktericide Wirkung des Sonnenlichtes. Archiv für Mikrobiologie 16:358–362.

Switalski, L., Hook, M., Beachey, E. H. 1989. Molecular mechanisms of microbial adhesion. New York. Springer-Verlag.

Tannock, G. W. 1990. The Microecology of *lactobacilli* inhabiting the gastrointestinal tract. Advances in Microbial Ecology 11:147–171.

Tansey, M. R., Brock, T. D. 1978. Microbial life at high temperatures: Ecological aspects. 159–216. Kushner, D. J. (ed.) Microbial life in extreme environments. London, Academic Press.

Tempest, D. W., Meers, J. L., Brown, C. M. 1970. Synthesis of glutamate in *Aerobacter aerogenes* by a hitherto unknown route. Biochemical Journal 117:405–407.

Tempest, D. W., Meers, J. L., Brown, C. M. 1973. Glutamate synthetase (Gogat): A key enzyme in the assimilation of ammonia by prokaryotic organisms. 167–182. Prusiner, S., and Stadtman, E. R. (ed.) The enzymes of glutamine metabolism. New York. Academic Press.

Tempest, D. W., Neijssel, O. M. 1976. Microbial adaptation of low-nutrient environments. 283–296. Dean, A. C. R., Ellwood, D. C., Evans, C. G. T., and Melling, J. (ed.) Continuous culture 6: Applications and new fields. Chichester, Ellis Horwood.

Tempest, D. W., Neijssel, O. M. 1979. Eco-physiological aspects of microbial growth in aerobic nutrient-limited environments. Advances in Microbial Ecology 2:105–153.

Thiele, H. H. 1968. Die Verwertung einfacher organischer Substrate durch Thiorhodaceae. Archiv für Mikrobiologie 60:124–138.

Tindall, B. J., Trüper, H. G. 1986. Ecophysiology of the aerobic halophilic archaebacteria. Systematic and Applied Microbiology 7:202–212.

Torma, A. E. 1977. The role of *Thiobacillus ferrooxidans* in hydrometallurgical processes. Advances in Biochemical Engineering 6:1–37.

Torsvik, V. L., Goksoyr, J. 1978. Determination of bacterial DNA in soil. Soil Biol. Biochem. 10:7–12.

Trüper, H. G. 1969. Bacterial sulfate reduction in the Red Sea hot brines. 262–271. Degens, E. T., and Ross, D. A. (ed.) Hot brines and recent heavy metal deposits in the Red Sea. New York. Springer-Verlag.

Trüper, H. G. 1976. Higher taxa of the phototrophic bacteria: Chloroflexaceae fam. nov., a family for the gliding filamentous, phototrophic "green" bacteria. International Journal of Systematic Bacteriology 26:74–75.

Tschech, A. 1989. Der anaerobe Abbau von aromatischen Verbindungen. Forum Mikrobiologie 12:251–264.

Tuovinen, O. H., Kelly, D. P. 1972. Biology of *Thiobacillus ferrooxidans* in relation to the microbiological leaching of sulphide ores. Zeitschrift für Allgemeine Mikrobiologie 12:311–346.

Tuttle, J. H., Randles, C. I., Dugan, P. R. 1968. Activity of microorganisms in acid mine water. I. Influence of acid water on aerobic heterotrophs of a normal stream. Journal of Bacteriology 95:1495–1503.

Uesugi, I., Yajima, M. 1978. Oxygen and "strictly anaerobic" intestinal bacteria. I. Effects of dissolved oxygen on growth. Zeitschrift für Allgemeine Mikrobiologie 18:287–295.

Umbreit, T. H., Pate, J. L. 1978. Characterization of the holdfast region of wild-type cells of holdfast mutants of *Asticcacaulis biprosthecum*. Archives of Microbiology 118:157–168.

Unterman, P. M., Baumann, P., McLean, D. L. 1989. Pea aphid symbiont relationships established by analysis of 16S rRNAs. J. Bacteriol. 171:2970–2974.

van Gemerden, H. 1974. Coexistence of organisms competing for the same substrate: An example among the purple sulfur bacteria. Microbial Ecology 1:104–119.

van Niel, C. B. 1932. On the morphology and physiology of the purple and green sulphur bacteria. Archiv für Mikrobiologie 3:1–112.

van Niel, C. B. 1936. On the metabolism of the Thiorhodaceae. Archiv für Mikrobiologie 7:323–358.

van Niel, C. B. 1955. The microbe as a whole. 3–12. Waksman, S. A. (ed.) Perspectives and horizons in microbiology. New Brunswick, Rutgers University Press.

van Veen, W. L., Mulder, E. G., Deinema, M. H. 1978. The *Sphaerotilus-Leptothrix* group of bacteria. Microbiological Reviews 42:329–356.

Vedder, A. 1934. *Bacillus alcalophilus* sp. nov., benevens enkle ervaringen met sterk alcalische voedingsbodems. Antonie van Leeuwenhoek Journal of Microbiology and Serology 1:141–147.

Veldkamp, H. 1970. Enrichment cultures of prokaryotic organisms. 305–361. Norris, J. R., and Ribbons, D. W. (ed.) Methods in microbiology, vol. 3A. London, Academic Press.

Veldkamp, H. 1976. Continuous culture in microbial physiology and ecology. Patterns of progress. Durham, Meadowfield Press.

Veldkamp, H., Jannasch, H. W. 1972. Mixed culture studies with the chemostat. Journal of Applied Chemistry and Biotechnology 22:105–123.

Veldkamp, H., van den Berg, G., Zevenhuizen, L. P. T. M. 1963. Glutamic acid production by *Arthrobacter globiformis*. Antonie van Leeuwenhoek Journal of Microbiology and Serology 29:35–51.

Voelz, H., Dworkin, M. 1962. Fine structure of *Myxococcus xanthus* during morphogenesis. Journal of Bacteriology 84:943–952.

Völker, H., Schweisfurth, R., Hirsch, P. 1977. Morphology and ultrastructure of *Crenothrix polyspora* Cohn. Journal of Bacteriology 131:306–313.

Walsby, A. E. 1970. The gas vesicles of aquatic prokaryotes. Regulations between structure and function in the prokaryotic cell. Society for General Microbiology Symposium 28:327–357. London, New York. Melbourne, Cambridge, University Press.

Walsby, A. E. 1975. Gas vesicles. Annual Review of Plant Physiology 26:427–439.

Walsby, A. E. 1977. The gas vacuoles of blue-green algae. Scientific American 237:90–97.

Wangersky, P. J. 1976. The surface film as a physical environment. Annual Review of Ecology and Systematics 7:161–176.

Watson, S. W., Waterbury, J. B. 1969. The sterile hot brines of the Red Sea. 272–281. Degens, E. T., and Ross, D. A. (ed.) Hot brines and recent heavy metal deposit in the Red Sea. New York. Springer-Verlag.

Weibull, C. 1960. Movement. 153–205. Gunsalus, I. C., and Stanier, R. Y. (ed.) The bacteria, vol. 1: Structure. New York. Academic Press.

Weimer, P. J., Zeikus, J. G. 1977. Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Applied and Environmental Microbiology 33:289–297.

Weiner, R. M., Devine, R. A., Powell, D. M., Dagasan, L., Moore, R. L. 1985. *Hyphomonas oceanitis* spec. nov., *H. hirschiana* spec. nov. and *H. jannaschiana* spec. nov. Int. J. System. Bact. 35:237–243.

Weiss, R. L. 1973. Attachment of bacteria to sulphur in extreme environments. Journal of General Microbiology 77:501–507.

Weller, R., Ward, D. M. 1989. Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. Appl. Environ. Microbiol. 55:1818–1822.

Whittaker, R. H., Levin, S. A., Root, R. B. 1973. Niche, habitat and ecotope. American Naturalist 107:321–338.

Whittenbury, R., Davies, S. L., Davey, J. F. 1970. Exospores and cysts formed by methane-utilizing bacteria. Journal of General Microbiology 61:219–226.

Wiegel, J., Schlegel, H. G. 1976. Enrichment and isolation of nitrogen fixing hydrogen bacteria. Archives of Microbiology 107:139–142.

Wiegel, J., Wilke, D., Baumgarten, J., Opitz, R., Schlegel, H. G. 1978. Transfer of the nitrogen fixing hydrogen bacterium *Corynebacterium autotrophicum* (Baumgarten et al.) to *Xanthobacter* gen. nov. International Journal of Systematic Bacteriology 28:573–581.

Wiley, W. R., Stokes, J. L. 1963. Effect of pH and ammonium ions on the permeability of *Bacillus pasteurii*. Journal of Bacteriology 86:1152–1156.

Williams, A. G. 1986. Rumen holotrich ciliate protozoa. Microbiological Review 50:25–49.

Windberger, E., Huber, R., Trincone, A., Fricke, H., Stetter, K. O. 1989. *Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental sulfataric springs. Arch. Microbiol. 151:506–512.

Winfrey, M. R., Zeikus, J. G. 1977. Effect of sulfate on carbon and electron flow during microbial methanogenesis in fresh-water sediments. Applied and Environmental Microbiology 33:275–281.

Winogradsky, S. N. 1925. Etudes sur la microbiologie du sol. I. Sur la m;aaethode. Annales de l'Institut Pasteur 39:299–354.

Winogradksy, S. N. 1926. Études sur la microbiologie du sol. Sur les microbes fixateurs d'azote. Annales de l'Institut Pasteur 40:455–520.

Winogradsky, S. N. 1947. Principles de la Microbiologie Ecologique. Antonie van Leeuwenhoek Journal of Microbiology and Serology 12:5–15.

Winogradsky, S. N. 1949. Microbiologie du sol: Problèmes et Méthodes. Paris, Masson et Cie.

Wirsen, C. O., Jannasch, H. W. 1975. Activity of marine psychrophilic bacteria at elevated hydrostatic pressures and low temperatures. Mar. Biol. 31:201–209.

Wirsen, C. O., Jannasch, H. W. 1978. Physiological and morphological observations on *Thiovulum* sp. Journal of Bacteriology 136:765–774.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Wolfe, R. S. 1960. Observations and studies of *Crenothrix polyspora*. Journal of American Water Works Association 52:915–918.

Wolin, M. J. 1976. Interactions between H_2-producing and methane-producing species. 141–150. Schlegel, H. G., Gottschalk, G., and Pfennig, N. (ed.) Microbial production and utilization of gases. Göttingen, Goltze.

Wolin, M. J., Miller, T. L. 1982. Interspecies hydrogen transfer: 15 years later. ASM-News 48:561–565.

Woodroffe, R. C. S., Shaw, D. A. 1974. Natural control and ecology of microbial populations on skin and hair. 13–34. Skinner, F. A., and Carr, J. G. (ed.) The normal microbial flora of man. London, Academic Press.

Yayanos, A. A., Dietz, A. S. 1983. Death of a hadal deep-sea bacterium after decompression. Science Washington. 220:497–498.

Yayanos, A. A., Dietz, A. S., Van Boxtel, R. 1979. Isolation of a deep-sea barophilic bacterium and some of its growth characteristics. Science Washington. 205:808–810.

Yayanos, A. A., Dietz, A. S., Van Boxtel, R. 1981. Obligately barophilic bacterium from the Marianas Trench. Proc. Nat. Acad. Sci. USA. 78:5212–5215.

Yayanos, A. A., Dietz, A. S., Van Boxtel, R. 1982. Dependence of reproduction rate on pressure as a hallmark of deep-sea bacteria. Appl. Environ. Microbiol. 44:1356–1361.

Zaitsev, Yu. P. 1971. Vinogradov. K. A. (ed.) Marine neustonology. Jerusalem, Keter Press.

Zebe, E. 1977. Anaerober Stoffwechsel bei wirbellosen Tieren. Vorträge der Rheinisch-Westfälischen Akademie der Wiseenschaften.

Zeikus, J. G., Wolfe, R. S. 1972. *Methanobacterium thermoautotrophicus* sp. n., an anaerobic, autotrophic, extreme thermophile. Journal of Bacteriology 109:707–713.

Zhao, H., Wood, A. G., Widdel, F., Bryant, M. P. 1988. An extremely thermophilic *Methanococcus* from a deep-sea hydrothermal vent and its plasmids. Arch. Microbiol. 150:178–183.

Zillig, W., Schnabel, R., Tu, J., Stetter, K. O. 1982. The phylogeny of archaebacteria, including novel anaerobic thermoacidophiles in the light of RNA polymerase structure. Naturwiss. 69:197–204.

Zillig, W., Stetter, K. O., Schäfer, W., Janekovic, D., Wunderl, S., Holz, I., Palm, P. 1981. Thermoproteales: a novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from Icelandic solfataras. Zlb. Bakt. Hyg., I. Abt. Orig. C 2:205–227.

ZoBell, C. E. 1946. Marine microbiology, a monograph on hydrobacteriology. Waltham, Massachusetts. Chronica Botanica.

ZoBell, C. E. 1970. Pressure effects of morphology and life processes. 85–130. Zimmermann, A. (ed.) High pressure effects on cellular processes. London, Academic Press.

ZoBell, C. E., Johnson, F. H. 1949. The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. J. Bacteriol. 57:179–189.

ZoBell, C. E., Morita, R. Y. 1957. Barophilic bacteria in some deep sea sediments. Journal of Bacteriology 73:563–568.

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CHAPTER 1.7

Morphological and Physiological Diversity

STEPHEN H. ZINDER AND MARTIN DWORKIN

Introduction

Over 5,000 species and nearly 1,000 genera of isolated prokaryotes were tabulated as of 1999 (Garrity and Holt, 2000). Moreover, studies examining 16S ribosomal DNA in natural populations have provided convincing evidence that these cultured organisms are just the "tip of the iceberg" with several entire phyla/divisions having no or few cultured representatives (Hugenholtz et al., 1998). Prokaryotic diversity is an immensely valuable resource—not only as a source of an almost infinite variety of metabolic capabilities, enzymes and genes, but also as a veritable cornucopia of strategies for dealing with the world. If what we wish to understand is not only how an organism operates, but also how what it does enables it to deal with an extremely variable and occasionally hostile environment, then the study of microbial diversity truly holds the answers.

Much attention has focused on understanding the properties of a relatively few organisms (such as *Escherichia coli* and some of its close relatives) in ever greater depth and detail that is unmatched in any other cellular organism. This understanding has tended to serve as a paradigm for understanding bacteria in general, and in a larger sense, all organisms. Its ultimate expression is attributed to Jacques Monod: "What was true for *Escherichia coli* would be true for the elephant" (Judson, 1979), although he was actually referring to an "old axiom" of A. J. Kluyver on the unity in biochemistry: "From elephant to butyric acid bacterium—it is all the same" (Singleton, 2000). In any event, we have come to understand that though some fundamental strategies are indeed universal, each organism has evolved its own combination of tactics to arrive at the solution to its peculiar problems. It is thus appropriate, indeed often even useful, to continue the pursuit of the unusual microbe, so eloquently referred to by Ralph Wolfe in the foreword to this treatise.

Among the many rewards of this pursuit has been the recent recognition that, in addition to the diversity among the familiar and commonly recognized bacteria, there is yet a higher level of diversity. The existence of the Archaea has led to the recognition of a group of prokaryotes that not only does not share many of the properties considered fundamental to bacteria (Stanier and van Niel, 1962), but also has some properties, most notably transcription, more closely resembling those of eukaryotes (Reeve et al., 1997).

To get a sense of the evolutionary diversity of prokaryotes, one need only examine the universal 16S rDNA tree (Fig. 1) to conclude that about two thirds of evolutionary diversity is prokaryotic, and indeed most of eukaryotic evolutionary diversity is also microbial, mainly consisting of protists (Sogin and Silberman, 1998). Some "macrobiologists," notably the eminent evolutionary biologist Ernst Mayr (Mayr, 1998), insist that the phenotypic diversity of prokaryotes pales before that of eukaryotes, which includes "jellyfish, butterflies, dinosaurs, hummingbirds, yeasts, giant kelp, and giant sequoias." Woese in his reply (Woese, 1998) justly states that Mayr is comparing apples and oranges, and that the human visual system has evolved to discern differences in plants and animals as a matter of survival. Whereas, as we will demonstrate presently, there is ample morphological variation in prokaryotes, their specialty is metabolic variation and adaptation to habitats and niches. Can eukaryotes use sulfur or a pesticide as their lunch, fix nitrogen, or grow in boiling water? Clearly not. Indeed, some of Mayr's naturalist colleagues at Harvard now appreciate the importance of prokaryotes. Stephen Jay Gould states (Gould, 1996) that "on any reasonable or fair criterion, bacteria are—and always have been—the dominant form of life on earth," and E. O. Wilson speculates at the end of his autobiography (Wilson, 1995) that were he to start over again in the twenty-first century, he would become a microbial ecologist.

It is not our intention in this essay to construct a bestiary. Nor is it our goal to provide an exhaustive description of prokaryotic diversity; in effect, that is the entire content of this book. Our choice of examples of diversity has been selective, and this essay shall be limited to using a variety of prokaryotes to illustrate the diversity

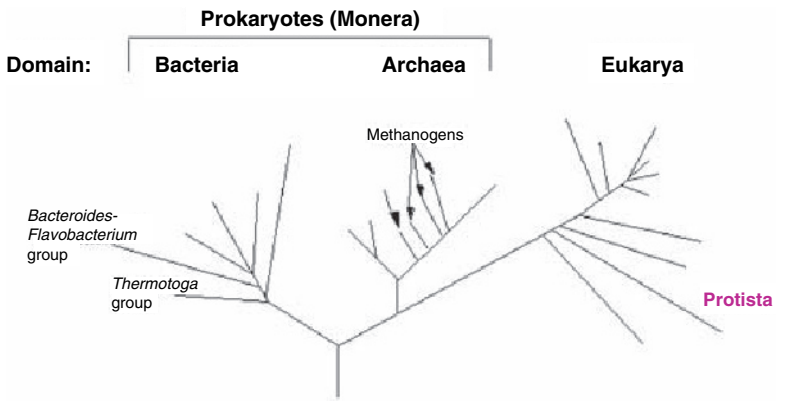


Fig. 1. Phylogenetic tree based on 16S rRNA. The three domains are shown, as well as the five classically defined kingdoms. (From Woese et al., 1990.)

of structural, physiological and metabolic strategies used by bacteria to adapt to the world.

Diversity in Cell Size

The conventional wisdom that prokaryotic cell diameters are typically near 1 μm, whereas eukaryotic cells are 10 μm or larger is generally true, but significant exceptions exist. Members of the Bacteria can show enormous diversity in cell size. For example, one of us works with a disk-shaped organism, *Dehalococcoides ethenogenes*, with a diameter of 0.4–0.5 μm and a height of 0.1–0.2 μm so that its volume is near 0.024 μm^3, about one twentieth that of *E. coli*. On the other hand, *Epulopiscium fishelsonii*, found in the gastrointestinal tracts of certain fish, are cigar-shaped cells with a diameter as great as 80 μm and lengths up to 600 μm (Angert et al., 1993). The volume of these cells is near 2 million μm^3, about eight orders of magnitude larger than of *D. ethenogenes*. Another example of prokaryotic gigantism, *Thiomargarita*, is a sphere (nearly a millimeter in diameter) composed of a thin layer of cytoplasm around a large internal vacuole in which nitrate, concentrated from the surrounding water, can reach concentrations as high as 0.8 M (Schulz et al., 1999). This nitrate-filled vacuole allows the organisms to oxidize sulfide under anaerobic conditions using the nitrate as a respiratory electron acceptor. Curiously, no examples of archaeal cells more than a few μm in diameter are known.

An advantage of existing as micrometer-sized cells is a greater surface-to-volume ratio. For example, if one compares spherical cells with 1 μm and 10 μm diameters, it requires 1,000 of the smaller cells (ca. 0.5 μm^3) to equal the larger cell (ca. 500 μm^3) in volume. Moreover, the smaller cells have 10 times greater total surface area (ca. 3,140 μm^2) than the larger one (ca. 314 μm^2), allowing them to present more surface to the environment for uptake of scarce nutrients. The smaller size apparently obviates the need for the organellar compartmentation and internal membrane systems generally found in eukaryotes because molecules can traverse the distances in the prokaryotic cell more easily by diffusion. Indeed, the cells of *Epulopiscium* contain an extensive internal membrane system (Clements and Bullivant, 1991), indicating that their large size necessitates such a system.

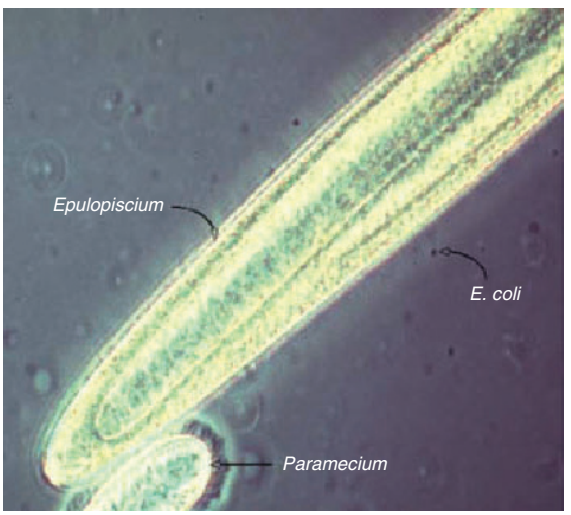


Fig. 2. Comparison of *Epulopiscium* with cells of the protist *Paramecium* and *E. coli*. Photo courtesy of E. Angert.

Diversity in Cell Shape

The controversy between the pleomorphists and the monomorphists led to a view of bacterial morphology first propounded by Ferdinand Cohn (Cohn, 1875) and hammered into a dogma by the subsequent work of Robert Koch. The classical textbook view of bacteria described them as spherical or cylindrical, the latter category divided between straight and curved rods. This view, based on the observations made on laboratory-grown cultures cultivated under conditions designed to result in a maximum growth

rate and morphological uniformity, came to represent a picture of the "true" morphology of bacteria. It obscured the spectrum of enormous morphological diversity manifested by the bacteria. It also obscured the fact that that diversity was often the result of life cycles that represented, for bacteria, survival strategies in addition to those of maximizing growth rates.

The shape of a cell is not a trivial or casual aspect of its adaptation to its environment but is instead a strategic consequence of this adaptation. Nor is the shape of a cell limited in any fundamental way to a rod, coccus or spiral. Ferdinand Cohn in 1875 divided bacteria into these three forms in one of the first systematic attempts to classify bacteria. It is now quite clear that the variety of cell shapes and groupings far exceeds those offered in early views. There are bacteria that are amorphous, ovoid, square, stellate, filamentous or stalked (Fig. 3). They may be grouped as pairs, clumps, chains, rosettes, cuboid packets, flat squares, networks, mycelia or fruiting bodies (Figs. 4, 5, 6).

What are the possible functions of cell shape and grouping? Certainly one of the most obvious relates to the surface-volume (S/V) ratio of a cell referred to above in the context of cell size. The sphere has the lowest possible S/V ratio, and as a cell becomes longer and thinner, its S/V ratio increases. One can imagine that a cell such as the oligotrophic *Caulobacter*, whose lifestyle involves the scavenging of extremely low concentrations of nutrients, will benefit from a rod shape, and from the consequent increase in surface area available for nutrient uptake. It has also been suggested (Pate and Ordal, 1965) that the characteristic stalks of *Caulobacter* (and perhaps also *Asticcacaulis*) function to increase the surface area of the cell yet further. Conversely, one may suggest that organisms that characteristically find themselves under conditions of ample nutrient levels would not be disadvantaged by low S/V ratios and may even find spherical shapes advantageous for other reasons. For example, one could imagine that an organism whose cell surface was subject to assault by antibodies or other host-defense factors might evolve a coccal shape that would minimize the amount of accessible surface.

It is conceivable that the shape of a cell may also be related to optimal hydrodynamic properties. It may not be a coincidence that motile organisms are rarely spherical; the number of flagellated cocci is quite small. Gliding bacteria are generally rod shaped (Fig. 6) although an interesting exception to this is found in *Isosphaera pallida*, a budding, chain-forming, gliding coccus (Fig. 4). In addition, the spiral shape of organisms such as spirochetes may be causally related to the ability of the organism to screw its way through a relatively highly viscous medium. In fact, it has been shown that the motility of spirochetes is enhanced at higher than normal viscosities (Canale-Parola, 1978).

It was appropriate at one time to view any departure from the rod—coccus—spiral morphology as a reflection of abnormal conditions. It is now quite clear that there are a variety of cell morphologies, each of which is uniquely appropriate for the ecological niche of that cell.

Diversity in Cell Grouping

It is also interesting to think about the functions of cell groupings. Is it possible that the characteristic clustering of the staphylococci further reduces the amount of accessible cell surface? And what is the conceivable function of the striking tendency of *Thiopedia* and *Lampropedia* (the latter a chemotrophic aerobe and the former a phototropic anaerobe) to grow as sheets of cells (Fig. 3)?

One instance where a characteristic grouping has been rationalized is in the myxobacteria. These organisms undergo a complex life cycle and feed on insoluble macromolecular debris by excreting a complex of hydrolytic enzymes. They are thus at the mercy of diffusion both of their enzymes away from the cell and of the hydrolyzed nutrients back to the cell. However, the complex life cycle of these organisms which causes them to periodically collect into densely packed fruiting bodies, as well as to form swarming, gliding masses of cells (Fig. 6b), guarantees a high local concentration of periodically excreted enzymes. This has been shown to optimize their feeding (Rosenberg et al., 1977) and has been referred to as a "microbial wolf-pack effect" (Dworkin, 1973).

Though the collection of resting cells compressed in the fruiting body represents the potential for releasing a high-density population once germination occurs, one can only speculate as to the function of fruiting body complexity. It is feasible, however, that each germinated cluster of cells has an optimal size, and that the number of cells in each of the sporangiole packages arrayed on the fruiting body determines this size.

There are other groupings that are familiar, e.g., chain formation by cocci or by rods (Fig. 7A, B), clusters (e.g., *Stomatococcus*, Fig. 7C), sheathed cells (e.g., *Sphaerotilus*, Fig. 7E), mycelium formation (e.g., *Streptomyces*, Fig. 7F), rosette formation (e.g., *Caulobacter*, Fig. 7D), cuboidal packets (e.g., *Sarcina*, Fig. 7G), and networks (e.g., *Rhodomicrobium*, Fig. 3). These groupings are probably not casual or accidental properties of the cells but are likely functional and should be viewed as other manifestations of

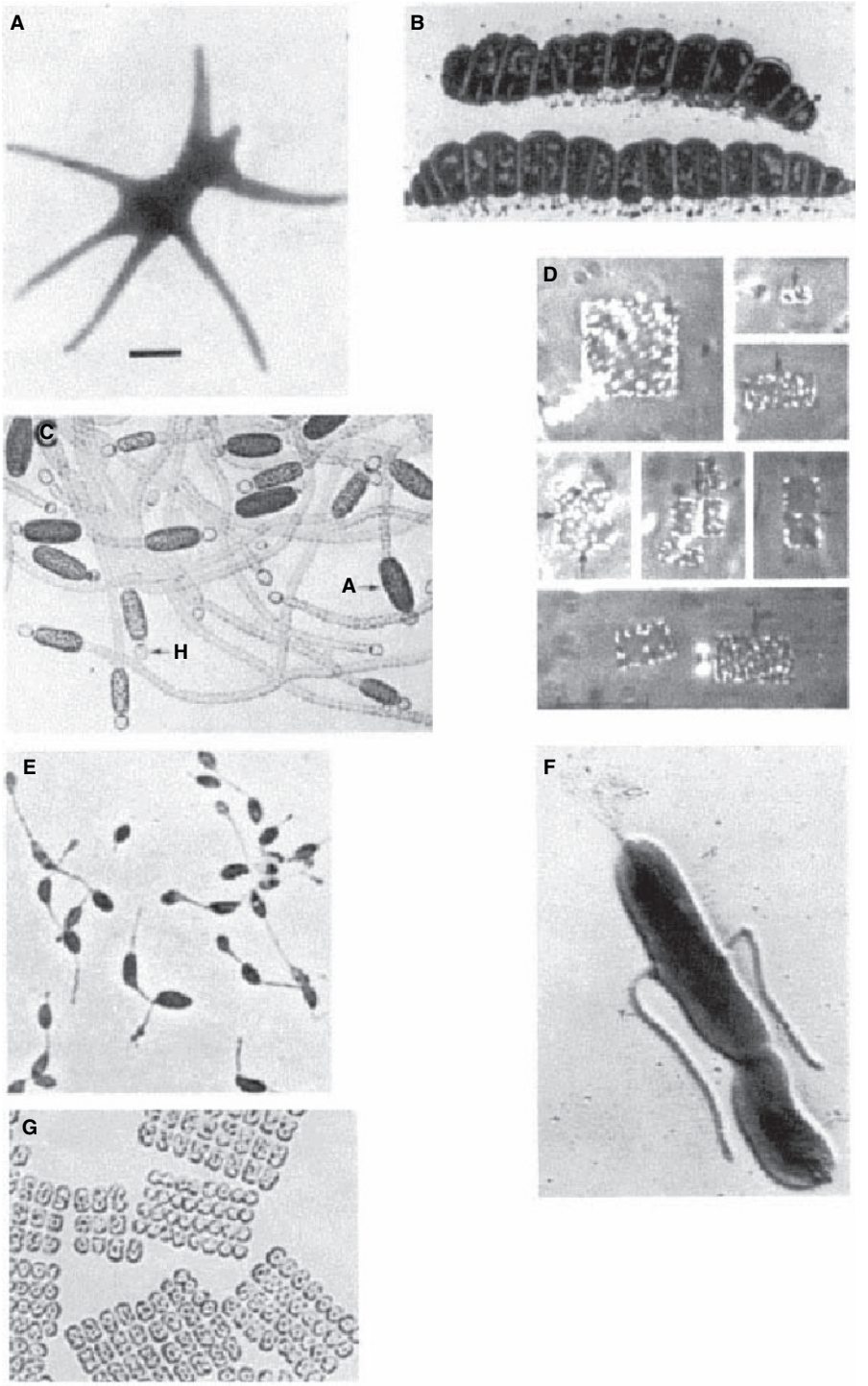
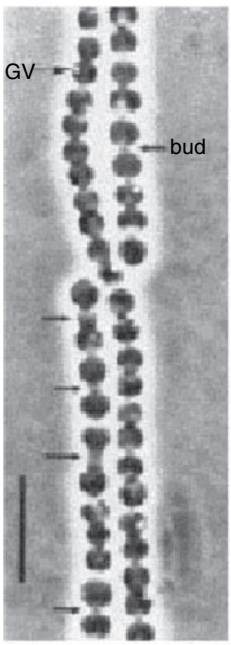


Fig. 3. A variety of cell shapes found among prokaryotes. A) *Prosthecomicrobium*. B) *Simonsiella*. C) *Cylindrospermum*. D) Square bacterium. E) *Rhodomicrobium*. F) *Asticcacaulis*. G) *Thiopedia rosea*. (A from Staley, 1968; B from Pangborn et al., 1977; C from Stanier et al., 1981; D from Walsby, 1980, with permission; F from Pate and Ordal, 1965, with permission; E courtesy of P. Hirsch; and G courtesy of N. Pfennig.)

Fig. 4. *Isosphaera pallida*. (From Giovannoni et al., 1987.) Arrows denote new cells growing as buds forming between cells, and gv denotes gas vesicles.



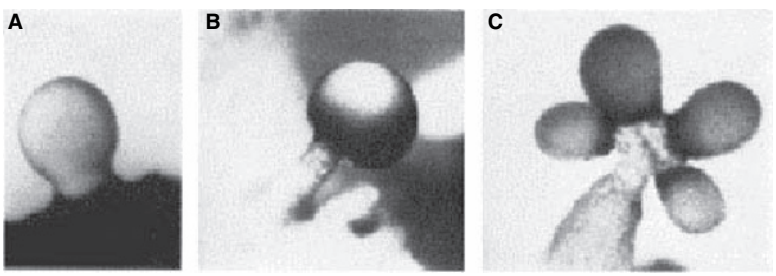


Fig. 5. Fruiting bodies of myxobacteria. A) *Myxococcus fulvus*. B) *Myxococcus stipitatus*. C) *Stigmatella aurantiaca*. (A and B from Reichenbach and Dworkin, 1981b; C courtesy of H. Reichenbach.)

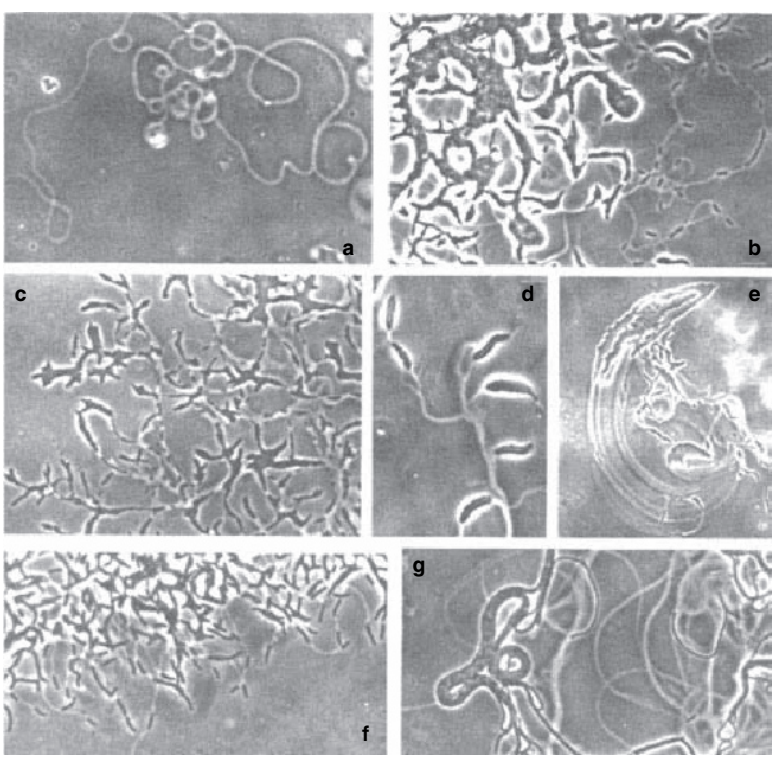


Fig. 6. Gliding bacteria. a) *Nannocystis exedens*. b) *Myxococcus fulvus*. c) *Cytophaga* sp. d) *Flexibacter elegans*. e) *Vitreoscilla stercoraria*. f) *Lysobacter* sp. g) *Herpetosiphon giganteus*. (From Reichenbach and Dworkin, 1981a.)

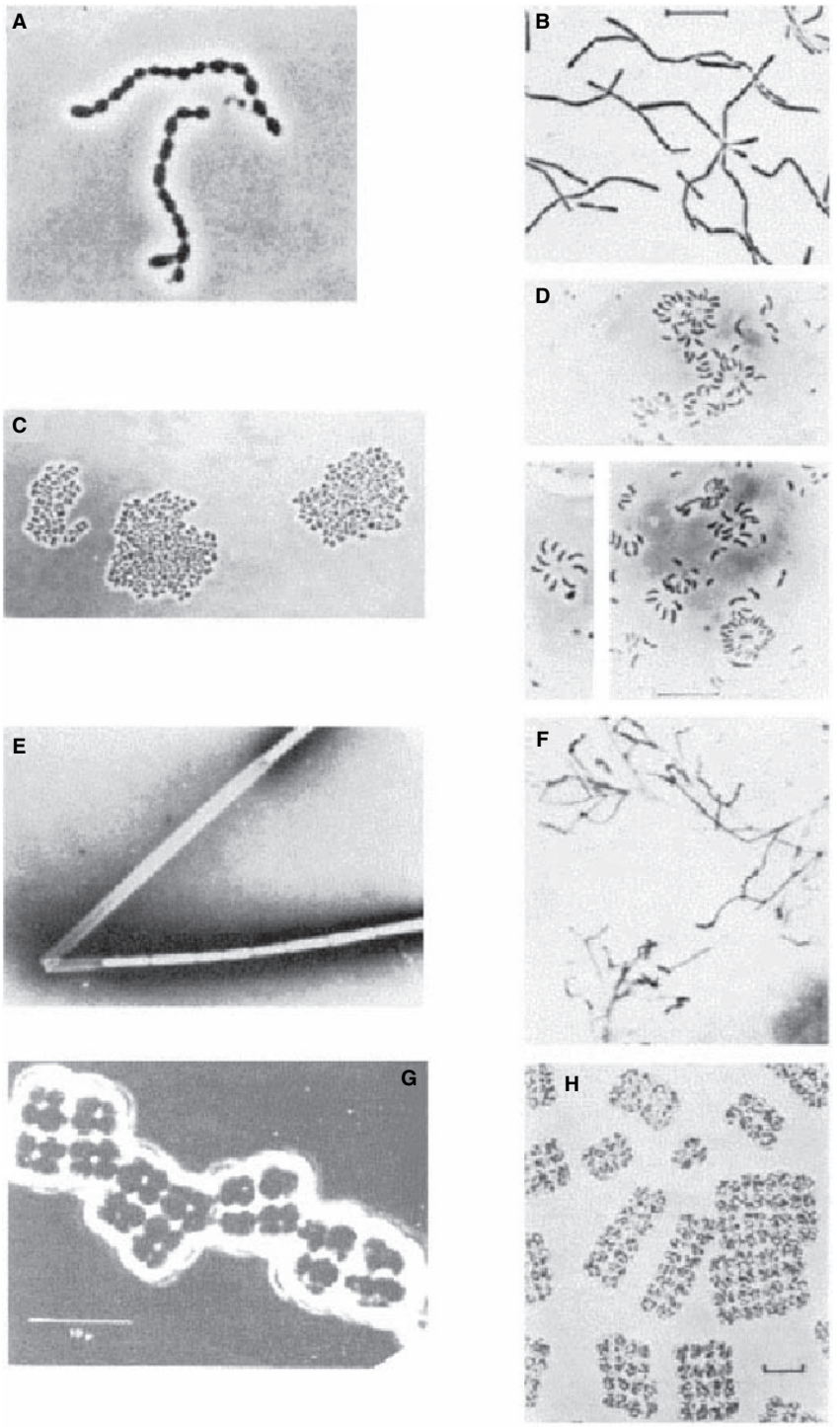


Fig. 7. A variety of cell arrangements among the prokaryotes. A) Phase-contrast micrograph of chains of *Streptococcus lactis*. B) Chain of rod-shaped cells of *Lactobacillus gasseri*. C) Irregular cluster of *Stomatococcus mucilaginosus*. D) Rosette of *Caulobacter*. E) Motile rods within a sheath, *Sphaerotilus natans*. F) Branched filament, *Streptomyces* sp. G) Three-dimensional packet of cocci, *Sarcina maxima*. H) Two-dimensional packet of cocci, *Amoebobacter pedioformis*. (A courtesy of T. D. Brock; B from Kandler and Weiss, 1986; C from Schleifer, 1986; D from Poindexter, 1964; E from Stokes, 1954; F from Kutzner, 1981; G from Holt and Canale-Parole, 1967; and H from Eichler and Pfennig, 1986.)

the diversity of prokaryotic adaptation to the environment. Understanding how these various groupings contribute to a cell's fitness lingers as one of the challenges of structure-function studies.

The Cell Surface

The interface between a bacterial cell and its surroundings is where the cell will first have to deal with a variable environment. It is thus the part of the bacterial cell where one might expect to find the greatest diversity. It is the cell surface that determines the nature of attachment to a substrate or to another cell. The cell surface also determines what gets into and out of the cell and the nature of that transport process.

Diversity in Capsules

Microbiologists have always had a fondness for working with organisms that grow in liquid in a nicely dispersed state. This has tended to obscure the fact that, in nature, most bacteria are not growing in a dispersed state but are clinging tenaciously to a substratum as a clump of cells or in a biofilm. Many organisms are surrounded by a more or less amorphous layer of slime, generally designated as the glycocalyx.

Furthermore, as was pointed out originally by Whittenbury and Dow (1977) and later emphasized by Costerton et al. (1986), the physiological properties of planktonic bacteria growing in a dispersed state are substantially different from those of the same cells when they are in their sessile state, embedded in a matrix of glycocalyces as pure or mixed culture biofilms (Costerton et al., 1999).

The principal component of the glycocalyx may be either polypeptide, as is the case with a number of species of *Bacillus*, or polysaccharide, characteristic of both Gram-positive and -negative organisms. These layers have a variety of functions depending on the organism and the nature of the layer. They serve in some cases to facilitate attachment to surfaces, either cellular or otherwise, and the nature of the capsule must, in some way, determine the kind of substratum to which the cell can attach.

The capsule has been shown to interfere with the function of the phagocytic cells of the immune system and may form a barrier to phage adsorption. Cook and Colvin (1980) have shown that the cellulosic fibers produced by *Acetobacter* serve to maintain these highly aerobic organisms at the liquid-air interface. Finally, it has been suggested that the hygroscopic quality of the glycocalyx may help a cell to resist desiccation. Based on such a variety of functions that a glycocalyx may play, it is no surprise that the composition of this structure varies so widely.

Another aspect of capsular diversity relates to the role of a capsule in interfering with the ability of phagocytic cells to engulf their bacterial prey. Certainly the ability of the pneumococcus to manifest a variety of capsular polysaccharide types plays an important role in the evasion of host defenses by the bacterium; the generation of diversity of the cell surface is a general strategy for compromising host-defense mechanisms.

Cell Wall Diversity

In Stanier and van Niel's classic description of the prokaryotic cell (Stanier and van Niel, 1962), one of the few positive characteristics of prokaryotes (as opposed to lack of nucleus, mitochondria, etc.) was the presence of a muramic acid-containing peptidoglycan cell wall. Indeed, because this structure is not present in eukaryotes, its synthesis is a primary target of several important antibiotics, such as the penicillin/cephalosporin group. At that time, cell walls were divided into Gram-positive, with its thick peptidoglycan layer, and Gram-negative, with a thin peptidoglycan layer and outer membrane (Beveridge, 1995). Stanier and van Niel were aware of mycoplasmas and similar organisms lacking a cell wall. It is now clear that though nearly all members of the Bacteria do indeed have a muramic acid-containing cell wall, many have protein cell walls, such as planktomycetes and chlamydia (Weisburg et al., 1986). The Gram-negative cell wall seems to be the default phenotype in the Bacteria, being found in most branches including the deeply branching Aquificae and Thermotogae. Moreover, even some deep branches of the Firmacutes contain organisms (such as *Sporomusa* and *Desulfotomaculum*) with Gram-negative cell wall structures. On the other hand, there are those who propose that the Gram-positive cell wall is the default structure (Gupta, 1998).

Arguably, the default cell wall architecture in the Archaea is the protein S-layer, a paracrystalline two-dimensional array of protein subunits (Sleytr et al., 1993). S-layers also are found outside the cell wall in many bacteria. In the genera *Methanosarcina* and *Halococcus*, there is a thick fibrous polysaccharide layer outside the S-layer, apparently serving as a corset to prevent lysis at relatively low osmolarities (Kandler, 1994; Sowers et al., 1993). In two methanogenic orders, the Methanobacteriales and Methanothermales, the cell walls consist of a thick peptidoglycan layer called "pseudomurein" which causes them to stain Gram-positive. Pseudomurein is similar to eubacterial murein but differs in a number of interesting aspects. It contains *N*-acetyl

talosamineuronic acid instead of *N*-acetyl muramic acid, the carbohydrate that, along with *N*-acetyl glucosamine, represents the carbohydrate backbone of murein. The amino acids in the peptide chain of the archaebacteria are all L-isomers and the glycosidic linkage is α -1,3 rather than -1,4.

Motility

Motility by multicellular eukaryotes serves a number of different functions. It enables an organism to escape from a predator or some other threat, it facilitates movement toward a potential mate, it allows developmental movement, and perhaps most importantly, it allows an organism to seek out and move toward a supply of food.

In the case of prokaryotes, it seems unlikely that cells can perceive the presence of predators such as protozoa, leucocytes, slime molds, phage or *Bdellovibrio* sufficiently in advance to allow them to exercise an avoidance strategy. Bacteria, however, can detect gradients of repellent chemicals and respond by moving away (see below).

With regard to mating, it does not seem that motility to facilitate mating interactions occurs among the bacteria. In those cases where a mating pheromone has, in fact, been demonstrated (Dunny et al., 1978), the participating organisms are nonmotile and depend on random motion to effect contact. The function of the pheromone is to prepare the organisms for the events involved in adhesion and gene transfer. Prokaryotic movement to accomplish a developmental event seems to be limited to the myxobacteria, where it allows the cells to maintain a high population density and to move into aggregation centers as a prelude to fruiting body formation (Spormann, 1999).

The most common function of motility among the prokaryotes seems to be to allow the organisms to position themselves optimally in their microenvironment, for example, to move along a concentration gradient toward a food source or away from a repellant (See Bacterial Behavior in Volume 2). The notion of a concentration gradient has implicit in it the condition that the substance in question is soluble rather than particulate, and that the milieu is thus an aqueous one. This serves to rationalize the observation that all the bacteria for which chemotaxis has been demonstrated are motile by means of flagella, with which they swim through an aqueous milieu. In fact, it is reasonable to suggest that the only conceivable function of swimming motility in bacteria is to allow the cell to travel along a perceived concentration gradient. The importance for chemotaxis and motility to the hunt for food is underlined by the finding that many naphthalene-degrading *Pseudomonas* strains possess plasmids which carry both the necessary biodegradation genes and genes encoding chemotaxis towards naphthalene (Grimm and Harwood, 1997), thereby forming a biodegradative package. Similarly, prokaryotes can position themselves in a gradient of light, a phenomenon called "phototaxis."

Swimming Motility

In all cases where they have been examined carefully, flagella in the Bacteria are fundamentally similar. They are usually composed of one, and in some cases two or three, species of a self-assembling protein, generically designated as "flagellin." This is attached, via a hook-shaped adaptor, to a motor-like basal body whereby the flagellar filament is inserted into the cell membrane. The flagellin subunits are translocated down the center of the flagellar shaft to the distal growth point where flagella are synthesized. An interesting adaptive use of this system is type III secretion found in pathogens, in which an assembly with protein subunits homologous to those in the flagellar filament is used to inject toxic proteins directly into eukaryotic host cells (Macnab, 1999). Diversity is manifested by the number and arrangement of the flagella around the cell. This varies from the polarly flagellated cell with a single flagellum (Fig. 8) to the profusely and peritrichously flagellated cells (Fig. 11). In between,

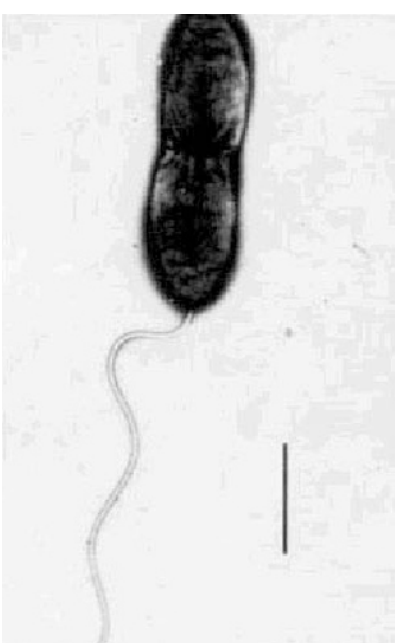


Fig. 8. Polarly flagellated cell of *Pseudomonas andropogonis*. Bar = 0.2 μm. (From Palleroni, 1984.)

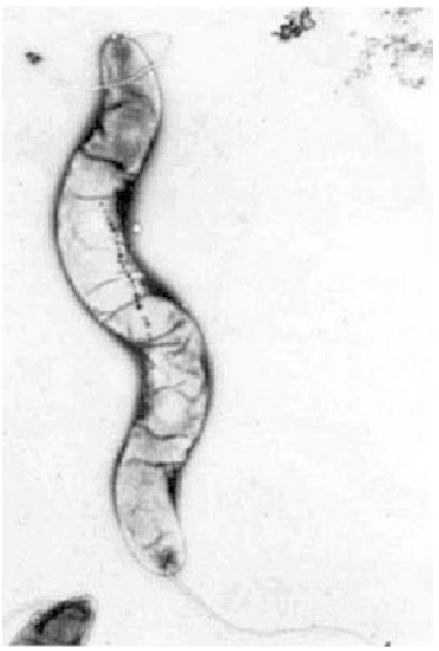


Fig. 9. Bipolarly flagellated cell of a magnetotactic spirillum. (From Balkwill et al., 1980.)

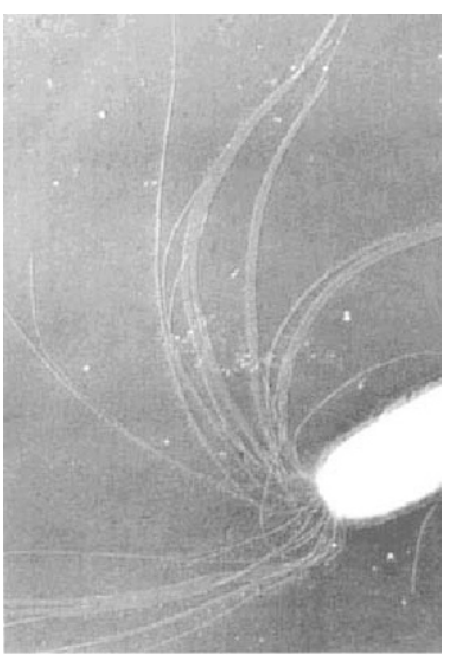


Fig. 10. Flagellar fascicle of *Spirillum volutans*. (From Krieg, 1984.)

there are cells with bipolar flagellation (Fig. 9) or with polar tufts of flagella (Fig. 10).

A most unusual style of flagellation is found among the spirochaetes. Rather than being freely turning propellers, the flagella are enshrouded by an outer cell envelope. It has been suggested (Berg, 1976) that their motion within this outer sheath generates a torque that, in combination with the helical structure of the cell, propels the cell in a screw-like fashion. It has been shown that, for certain of the spirochaetes (Canale-Parola, 1978), flagellar motility is enhanced in a high viscosity medium. This peculiar variation on flagellar organization may have evolved in response to high-viscosity environments that would hamper or prevent ordinary flagellar motility, allowing the spirochaetes to move through viscous media like a corkscrew through a cork. Interestingly, spirochaete motility seems to have at least three modes: swimming forward, swimming backward, and flexing (Fosnaugh and Greenberg, 1988).

Only flagellated prokaryotes move, in effect, via a rotating propeller principle. In this sense it is completely unlike the movement of conventional eukaryotic flagella, whose action is based instead on the sinusoidal beating of the flagellum within a single plane. One might ask either how the bacterium manages to operate an intracellular, rotating structure or, on the other hand, why there are no fish with propellers. The answer may lie in two features of the biophysics of movement. First, the efficiency of converting input energy to thrust is about 96–98% for an oscillating, flexible foil (such as the eukaryotic flagellum; Katz and Weihs, 1979); this is substantially higher than what can be obtained with a propeller-type mechanism. Second, the bacterium does not have this option; the tremendous viscous drag exerted on a small cell would damp out the oscillations of a sinusoidally beating flagellum. A screw-like motion is its solution to the need to move through an aqueous but (from the viewpoint of the bacterium) tar-like milieu.

Swarming Motility

Certain species of *Proteus* and of *Vibrio* carry out a kind of motility called "swarming" (Williams, 1978) that is an alternative to their normal, swimming motility. Under certain conditions, usually the perception of a solid surface or of an increased viscosity (in the case of *Vibrio*), the cell morphology and the manner of flagellation change. The cells become longer and, in the case of *Proteus*, filamentous, and the sparsely or polarly flagellated cells become profusely peritrichous (Fig. 11). In *Proteus*, when the cells are placed on an agar surface under the appropriate conditions, the swarming phase is periodic and alternates with a growth phase, during which shorter, less extensively flagellated swimming cells are formed (Matsuyama et al., 2000).

Twitching Motility

Certain of the Gram-negative cocci and coccobacilli, such as *Neisseria*, *Moraxella* and *Acineto-*

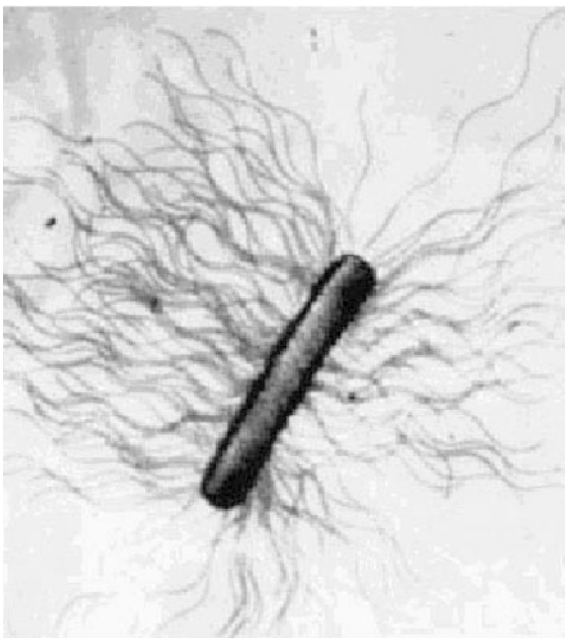


Fig. 11. Peritrichously flagellated swarmer cell of *Proteus mirabilis*. (From Hoeniger, 1965.)

bacter, manifest a peculiar type of movement known as "twitching" (Henrichsen, 1983). The cells exhibit jerking or jumping movements of a few micrometers in any direction. It now appears that retraction of type IV pili is responsible for this motility (Merz et al., 2000).

Gliding Motility

There are, however, alternative mechanisms for motility in prokaryotes. An entire group of bacteria, colloquially termed "the gliding bacteria," move by an undefined mechanism. These organisms are able to glide across solid surfaces such as agar and occasionally glass or plastic. The mechanism or mechanisms of such motility are not understood. The cells do not inch, wiggle or sidle. There seem to be no organelles of locomotion, although various authors have, on occasion, seen structures that they have interpreted as locomotory organelles (Lünsdorf and Reichenbach, 1989; Pate, 1979). Various mechanisms for gliding motility have been proposed (Burchard, 1984). They range from moving tracks (Lapidus and Berg, 1982), rotating organelles (Pate and Chang, 1979), polarized excretion of surfactants (Keller et al., 1983), and contractile fibers (Burchard, 1984). No one of these proposals is supported by other than those who have suggested them and it now appears that there may be more than one mechanism of gliding (Spormann, 1999).

The gliding bacteria, though customarily grouped together, have in common only the facts that they move by gliding and that they all have Gram-negative cell wall architecture. The motility mechanisms seem to be different, and the organisms cross the entire spectrum of physiological types.

While there is no hard evidence, it seems appropriate that those organisms whose nutrition depends on the extracellular solubilization of particulate material would glide rather than swim to gain access to their substrates. Though this seems to be the case for the myxobacteria, it is not clearly the case for the cyanobacteria or other gliding phototrophs.

Motility in Archaea

Some archaea show a flagellar-based motility, though gliding motility has never been detected in the Archaea. Whereas the archaeal flagellar assembly superficially resembles those of the Bacteria, it lacks a hook region, and, interestingly, the amino acid sequences of the subunits of the flagellar shaft resemble those of type IV pili in bacteria, rather than those of flagellins (Jarrell et al., 1996). Moreover, whereas bacterial flagellins are translocated outside the cell via a sec-independent transport system involving transport through the interior of the flagellum shaft, the flagellar subunits in the Archaea are transported using a standard sec-dependent system (Jarrell et al., 1996). Homologues of methyl-accepting chemotaxis proteins and the CheA-CheY two-component signal transduction system (Rudolph and Oesterhelt, 1996) have been found in the haloarchaea. Indeed, the haloarchaea have special sensory rhodopsins that, instead of pumping ions as do bacteriorhodopsin and halorhodopsin, couple with the methyl-accepting chemotaxis proteins to form a phototaxis system (Perazzona and Spudich, 1999).

Cell Division Strategies

Bacterial multiplication is usually thought of as being synonymous with binary transverse fission, the division strategy used by most of the commonly studied bacteria. This process has been intensively investigated, and, though many of the regulatory processes of division remain unknown, the descriptive details of the process are clearly defined (Ingraham et al., 1983). There is, however, a variety of other division strategies found among the bacteria, once one strays from the old laboratory standbys. Thus, division by budding among such organisms as *Hyphomicrobium* and *Rhodomicrobium* (Fig. 3E), by mycelial extension and subsequent fragmentation among the filamentous actinomycetes (Fig. 7F),

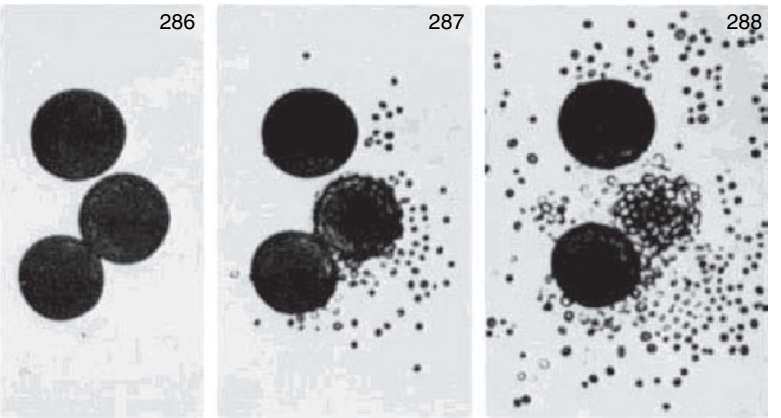


Fig. 12. Multiple fission and release of baeocytes from *Dermocarpa*. The numbers represent the hours that have elapsed during the growth of the initial, small baeocyte. (From Stanier et al., 1981.)

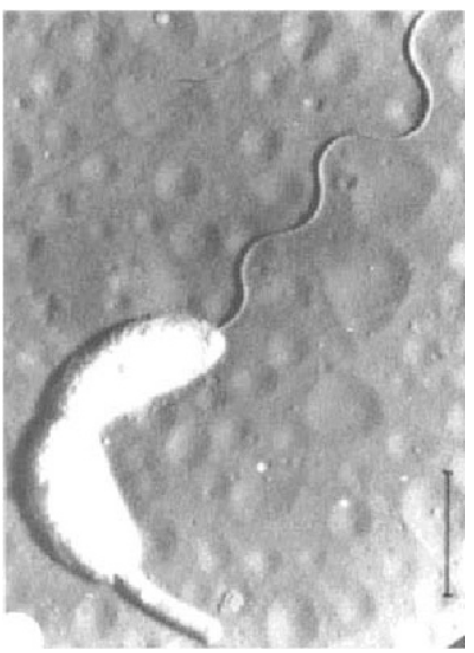


Fig. 13. Generation of swarmer cell from stalked cell of *Caulobacter crescentis*. (From Poindexter, 1964.)

and by multiple fission among the pleurocapsalean cyanobacteria (Fig. 12) has been described among the bacteria.

While it does seem a useful and interesting exercise to wonder what particular advantages are associated with the different strategies for cell division, this is not a subject that has received a great deal of attention from microbiologists. Nevertheless, it does seem obvious that these different mechanisms of cell division must be functionally related to the larger aspects of the biology of the cell. Thus, for example, mycelial extension followed by fragmentation seems a reasonable way to alternate filamentous and single-celled modes. And the unequal division of the stalked cell of *Caulobacter* (Fig. 13) leading to the generation of a flagellated swarmer cell must certainly reflect the division of the organism's activity between a sessile, stalked stem cell and a motile cell that may expand the territory occupied by that clone. Certainly, other speculations are possible to rationalize other modes of prokaryotic cell division.

Developmental Diversity

The chapter Prokaryotic Life Cycles in Volume 2 presents a more extensive discussion of the traditional view (and a more detailed description) of development among bacteria. The purpose of this section is to point out that development represents an additional set of strategies for diversity.

Development implies a set of alternative states. These alternatives may be expressed as a function of time, in which case we think of the alternative states as parts of a life cycle; on the other hand, the heterogeneity may be spatial, with different parts of the cell differentiated so as to fulfill specific functions. An example of the latter would be the stalked cell of *Caulobacter* (Fig. 13). In the case of either temporal or spatial alternatives, prokaryotic development generates considerably greater diversity than was afforded by the traditional nineteenth-century view of bacteria as rods, cocci or spirilla.

The generation of diversity among bacteria by developmental morphogenesis or by differentiation expands the kinds of niches that the bacteria can occupy. As an example, the ability of *Caulobacter* to form both a swarmer cell and a stalked cell allows it to separate the processes of growth and reproduction from dispersal. As an oligotroph, that is, an organism designed to feed on relatively low concentrations of nutrients, its ability to seek out, detect, and move toward such low concentrations must be optimized. That is the presumptive function of the swarmer cell. Once such a site has been discovered, it is the

function of the stalked cell to attach to a surface and begin the process of feeding, growth and reproduction. This sort of alternation between a sessile, feeding and reproductive stage and a nongrowing, swarming stage is also characteristic of organisms such as the actinoplanes, in which a mycelial phase alternates with a motile zoospore-like cellular stage. This type of developmental strategy facilitates the colonization of sites that might otherwise be inaccessible or inappropriate.

The characteristic swarm of the myxobacteria is another developmental strategy used by a bacterium to optimize its feeding. The myxobacteria feed by excreting a battery of potent hydrolytic enzymes that degrade proteins, peptidoglycan, polysaccharides, lipids and nucleic acids. The cells are thus at the mercy of the process of diffusion of enzymes away from the cell and of low-molecular-weight products of hydrolysis toward the cell. The myxobacteria optimize this process by traveling as a swarm—a high population density of cells (Fig. 14). The rate of cell growth on such substrates increases as a function of the cell density—a reflection of the fact that the cells are feeding cooperatively (Rosenberg et al., 1977). This sort of microbial "wolf-pack effect" has been suggested to rationalize the overall life cycle of the myxobacteria (Dworkin, 1973).

The ability of organisms to convert from a vegetative, growing stage to a resting stage allows them to persist in environments that might periodically become inhospitable to normal, prokaryotic life. As was pointed out earlier, the ability to grow in an extreme environment is invariably at the expense of the ability to grow in the so-called "normal environment." However, morphogenesis to a resistant, metabolically quiescent resting cell allows the organism to survive in the absence of growth until conditions are once again suitable for growth. A remarkable feature of this adaptation is that the state of almost total quiescence is juxtaposed with the ability to respond to the reappearance of conditions suitable for growth by germinating in an almost hair-trigger fashion.

Fig. 14. Swarm edge of *Stigmatella erecta* at low magnification. Bar = 110 μm. (From Reichenbach, 1984.)

One particular type of spore is referred to as an "endospore" and is found among the Gram-positive bacteria. This resting cell is formed within the vegetative cell and is usually released as a free spore as the process of sporulation is completed. Endosporulation occurs widely across traditional taxonomic lines and is found among the genera Bacillus, *Clostridium*, *Sporolactobacillus*, *Sporosarcina* and *Thermoactinomyces* (Fig. 15). In all of these organisms, the essential features of the spore and of the process of sporulation are quite similar. Another type of sporulation among the Gram-positive bacteria is found among the actinomycetes. Actinospores include the nonmotile conidial spores of *Streptomyces* (Fig. 16), the sporangiospores of *Actinoplanes* (Fig. 17) and the endospores of *Thermoactinomyces* (Fig. 18), which, despite their traditional taxonomic distance from the Bacillaceae, share many of the properties of *Bacillus* endospores. In this context, it is interesting that molecular techniques have revealed a moderate but distinct phylogenetic relationship between *Bacillus stearothermophilus* and *Thermoactinomyces vulgaris* (Stackebrandt et al., 1987).

The exospores of Gram-negative bacteria are, in general, less resistant to environmental extremes than are endospores. They are, however, metabolically quiescent and substantially more resistant to desiccation, physical breakage, and environmental extremes than the corresponding vegetative cells. Among the Gram-negative bacteria, resistant resting cells that have been fairly well characterized are found among the myxobacteria, as myxospores; among the cyanobacteria, as akinetes; and among *Azotobacter* and related genera, as azotocysts. Less well characterized resting cells have been described for the photosynthetic bacterium *Rhodomicrobium* (Dow and Whittenbury, 1979) and some of the methylotrophic bacteria (Whittenbury et al., 1970). Myxobacterial resting cells are contained in the characteristic fruiting bodies formed by the myxobacteria (Fig. 5), and their shapes vary from the round, resistant, optically refractile cells formed by the genus

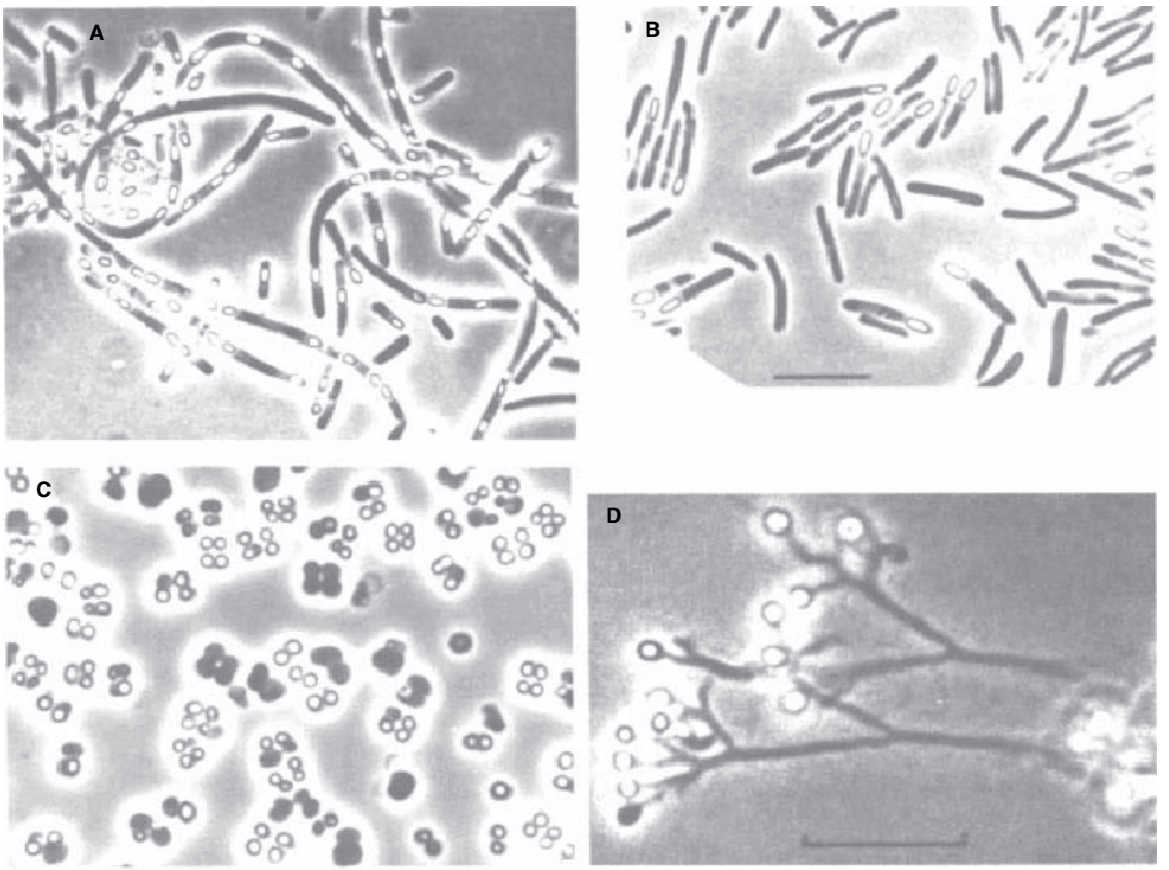


Fig. 15. Endospores of A) *Bacillus fastidiosus*, B) *Clostridium butyricum*, C) *Sarcina ureae*, and D) *Thermoactinomyces dichotomicus*. (A courtesy of S. C. Holt; B from Gottschalk et al., 1981; C from Sneath, 1986; and D from Cross, 1981.)

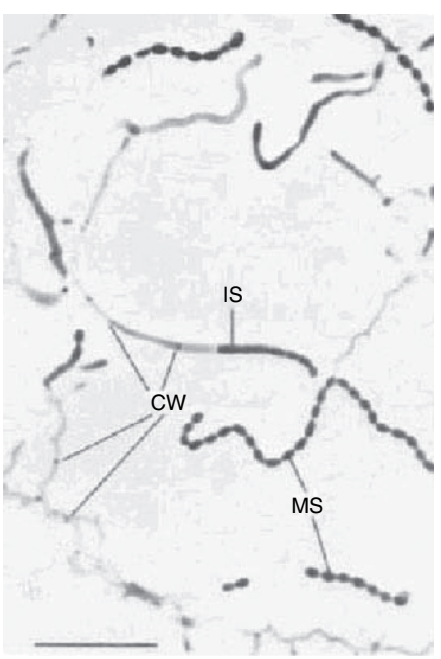


Fig. 16. Phase contrast micrographs of vegetative hyphae and conidiospores of *Streptomyces coelicolor*. CW, cross walls; IS, immature spores; and MS, mature spores. Bar = 10 µm. (From Chater and Hopwood, 1973.)

Myxococcus (Fig. 19A) to the slightly shortened, oval rods formed by *Stigmatella* (Fig. 19B). The myxospore of *Myxococcus xanthus* is formed by the shortening and rounding up of the entire rod-shaped vegetative cell and is finally enclosed by a multilayered spore coat/capsule (White, 1984). The *Myxococcus xanthus* myxospore is the only myxobacterial resting cell that has been well characterized both structurally and biochemically.

The cyanobacteria form a variety of cell types that have been considered to be spores or resting cells. These include the hormocysts of *Westiella*, the exospores of *Chamaesiphon*, the endospores or baeocytes of the Pleurocapsales (Fig. 12), and the akinetes of *Anabaena* and other genera. The cyanobacteria, in general, have not been characterized from a physiological or biochemical point of view with the same intensity that many of the eubacteria have. Thus, information about the properties of cyanobacterial resting cells is quite sparse. Most of the available information centers on the akinetes of *Anabaena*, and even in that case, the difficulty of obtaining large populations of relatively pure akinetes has limited the available information

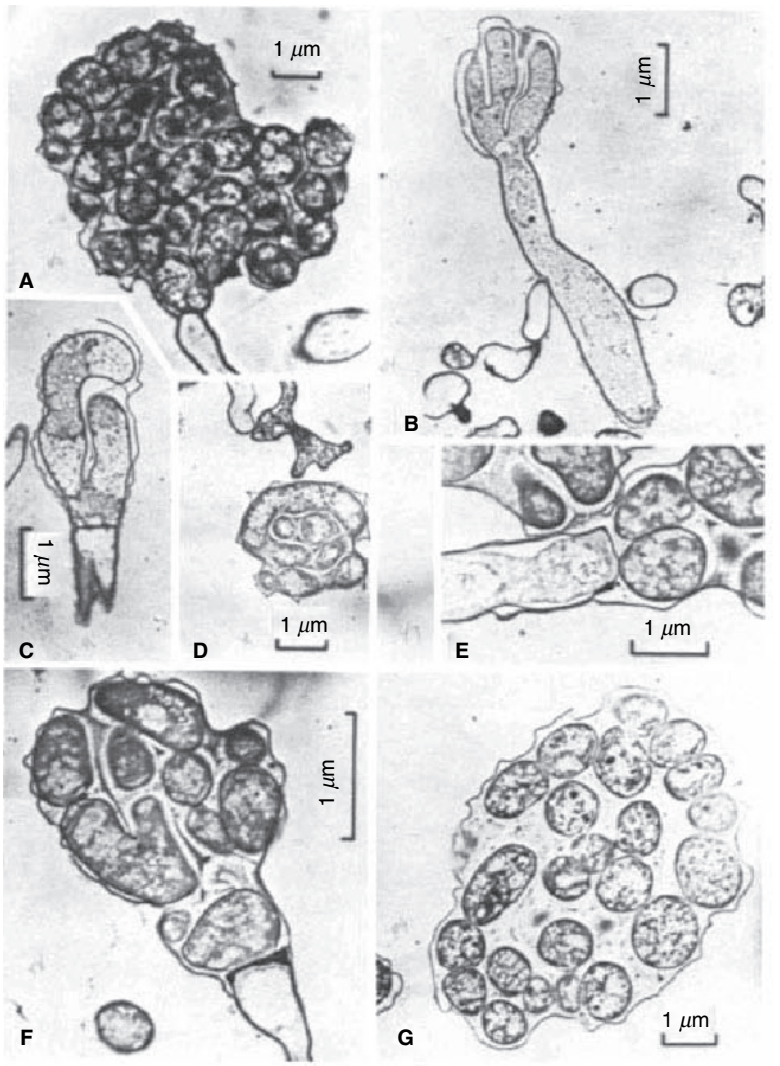


Fig. 17. Electron micrographs of thin sections of sporangiospores and sporangia of *Actinoplanes*. A) Mature sporangium, four days old. B), C) and D) Immature sporangium, two days old. E), F) and G) Mature sporangium, four days old. (From Lechevalier and Holbert, 1965.)

(Nichols and Adams, 1982). While the akinetes of *Anabaena* are more resistant to extreme low temperature and desiccation than the corresponding vegetative cells, their respiratory rate is twice that of the vegetative cells. Figure 20 illustrates the akinetes of *Anabaena*.

Cells of the Gram-negative, nitrogen-fixing genus *Azotobacter* are able to convert to cysts when the cells have exhausted the nutrients of the growth medium (Sadoff, 1976). The resting cells also can be induced by placing the cells in a medium containing hydroxybutyrate as the carbon source. *Azotobacter* cysts (Fig. 21) are superficially similar to myxospores, in that they are only slightly more resistant to temperature extremes than the corresponding vegetative cells but are considerably more resistant to desiccation and seem to be truly metabolically quiescent. (See Sudo and Dworkin, 1973 for an extensive, comparative survey of prokaryotic resting cell properties.)

Another interesting type of life cycle is shown by intracellular pathogens of the chlamydia group. Cells of the chlamydia must be able to carry out three very different kinds of processes. Because they are not transmitted by any sort of a vector, but may exist free in the environment, they must be able to persist in a nutrient-free, desiccated environment, subject to the normal variations of environmental conditions. Second, they must be able, from this state, to infect a specific host and to enter the host cell. Third, they must then be able to grow and reproduce intracellularly. Chlamydia accomplishes these processes by alternating between two states—the elementary body (a small, dense, resistant, and nongrowing cell) and the reticulate body (the vegetative form of the cell that can grow and reproduce).

Readers interested in a more detailed description of the various groups of prokaryotes that undergo development are referred to the excel-

lent recent monograph on the subject (Brun and Shimkets, 2000).

Metabolic Diversity

Catabolism is the part of metabolism involved in conservation of energy that can be used for biosynthesis and other cellular functions. Energy can be conserved from chemical reactions (chemotrophy) or from light (phototrophy). Catabolic diversity in prokaryotes greatly exceeds that in eukaryotes. There are many modes of metabolism, including anaerobic respiration, or lithotrophy, which eukaryotes cannot perform; even in those that the two cell types share, such as fermentation or photosynthesis, the eukaryotes are greatly outstripped by the prokaryotes in terms of substrates utilized and metabolic modes. Majorie Stephenson (Stephenson, 1949) expressed this concept presciently in the introduction to the first edition of her textbook on bacterial metabolism (also in the third edition), written well before the modern era of

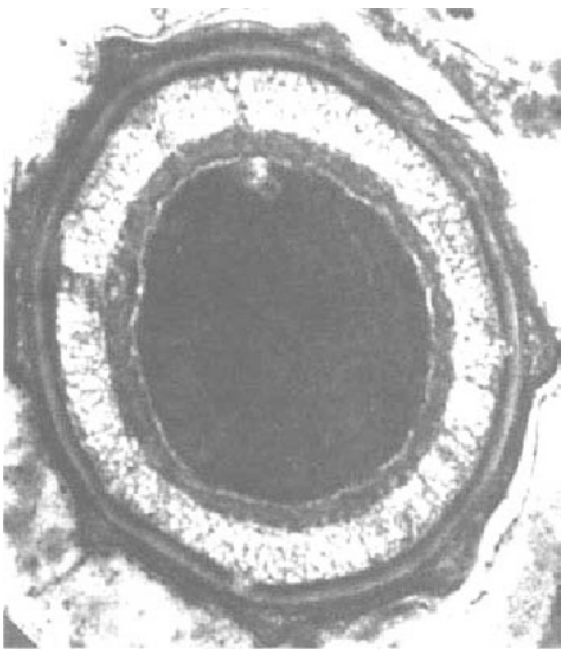


Fig. 18. Electron micrograph of a thin section of a spore of *Thermoactinomyces sacchari*. (From Lacy, 1971.)

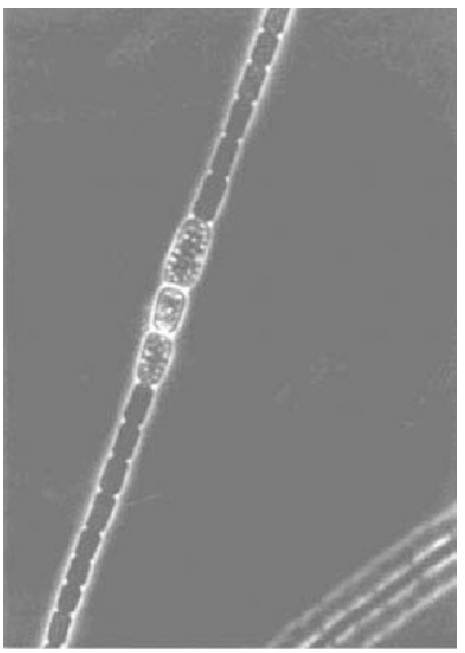


Fig. 20. Akinetes of *Anabaena*, positioned on both sides of a heterocyst. (From Carr, 1979.)

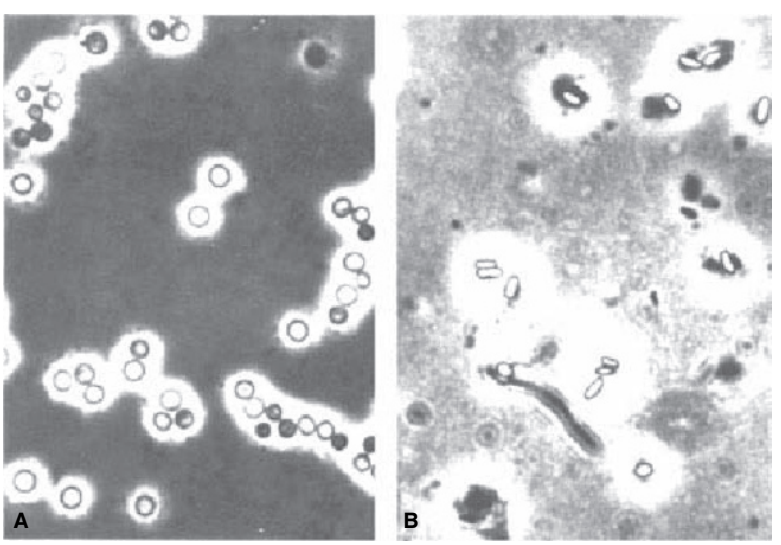


Fig. 19. Myxospores of myxobacteria. A) *Myxococcus xanthus*. B) *Stigmatella aurantiaca*. (Courtesy of H. Reichenbach.)

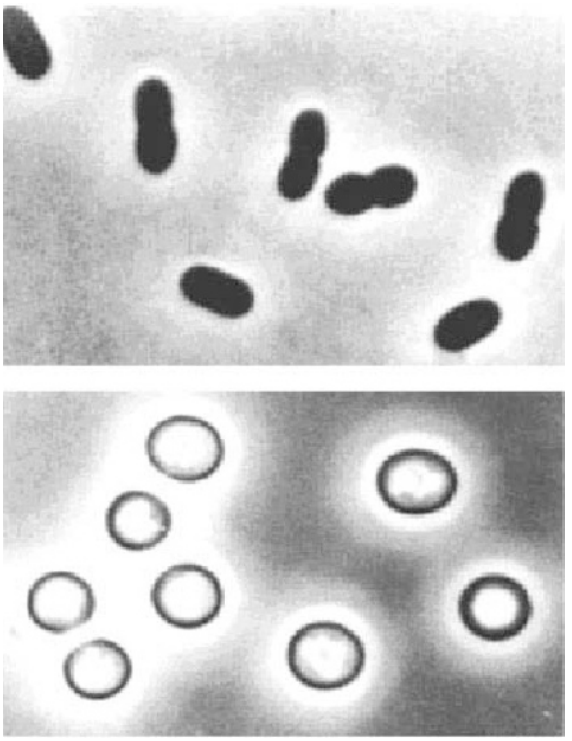


Fig. 21. Vegetative cells (top) and azotocysts (bottom) of *Azotobacter vinelandii*. (Courtesy of H. Sadoff.)

molecular biochemistry or bacterial genetics. In it she states:

"Bacteria may be tentatively regarded as biochemical experimenters; owing to their small size and rapid growth, variations must arise very much more frequently than in more differentiated forms of life, and they can in addition afford to occupy more precarious positions in the natural economy than larger organisms with more exacting requirements."

In terms of anabolism, all organisms share essentially the same pathways for biosynthesis of protein, nucleic acids, carbohydrates and lipids. Prokaryotes can biosynthesize certain compounds like vitamin B_{12} or certain antibiotics not found in the eukaryotes, but one must also give due credit to the biosynthetic capabilities of the fungi, and especially those of the plants that biosynthesize an incredible diversity of chemical compounds including hydrocarbons, aromatic compounds, heterocyclics and alkaloids. Indeed the formidable biodegradative abilities of soil microorganisms can be partially attributed to the selective pressures of diverse plant compounds in their environment serving as potential growth substrates.

Two crucial aspects of anabolism are the fixation of carbon and nitrogen, processes that are essential for primary production of biomass on Earth. It has long been known that fixation of nitrogen is a solely prokaryotic process. Moreover, if one considers chloroplasts to be descended from cyanobacteria, which is clearly the case (Moreira et al., 2000), then essentially all fixation of carbon on earth is also prokaryotic.

In the following sections, we will give an overview of various metabolic modes found in prokaryotes, discussing catabolic diversity and the fixation of carbon and nitrogen.

Anaerobic Fermentation

Fermentation can be defined as the utilization of an organic compound in the absence of external electron acceptors, including oxygen. Microbes are unique in their ability to exploit anaerobic environments, and Louis Pasteur's insight into "La vie sans l'air" opened up a whole new area of metabolism. Among the microbes, the prokaryotes have most extensively exploited this modus vivendi. As is the case with almost all aspects of biological diversity, there are few clear-cut categories but rather a spectrum of differences that diminish as one learns more about the property. Thus, the spectrum of relations to oxygen includes those organisms that cannot use oxygen as a terminal electron acceptor and are in fact damaged by exposure to oxygen, those that are likewise obligately anaerobic but are indifferent to the presence of oxygen, those facultative organisms that have the option of metabolizing either aerobically or anaerobically, and finally those organisms that are obliged to use oxygen as a terminal electron acceptor.

Eukaryotes can ferment a few common carbohydrates such as starch, cellulose, glucose or sucrose to a limited number of products: lactate or ethanol and CO_2, or, in the case of hydrogenosome-containing anaerobic protists, to ethanol, acetate and CO_2 and H_2. The fermentative abilities of prokaryotes are more extensive.

Carbohydrates often are the main organic substrate available for fermentation. The canonical pathway for utilization of carbohydrates is the Embden-Meyerhof-Parnas (EMP) pathway found in eukaryotes and many prokaryotes. The Entner-Doudoroff (ED) pathway involves oxidation of glucose-6-phosphate to glucuronic acid-6-phosphate and leads to the conservation of only a single ATP per hexose via substrate-level phosphorylation (Conway, 1992; Fig. 22). The ED pathway is common in the Proteobacteria, especially aerobes, but can be found in some clostridia and bacilli and even some eukaryotic microbes such as *Entamoeba histolytica* and *Penicillium notatum* (Conway, 1992). Most of the organisms that use the ED pathway are aerobes, but the Proteobacterium *Zymomonas mobilis* uses it as a primary fermentative pathway for

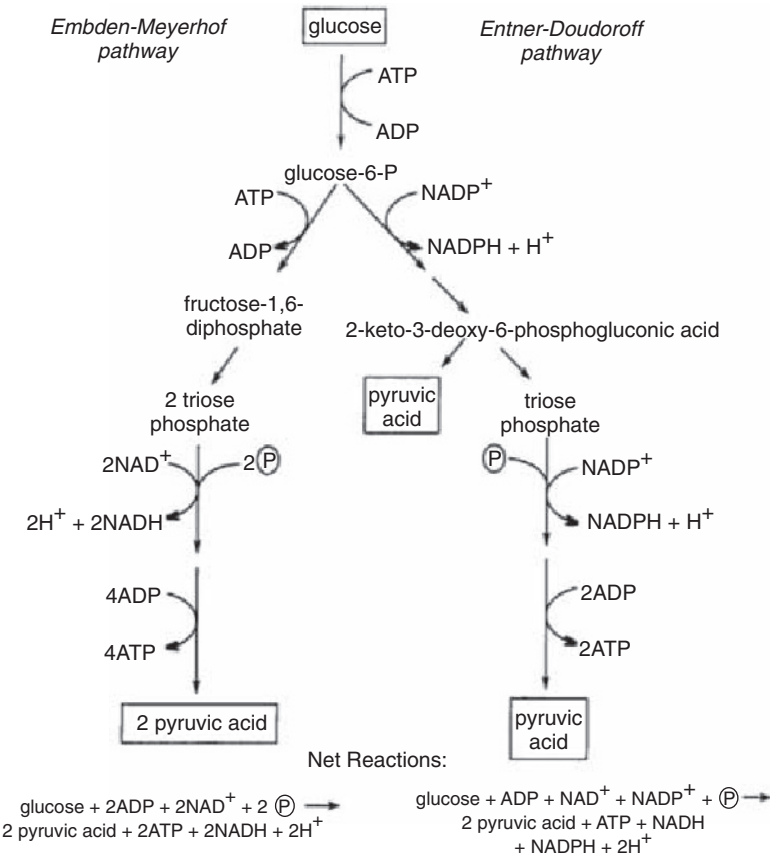


Fig. 22. The Embden-Meyerhof and Entner-Doudoroff pathways. (Adapted from Stanier et al., 1979.)

hexoses. *Escherichia coli* possesses both the EMP and the ED pathways and apparently uses the latter when dining on uronic acids in the gut (Peekhaus and Conway, 1998). Other glycolytic pathways in the Bacteria include that used by heterolactic Gram-positive bacteria (also leading to the conservation of a single ATP per hexose), the pathway found in bifidobacteria, and the pentose phosphate pathway, which is generally used for biosynthesis of five carbon sugars for nucleic acids (Gottschalk, 1986).

When sugar-fermenting Archaea were examined for glycolytic pathways, they were shown to have interesting modifications of both the EMP and the ED pathways (Kengen et al., 1996; Selig et al., 1997). For example, the hyperthermophile Pyrococcus furiosis, which ferments hexoses to acetate, hydrogen, CO_2 and alanine, was found to utilize a modified EMP pathway in which ADP rather than ATP serves as the phosphoryl donor for hexokinase and phosphofructokinase (Fig. 23). Moreover, instead of the standard glyceraldehyde 3-phosphate dehydrogenase, which uses NAD^+ as an electron acceptor and produces 1,3-diphosphoglycerate, *Pyrococcus* and certain other Archaea possess a novel tungsten-containing glyceraldehyde 3-phosphate/ferredoxin oxidoreductase (Adams, 1993) that produces 3-phosphoglycerate. Thus the net high-energy phosphodiester bonds conserved from hexose to pyruvate by this pathway are zero. The ATP from acetyl-CoA produced from pyruvate, however, is conserved by a novel ADP-dependent acetyl-CoA synthetase (previously described acetyl Co-A synthetases cleave ATP to AMP and pyrophosphate in the acetyl-CoA synthesis direction).

In contrast, *Thermotoga maritima*, a phylogenetically deep branching hyperthermophilic fermentative bacterium, converts hexoses to pyruvate using the conventional EMP pathway, and conserves ATP from acetyl-CoA using phosphotransacetylase and acetate kinase, as is found in mesophilic bacteria. The advantage of the glyceraldehyde 3-phosphate/ferredoxin oxidoreductase over the NAD^+-utilizing dehydrogenase is unclear, but one possibility is that ferredoxin is a very strong electron donor capable of reducing protons to H_2. In contrast, H_2 production from NADH is thermodynamically unfavorable at H_2 partial pressures above 10^{-3} atmospheres (Wolin and Miller, 1982). Therefore, the modified EMP pathway is less likely to be inhibited under conditions of high H_2 partial pressures. Indeed, growth of *T. maritima* on sugars becomes inhibited unless an electron

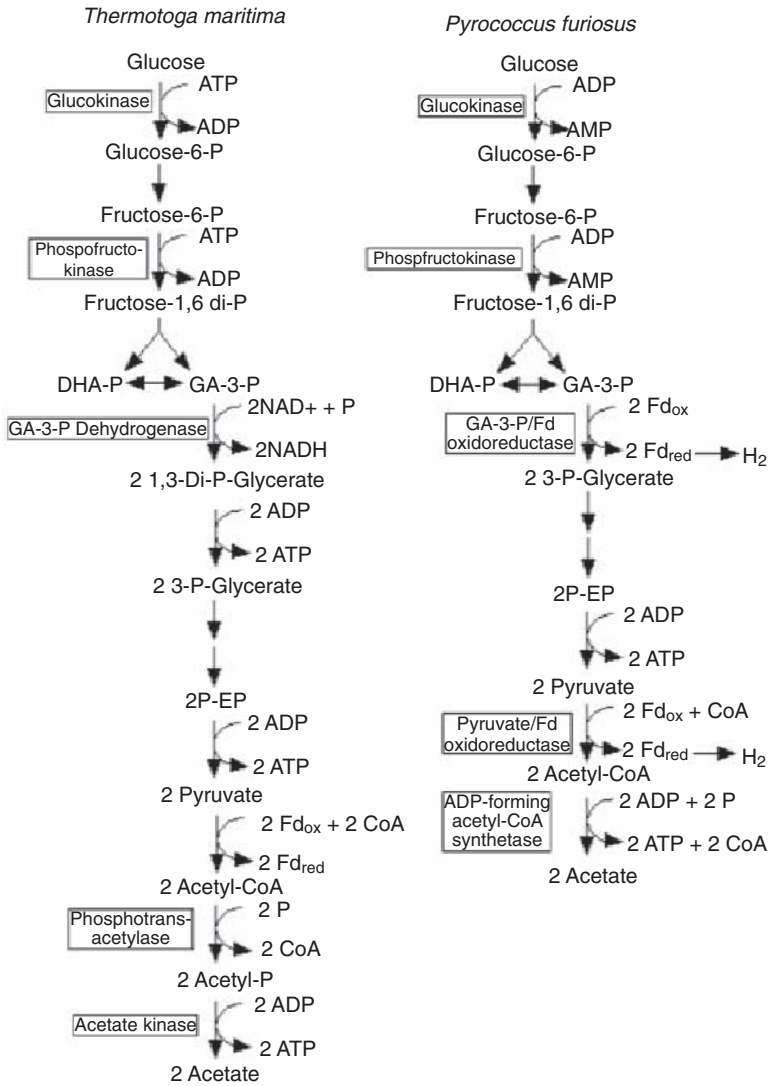


Fig. 23. Comparison of glycolytic pathways in *Thermotoga maritima* and *Pyrococcus furiosis*. P, phosphate; DHA, dihydroxyacetone; GA, glyceraldehyde; P-EP, phosphoenolpyruvate; Fd, ferredoxin; and CoA, coenzyme A.

acceptor such as elemental sulfur or a hydrogen-utilizing methanogen is added to remove H_2.

Archaea possess other variants on the EMP pathway (Menendez et al., 1999). In *Desulfurococcus*, ATP is used in both hexokinase and phosphofructokinase as in the classic EMP pathway, whereas in *Thermoproteus*, ATP is used by hexokinase and pyrophosphate is used by a phosphofructokinase, as has been found in some eubacteria and eukaryotes. All EMP pathways studied in the Archaea thus far utilize glyceraldehyde 3-phosphate/ferredoxin oxidoreductase instead of the NAD^+-utilizing dehydrogenase. A modification of the ED pathway in certain Archaea (including *Sulfolobus*, *Thermoplasma* and *Halobacterium*) has been described in which glucose is not phosphorylated before oxidation to gluconic acid, and phosporylation occurs at a later step in the ED pathway (Conway, 1992).

Besides carbohydrates, amino acids, derived from the hydrolysis of proteins by proteases, are fermented by a variety of prokaryotes, particularly the clostridia, either singly or in pairs, as in the Stickland reaction (Gottschalk, 1986). The products of amino acid fermentation, amines (including putrescine and cadaverine), branched-chain fatty acids, and sulfide and mercaptans from sulfur-containing amino acids, essentially define the word putrid. Our great olfactory sensitivity to these compounds no doubt has played a selective role in protecting us from eating spoiled food potentially containing botulin and other toxins. The purines and pyrimidines derived from nucleic acids are also readily fermented by prokaryotes.

Originally it was thought that only a small number of sugars and amino acids were fermented by prokaryotes. As anaerobic culture techniques have improved, an astounding diversity of compounds has been found to serve as substrates for fermentation, including aromatic compounds and even saturated alkane hydrocar-

bons (Zengler et al., 1999). Many of these fermentations take place in the presence of a hydrogen-consuming anaerobe such as a methanogen. The removal of hydrogen (or acetate in some cases) allows the fermentation to be energetically favorable. This syntrophic interaction is called "interspecies hydrogen transfer" (Schink, 1997).

Indeed, our concepts of fermentation have had to change over the past few decades. It was generally considered that fermentations involved substrate-level phosphorylation. However, consider the example of *Propiogenium modestum*, which grows by decarboxylating succinate to propionate by the following reaction:

There is no net redox reaction, only a decarboxylation. Readers might rightly guess that the pathway is not a simple decarboxylation, but rather involves coenzyme A derivatives and includes a rearrangement to methylmalonyl-CoA. However, the substrate-level phosphorylations in this pathway provide a net ATP yield of zero. Instead, it was shown that membrane-bound methylmalonyl-CoA decarboxylase pumps sodium ions with each decarboxylation it carries out. A sodium-dependent version of an F_1F_0-ATPase can then conserve ATP from the sodium gradient across the cell membrane. Thus, in the absence of electron transport, a chemiosmotic potential is generated in this organism, as well as in others carrying out similar decarboxylations (Dimroth and Schink, 1998).

Anaerobic Respiration

For aerobic respiration, eukaryotes are dependent upon mitochondria, clearly derived from endosymbiotic prokaryotes in the α subphylum of the Proteobacteria (Gray et al., 1999). If the mitochondria were once capable of using electron acceptors other than oxygen, they no longer are, nor have the eukaryotes evolved other mechanisms to use alternate electron acceptors. Thus, anaerobic respiration is solely the domain of prokaryotes. Moreover, prokaryotes can utilize a large range of electron acceptors other than oxygen (Table 1).

Nitrate and nitrite are produced aerobically from ammonia by nitrifyers (see below) and can be used as electron acceptors by diverse members of both the Bacteria and the Archaea (Zumft, 1997). Nitrate is initially reduced to nitrite, and nitrite can be reduced either to N_2 and N_2O gas via the denitrification pathway or to ammonia. The former process is more common as a respiratory process whereas the latter is often used to assimilate nitrate and nitrite but

Table 1. Electron acceptor utilization for respiration.

Reactants	Products	$\Delta G^{\circ\prime}/H_2$ (kJ)	Representative organisms
$O_2 + 2\ H_2$	$2\ H_2O$	–237	*Homo sapiens* *Escherichia coli* *Sulfolobus acidocaldarius*
$NO_3^- + H_2$	$NO_2^- + H_2O$	–163	*Eschericha coli* *Pyrobaculum aerophilum*
$NO_2^- + 2\ H^+ + 3\ H_2$	$NH_4^+ + 2\ H_2O$	–145	*Escherichia coli*
$2\ NO_2^- + 2\ H^+ + 3\ H_2$	$N_2 + 4\ H_2O$	–265	*Pseudomans stuzeri* *Pyrobaculum aerophilum*
$2\ Fe(OH)_3 + 2\ HCO_3^- + 2\ H^+ + H_2$	$2\ FeCO_3 + 6\ H_2$	–118	*Geobacter metallireducens* *Shewanella putrefaciens*
$SO_4^{2-} + H^+ + 4\ H_2$	$HS^- + 4\ H_2O$	–38	*Desulfovibrio desulfuricans* *Archaeoglobus fulgidus*
$S + H_2$	$HS^- + H^+$	–28	*Desulfuromonas acetoxidans* *Pyrodictium brockii*
$HCO_3^- + 4\ H_2 + H^+$	$CH_4 + 3\ H_2O$	–34	*Methanococcus jannaschii* *Methanospirillum hungatei*
$2\ HCO_3^- + 4\ H_2 + H^+$	$CH_3COO^- + 4\ H_2O$	–26	*Acetobacterium woodii*
$Fumarate^{2-} + H_2$	$Succinate^{2-}$	–86	*Escherichia coli*
$(CH_3)_2SO + H_2$	$(CH_3)_2S + H_2O$	–124	*Escherichia coli* *Rhodobacter capsulatus*
R–Cl	R–H	–170	*Desulfomonile tiedjei* *Dehalococcoides ethenogenes*
$ClO_4^- + 4\ H_2$	$Cl^- + 4\ H_2O$	–268	*Ideonella dechloratans*

[a]H_2 is used as a model electron donor for the sake of comparisons of the reactions and its use does not imply that the representative organisms use H_2. Most $\Delta G^{\circ\prime}$ values are taken from Thauer et al., 1977.

can be used as a respiratory process by *E. coli* for example (Stewart, 1994). The energetics of using nitrate and nitrate compounds as electron acceptors is comparable to that of using O_2, and most of the organisms reducing nitrogen oxides are facultative aerobes.

In the final decade of the twentieth century, recognition of the importance of Fe(III) as an electron acceptor in anaerobic habitats increased. Whereas at low pH, Fe(III) mainly exists as the free ion and the Fe(III)/Fe(II) oxidation-reduction potential is near + 0.77v, at circumneutral pH values, Fe(III) exists primarily as hydroxide precipitates, which causes the oxidation/reduction potential to be between +0.2 and –0.2v (Widdel et al., 1993). Amorphous ferric hydroxide precipitates are much more bioavailable than are more crystalline ones. Most known Fe(III) reducers are members of the Proteobacteria, but even certain deep-branching thermophilic Bacteria and Archaea can reduce Fe(III) (Vargas et al., 1998), suggesting a role for this process early in life's history on Earth. Also, certain purple photosynthetic bacteria oxidize Fe(II) to Fe(III) (Widdel et al., 1993), making for a photosynthetic producer-consumer cycle analogous to those for oxygen or sulfur.

Sulfur compounds can serve as electron acceptors, and it is apparent from Table 1 that the amount of energy available from their reduction is considerably less than that of the preceding compounds. Sulfate is an abundant form of sulfur, especially in seawater, where its concentration is near 28 mM. Two genera, *Desulfovibrio* and *Desulfotomaculum*, were essentially the only ones known until better anaerobic techniques were applied, revealing an enormous diversity of organisms. Most sulfate reducers belong to the δ and ε subphyla of the Proteobacteria or to the Firmicutes (Gram-positive bacteria) in the Bacteria and to the genus *Archaeoglobus* in the Archaea. Sulfate reducers and other organisms can generally utilize sulfite or thiosulfate, and these two compounds can be used even in the absence of organic compounds in an inorganic "fermentation" to sulfide and sulfate (Bak and Pfennig, 1987).

Elemental sulfur also is used as an electron acceptor by diverse anaerobes, either as a respiratory electron acceptor leading to energy conservation by an electron transport chain or simply as a way to recycle NADH to NAD^+ for certain fermentative organisms such as *Thermotoga*. Elemental sulfur is reduced to sulfide by many hyperthermophiles growing under conditions of "fire and brimstone," and sulfur reduction is a good candidate for the first respiratory process on earth.

When other electron acceptors in anaerobic habitats are absent or depleted, the acceptor remaining is carbon dioxide, which can be used by either methanogens or acetogens. Methanogens represent the predominant phenotype in the Euryarchaeota phylum in the Archaea, demonstrating an enormous phylogenetic and morphological diversity. Methanogens use only a small number of simple substrates (the most complex is acetic acid) and an intricate pathway for reduction of one-carbon units to methane (DiMarco et al., 1990). This pathway was thought to contain many unique enzymes and cofactors, but a large portion of the pathway is now known to be used to oxidize formaldehyde to CO_2 in aerobic methylotrophic bacteria (Chistoserdova et al., 1998). Moreover, coenzyme M, the immediate precursor of methane in methanogens, has been found in an aerobic alkane-oxidizing *Xanthobacter* species (Allen et al., 1999). Thus, there is indeed unity in biochemistry (Singleton, 2000).

Acetogens are anaerobic eubacteria generally in the Firmicutes capable of reducing two CO_2 moieties to the methyl and acetyl groups of acetyl-CoA (see "Carbon Dioxide Fixation" in this Chapter) by a pathway utilizing an enzyme complex sometimes called the "carbon monoxide dehydrogenase/acetyl coenzyme A synthetase complex" (Ragsdale, 1997). A variant on this enzyme complex is used by methanogenic Archaea to fix carbon dioxide for autotrophic growth and by acetate-utilizing methanogens to split acetyl-coenzyme A. Unlike the methanogens, the acetogens are metabolically versatile, using a wide variety of substrates for acetogenesis.

In most anaerobic habitats, the amount of electron donor is limiting and prokaryotes capable of anaerobic respiration are in fierce competition for those electrons. It has long been known that the outcome of this competition correlates with the free energy available from the reactions, so that denitrifiers could outcompete sulfate reducers, which, in turn, outcompete methanogens, which generally outcompete acetogens. This hierarchy of competitive exclusion is best explained by a threshold model based on thermodynamic principles, so that if the substrate is H_2, sulfate reducers are capable of utilizing that H_2 at concentrations below that at which methanogens can conserve energy from it (Cord-Ruwisch et al., 1988).

Besides these biogeochemically important electron acceptors, a wide diversity of others can be utilized, and we will touch on only a few. It is generally considered that electron acceptors in anaerobic respiration are inorganic, but, for example, *E. coli* and many other organisms capable of anaerobic growth can use fumarate or dimethyl sulfoxide (Weiner et al., 1988) as respiratory electron acceptors. Indeed, during its "fermentation" of glucose, *E. coli* produces suc-

cinate, which is the product of the respiratory reduction of fumarate.

Besides its importance to the biogeochemical cycles of carbon, nitrogen, and sulfur, anaerobic respiration plays an important role in biodegradation of pollutants. One important set of reactions is that involving reductive dechlorination of chlorinated organics, which are among the most important of pollutants. The most highly chlorinated organics are often resistant to aerobic attack, but there is considerable energy available for conservation if they can use the chlorinated organic compound as a respiratory electron acceptor (Mohn and Tiedje, 1992), a process sometimes called "dehalorespiration." It was first demonstrated that 3-chlorobenzoate could serve as an electron acceptor for energy conservation via reductive dechlorination for an organism called *Desulfomonile tiedjei* (DeWeerd et al., 1990; Suflita et al., 1982), a member of the δ-Proteobacteria. Since then, organisms have been shown to conserve energy by reductive dechlorination of chlorophenols and chlorinated ethenes (Holliger et al., 1998; Maymó-Gatell et al., 1997; Mohn and Tiedje, 1992), the latter including the solvents tetrachloroethene and trichloroethene, which are particularly pervasive ground water pollutants. Curiously, although evidence for anaerobic breakdown of less chlorinated methanes is ample (Mägli et al., 1996; Messmer et al., 1993), respiratory utilization of chloroform and carbon tetrachloride has not been described.

Other environmental applications utilizing anaerobic respiration involve reduction of various metals besides Fe(III) (Lovley and Coates, 2000). For example, *Desulfovibrio desulfuricans* can reduce U(VI) to U(IV), which is a precipitate, and allows immobilization of radioactive wastes (Lovley and Phillips, 1992). Another novel reaction with environmental potential is the respiratory reduction of perchlorate, which is part of rocket propellant mixtures and has contaminated several groundwater aquifer sites, to chloride (Coates et al., 1999; Malmquist et al., 1994).

Aerobic Metabolism

In aerobic eukaryotes, respiration is carried out by mitochondria, which are now known to be descended from the α-Proteobacteria (Gray et al., 1999). Thus, all respiration on Earth is prokaryotic. In essentially all aerobic organisms, electrons travel down an electron transport chain from the organic compound to oxygen in a manner such that protons, or sometimes sodium ions, are pumped out of the cell or mitochondrion, leading to development of an electric potential that can be converted to ATP by membrane-associated ATPases (Saraste, 1999).

Diversity of prokaryotic electron transport chains can be considerable. For example, the α-Proteobacterium *Paracoccus denitrificans* utilizes a complex transport chain very closely resembling that in mitochondria consisting of three large membrane-bound enzyme complexes (Fig. 24A). The first is a NADH/ubiquinone

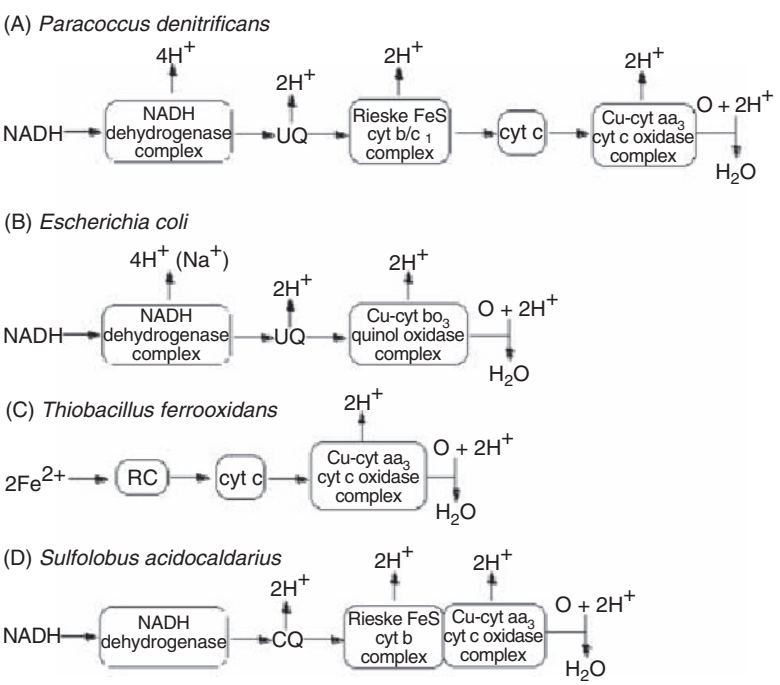


Fig. 24. Electron transport in various prokaryotic aerobes: A) *Paracoccus denitrificans*; B) *Escherichia coli*; C) *Thiobacillus ferrooxidans*; and D) *Sulfolobus acidocaldarius*. UQ, ubiquinone; cyt, cytochrome; RC, rusticyanin; and CQ, caldariellaquinone.

oxidoreductase complex (NADH dehydrogenase or Complex I) containing a bound flavin and several iron/sulfur centers. The second is a ubiquinol/cytochrome *c*-oxidoreductase complex (cytochrome bc_1 complex or Complex III), which contains heme groups, and the high potential Rieske iron/sulfur center carries out a Q cycle (Saraste, 1999), thereby conserving energy from ubiquinone transport. Cytochrome c is reduced in the periplasm and transfers its electrons to a copper- and heme-containing cytochrome c oxidase (Complex IV), which passes the electrons from a periplasmic cytochrome *c* to oxygen. Protons are pumped out of the cell by each of the complexes, and by the asymmetric reduction of ubiquinone on the inside of the cell membrane and its oxidation of the outside (the Q loop) and by the consumption of protons inside the cell by the reduction of oxygen to water. It should be mentioned that *P. denitrificans* actually has several different terminal oxidases that it regulates in response to growth conditions (De Gier et al., 1994).

Escherichia coli has a simpler electron transport chain that lacks Complex III, so that electrons are passed directly from the quinol to an oxidase complex (Fig. 24B). Thus, it does not pump as many protons per oxygen as does *P. denitrificans*. Moreover, under low oxygen conditions, *E. coli* induces more of a quinol oxidase complex containing hemes *b* and *d*. This complex does not pump protons at all, but has a lower K_m for oxygen, which is a useful tradeoff.

Thiobacillus ferrooxidans is an acidophile which transports electrons from Fe(II) to oxygen, and inasmuch as the Fe(II)/Fe(III) couple is near +0.77 v, Fe(II) cannot reduce NADH (–0.32 v) or quinones (ca. 0.0 v). Therefore *T. ferrooxidans* has a truncated electron transport chain in which electrons flow from Fe(II) to a small periplasmic copper-containing protein called "rusticyanin," then to cytochrome c and finally to a typical oxidase (Fig. 24C). Thus *T. ferrooxidans* conserves less energy per electron than do organisms using reactions in which NADH is the electron donor.

As an example of an archaeal electron transport chain, the thermoacidophile *Sulfolobus acidocaldarius* can use elemental sulfur or organic compounds as electron donors for its aerobic growth. When growing on organic compounds, it transports electrons from NADH to oxygen using an electron transport chain that is similar to those in eubacteria, but with some interesting differences (Fig. 24D). Its NADH dehydrogenase complex is relatively small and does not appear to pump protons; it uses a sulfur-containing quinone called "calderiellaquinone," and the electrons then go through a supercomplex equivalent to the two found in *Paracoccus*, but lacking cytochrome *c* (Schäfer et al., 1996). The main subunit of the terminal oxidase complex shows genetic relatedness to other copper-heme oxidases, which form a gene family (Garcia-Horsman et al., 1994) that also includes nitric oxide reductase from denitrifiers, suggesting that the different reductases had a common origin.

The energetics of aerobic respiration is so energetically favorable that the oxidation of essentially any organic compound is thermodynamically feasible, and the only limitation for an organism to utilize a particular organic compound is devising an energy-conserving breakdown pathway. Typically, microorganisms can break down any compound made by nature, as well as many non-natural synthetic compounds (xenobiotics). There are exceptions to this doctrine of microbial infallibility (Alexander, 1981) including some polymers and some smaller molecules that do not resemble natural substrates, leading to persistence of some toxic chemicals in the environment. Nevertheless, a novel compound existing in an environment at a reasonably high concentration may eventually select for organisms capable of using it. For example, *Burkholderia cepacia* strain AC1100 has, apparently by mutation and genetic exchange, developed a pathway to utilize the herbicide 2,4,5-T, originally considered nondegradable, as a growth substrate (Haugland et al., 1990; Huebner et al., 1998).

Lithotrophy and Methanotrophy

Another metabolic capability found uniquely in prokaryotes is lithotrophy, the ability to use inorganic electron donors for energy conservation. Their ability to oxidize inorganic nitrogen and sulfur compounds makes prokaryotic lithotrophs important components of the biogeochemical cycles of these elements. Both aerobes and anaerobes can oxidize inorganic substrates, such as hydrogen oxidation by methanogens, but we will confine our discussion mainly to aerobes. Because they use inorganic substrates for energy conservation, lithotrophs are often autotrophs, i.e., they fix carbon dioxide, but some can incorporate organic carbon. Methanotrophy, the ability to utilize methane as an electron donor, has only been found in certain aerobic members of the Proteobacteria.

The nitrifyers, bacteria that oxidize ammonia and nitrite to nitrate, were originally characterized in the classic studies of Winogradsky. One set of organisms, sometimes called "nitrosofiers," oxidizes ammonia to nitrite, followed by oxidation of nitrite to nitrate. The reason that oxidation of ammonia to nitrate requires two separate microbial groups is not understood. Most extant

Table 2. Reactions carried out by aerobic lithotrophs and methanotrophs.

Reaction	$\Delta G^{\circ\prime}/2e^-$ (kJ)	Organism type	Example species
$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + 2\ H_2O$	–137	Nitrosofying bacteria	*Nitrosomonas europea*
$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$	–76	Nitrifying bacteria	*Nitrobacter winogradsii*
$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$	–238	Anammox organisms	*Brocadia ammanoxidans*
$S^\circ + 1.5\ O_2 \rightarrow SO_4^{2-} + 2\ H^+$	–196	"Colorless" sulfur bacteria	*Thiobacillus thiooxidans* *Sulfolobus acidocaldarius*
$2\ Fe^{2+} + 2\ H^+ + 0.5\ O_2 \rightarrow 2\ Fe^{3+} + H_2O$	–66[a]	Iron bacteria	*Thiobacillus ferrooxidans* *Sulfolobus acidocaldarius*
$2\ FeS_2 + 7.5\ O_2 + 7\ H_2O \rightarrow 2\ Fe(OH)_3 + 8\ H^+ + 4\ SO_4^{2-}$	–164[a]	Iron bacteria	*Thiobacillus ferrooxidans* *Metallosphaera sedula*
$H_2 + 0.5\ O_2 \rightarrow H_2O$	–237	Hydrogen bacteria	*Ralstonia eutropha*
$CH_4 + 2\ O_2 \rightarrow HCO_3^- + H^+ + H_2O$	–203	Methanotrophs	*Methylococcus capsulatus*

Abbreviation: Anammox, anaerobic ammonium oxidation.
[a]Values for pH = 2.

nitrifiers are members of the Proteobacteria, although the nitrite oxidizer *Nitrospira* is in a distinct phylum, and all can fix CO_2 by the Calvin cycle. Because organic matter generally inhibits nitrofiers, Winogradsky used silica gel plates rather than agar to isolate them.

The aerobic oxidation of ammonia begins with oxidation to hydroxylamine by ammonia monooxygenase, making that process obligately aerobic. However, an anaerobic oxidation process, called the "anammox reaction," has been found in which ammonia is the electron donor and nitrite is the electron acceptor (Table 2). The organism responsible for this reaction has not been isolated but strong molecular biological evidence shows that the predominant organism in the enrichment culture is a member of the phylum Planctomycetes (Strous et al., 1999).

The "colorless" sulfur bacteria are so named to differentiate them from the purple sulfur and green sulfur photosynthetic bacteria. They oxidize reduced sulfur compounds, often the product of sulfate reducers in anaerobic zones, so they are often found at anaerobic/aerobic interfaces. Typically they can oxidize sulfide, elemental sulfur (to which they can sometimes be attached or which they store in cellular vacuoles), or thiosulfate. Because their main metabolic product is sulfuric acid, some of the sulfur oxidizers are acidophiles. Many of the Gram-negative rods, both acidophilic and neutrophilic, which carry out this reaction, were named "*Thiobacillus*." However, these organisms are scattered throughout the Proteobacteria and many will require new generic assignments. There are also some filamentous sulfur-oxidizing bacteria such as *Beggiatoa* and *Thiothrix*, which are also in the proteobacteria (although *Beggiatoa*, because of its close morphological resemblance to the filamentous cyanobacterium *Oscillatoria*, was once considered by some to be a colorless cyanobacterium). There are also archaeal sulfur oxidizers, such as *Sulfolobus*.

Some sulfur-oxidizers can use nitrate as an electron acceptor. One interesting example is the large (ca. 50 μm in diameter) gliding filaments called *Thioploca*, which form large mats in upwelling zones. When these filaments are in the aerobic zone, they concentrate nitrate from seawater, where its concentration is near 25 micromolar, into intracellular vacuoles, where the nitrate concentrations can reach the remarkably high concentration of 5 M. They can then glide down into the anaerobic parts of the mat, where they can then use the nitrate for oxidation of sulfide (Fossing et al., 1995). An even larger organism, up to 1 mm in diameter, is the spherical organism, called "*Thiomargarita*" (sulfur-pearl), which consists of a thin film of cytoplasm around a large nitrate-filled vesicle. These organisms are uniquely poised to exploit the anaerobic/aerobic interface by using an electron acceptor that allows them to use sulfide in anaerobic layers where aerobic metabolism is excluded.

Ferrous iron spontaneously oxidizes at pH 7, which has hampered studies on neutrophilic iron oxidizers. Several organisms, such as the sheathed bacterium *Leptothrix* and the stalked bacterium *Gallionella*, are known to precipitate iron and manganese oxides, but it is still unclear what role they play in their metabolism. More recently, Emerson and Moyer (1997) used agar gradient cultures in which reduced iron diffused from an agar plug at the bottom while oxygen diffused from the top. Microorganisms grew as a band at the iron/oxygen interface in these cultures, from which neutrophilic iron oxidizers were isolated.

At low pH, ferrous iron is stable in the presence of oxygen, and *Thiobacillus ferrooxidans* and other iron-oxidizing organisms such as *Leptospirillum ferrooxidans* can be readily isolated from acidophilic environments. Acidophilic Archaea such as *Sulfolobus acidocaldarius* and the newly described mesophile *Ferroplasma aci-*

darmanus (Edwards et al., 2000) can also oxidize ferrous iron. Most organisms that can oxidize ferrous iron and sulfur compounds play a role in the leaching of metal sulfide minerals such as pyrite (FeS_2). The exposure of pyrite minerals to oxygen during coal mining leads to their oxidation to sulfuric acid, resulting in acid mine drainage. On the other hand, this ability has been taken advantage of to recover metals such as copper from low-grade sulfide ores. Thus, the iron-oxidizing prokaryotes can have positive or negative economic effects.

The ability to oxidize hydrogen (which only requires the ability to link a hydrogenase with an electron transport chain) is widespread in the Bacteria and Archaea. Because of labile iron/sulfur centers in hydrogenases, often hydrogen oxidation occurs under microaerophilic conditions in aerobes. Some hydrogen bacteria can grow as autotrophs, but many will incorporate organic carbon when available.

Methanotrophy, the ability to oxidize methane, has only been found in two clusters in the Proteobacteria. Methylotrophy, the ability to use single-carbon compounds such as methanol is more widespread, occurring in many bacteria and archaea, as well as in certain yeasts. The first step in aerobic methanotrophs is the hydroxylation of methane to methanol by methane monooxygenase, making the process obligatorily aerobic. However, there is considerable evidence that methane may be oxidized under anaerobic conditions (Boetius et al., 2000), but the organisms involved have thus far proved elusive. It was long believed that the intermediates after methanol in the pathway of methane oxidation were free one-carbon compounds such as formaldehyde and formate (Dworkin and Foster, 1956), but it has recently been shown that the pathway in many methanotrophs and methylotrophs closely resembles that in methanogenic Archaea in which intermediates are bound to tetrahydromethanopterin and methanofuran and which involves enzymes homologous to those in methanogens (Chistoserdova et al., 1998).

Eukaryotes are unable to utilize inorganic energy sources or methane, and therefore, some living in habitats in which these energy sources are abundant, such as anaerobic/aerobic sediment interfaces or near undersea spreading centers, have entered symbioses with lithotrophs or methanotrophs. One of the best known examples is the giant tubeworm *Riftia* living near undersea spreading centers and using intracellular bacteria that oxidize sulfide and fix CO_2 via the Calvin cycle (Robinson et al., 1998). A wide variety of marine invertebrates, especially clams and mussels, have either sulfide-oxidizing or methane-oxidizing symbionts, sometimes both (Distel and Cavanaugh, 1994). Thus, the eukaryotic hosts have used the prokaryotes to augment their metabolic capabilities, much as eukaryotic hosts did when entering symbioses with the ancestors of the mitochondria and chloroplasts or with symbiotic bacteria providing needed nutrients (Moran and Baumann, 2000).

Phototrophy

Phototrophy, the ability to use light as an energy source, is yet another invention of prokaryotes. Photosynthesis in eukaryotes is carried out in chloroplasts, conclusively shown to be derived from cyanobacteria and probably the result of a single endosymbiotic event (Moreira et al., 2000). There are two general classes of phototrophy in prokaryotes. The first is one based on the now inappropriately named "bacteriorhodopsin" (it should be "archaeorhodopsin") found in halophilic Archaea. Bacteriorhodopsin is a polypeptide with a retinal prosthetic group, which allows it to pump protons, thereby generating a protonmotive force. This system seems to be supplemental to normal heterotrophic growth in these organisms, although its actual ecophysiological role is unclear. Besides bacteriorhodopsin, the halobacteria can possess halorhodopsin, a light-driven chloride pump, and two types of sensory rhodopsins used in phototaxis (Spudich, 1993). Recently, a DNA fragment cloned from ocean water was found to have, besides a 16S rDNA gene, which showed it to be from a proteobacterium, a gene encoding rhodopsin-like protein (Beja et al., 2000). When expressed in *E. coli* and provided with retinal, this protein pumped protons, and has been given the name "proteorhodopsin."

The major type of phototrophy is that based on chlorophylls. Among the prokaryotes, there is considerable diversity of pigments, photosystems and electron donors, all adapted to a variety of ecological niches. The photosynthetic bacteria can be placed into five groups, the purple bacteria (traditionally divided into the purple sulfur and nonsulfur bacteria, based on their use of reduced sulfur or organic compounds as the preferred electron donor), the heliobacteria, the green sulfur bacteria, the green nonsulfur bacteria and the cyanobacteria (Table 3). Phylogenetic analysis of the 16S rDNA of the photosynthetic bacteria (Stackebrandt et al., 1996) shows that these groups occupy different phyla.

The purple bacteria are found in three subphyla of the diverse phylum Proteobacteria, which includes many typical "Gram-negatives" such as *E. coli*, *Pseudomonas* and *Rhizobium*. The purple nonsulfur bacteria are spread within the α and β subphyla and the purple sulfur bacteria are associated with the γ-subphylum. This

Table 3. Properties of photosynthetic bacteria and chloroplasts.

	Phylogenetic group	Reaction center chlorophyl	Accessory pigments	Electron donors	Primary electron acceptor	CO_2 Fixation pathway
Purple nonsulfur bacteria	α and β Proteobacteria	bcl *a* or *b*	chl *a* or *b*	Organic compounds, H_2, Fe^{2+}, H_2S (at low concentration)	Q	Calvin cycle
Purple sulfur bacteria	γ Proteobacteria	bcl *a*	chl *a* or *b*	H_2S, etc., H_2, some organic compounds	Q	Calvin cycle
Heliobacteria	Firmacutes	bcl *g*	—	Organic compounds	Fd	Calvin cycle
Green sulfur bacteria	Chlorobi	bcl *a*	bcl *c*,*d*,*e* (chlorosomes)	H_2S, $S_2O_3^{2-}$, H_2	Fd	Reverse TCA cycle
Green nonsulfur bacteria	Chloroflexi	bcl *a*	bcl *c*,*d*,*e* (chlorosomes)	Organic compounds, H_2, H_2S	Q	Hydroxypro-pionate cycle
Cyanobacteria	Cyanobacteria	chl *a*	phycobilins or chl *b*	H_2O	Q (PSI) Fd (PSII)	Calvin cycle
Chloroplasts	Cyanobacteria	chl *a*	phycobilins or chl *b*	H_2O	Q (PSI) Fd (PSII)	Calvin cycle

Abbreviations: Q, quinone; Fd, ferredoxin; TCA, tricarboxylic acid; and PS, photosystem.

distribution strongly suggests that the ancestor of these groups was itself a "purple bacterium" and that the current nonphotosynthetic organisms in the Proteobacteria, like *E. coli*, evolved from photosynthetic ancestors.

Similarly, it was a surprise when the heliobacteria, a group physiologically similar to the purple nonsulfur bacteria, were found to be members of the Gram-positive phylum Firmacutes, although in this case only a single small branch within the phylum contains photosynthetic organisms. The green sulfur bacteria form essentially their own separate phylum, the Chlorobia. According to 16S rDNA phylogeny, the green nonsulfur bacteria, despite similarities of their photopigments, are not closely related to the green sulfur bacteria and are the founding members of the phylum Chloroflexus. Interestingly, the phylogeny of genes for chlorophyll synthesis in the green sulfur and nonsulfur bacteria indicates a relatively close affinity of the two groups, suggesting horizontal gene transfer of the photosynthesis genes (Xiong et al., 2000). Finally, the cyanobacteria also form a separate phylum, which also includes chloroplasts.

The cyanobacteria and chloroplasts contain chlorophyll *a* (chl *a*) as their primary photosynthetic pigment in their reaction centers, which absorbs light maximally at 680–700 nm, depending on whether it is in photosystem I or II. The other photosynthetic bacteria possess only a single photosystem, most containing mainly bacteriochlorophyll a (bcl *a*), which absorbs maximally in the infrared at 800–870 nm. Exceptions include certain purple nonsulfur bacteria which utilize bcl *b*, which absorbs maximally in the far infrared at 1,050 nm, and the heliobacteria, which utilize the interesting pigment bcl g, which absorbs maximally at 790 nm and breaks down to chl *a* upon exposure to oxygen.

All phototrophs contain carotenoids (which make the purple bacteria "purple" and green bacteria green) as accessory pigments to widen the spectrum of light used. In the purple bacteria, the structure of the antenna complex in the membrane, which feeds excitation energy into the reaction center, forms a set of concentric circles around the reaction center (Cogdell et al., 1999). In the green sulfur and nonsulfur bacteria, light energy is transmitted to the reaction center in the cell membrane by the chlorosome (a macromolecular complex containing protein and bacteriochlorophylls *c*, *d*, and *e*), which is associated with the inside face of the cell membrane. In most cyanobacteria, the main accessory pigments that transmit energy to photosystem II are the phycobilins (tetrapyrrole-containing proteins, phycocyanin being the one that confers the blue part of the blue green color of most members of this group). Chloroplasts can contain either phycobilins, as in the case of the red algae, or chl *b*, as found in plants and green algae, as the main accessory pigment. Finding cyanobacteria with chl *b* suggested that they were the ancestors of the green plant chloroplasts; however, phylogenetic analyses have demonstrated that these "prochlorophytes" are simply distinct strains of cyanobacteria with no direct relationship with chloroplasts. The genes for chl *b* synthesis in cyanobacteria and chloroplasts appear to share a common evolutionary origin (Tomitani et al., 1999), but whether that is due to the ancestor of the cyanobacteria and chloroplasts containing chl *b* or horizontal gene transfer has yet to be determined.

Van Niel's insight that the photosynthetic reduction of CO_2 in green plants was a consequence of [H] released by the photolysis of water (van Niel, 1949) was a stroke of genius and led to a unifying equation of photosynthesis: $CO_2 + 2\ H_2A \rightarrow CH_2O\ H_2O + A$, where A could be O, S, an organic constituent, or even nothing in the case of H_2. It also rationalized the diversity among the photosynthetic bacteria, as it became clear that water in the cyanobacteria reduced sulfur in the green and purple sulfur bacteria, and organic molecules in the purple nonsulfur bacteria all served the same purpose—that of providing reducing power for biosynthesis. And each of these sources of reducing power made it possible for that particular group of photosynthetic prokaryotes to exploit a particular ecological niche: water in the case of the cyanobacteria, areas rich in reduced sulfur for the green and purple sulfur bacteria, and areas with organic substrates for the purple and green nonsulfur bacteria.

Photosynthetic pigments have adapted further. All of the chlorophyll molecules have the same basic ground plan—a substituted Mg-tetraporphyrin. However, the nature of the substitutions and of the sequence of conjugated double bonds generate a series of absorption spectra that span from 680 nm for the major absorption peak of chlorophyll *a* in the cyanobacteria to 1,035 nm for the major absorption maximum for bacteriochlorophyll *b* in certain purple bacteria. Thus, the photosynthetic prokaryotes are able to span a broad range of photic zones. The green and purple bacteria usually occupy shallow, aquatic areas, underlying the oxygenic cyanobacteria that filter out the relatively short, visible wavelengths of light and transmit the longer, near-infrared wavelengths. This orientation is consistent with the fact that photosynthesis in the green and purple bacteria is an anoxygenic process and their photosynthesis is obligately anaerobic.

Autotrophic CO_2 Fixation

Carbon dioxide is an abundant and available source of carbon, but it must be reduced to cellular organic carbon at approximately the level of CH_2O, a process that requires reducing power and usually requires energy. Organisms that use CO_2 as their primary carbon source are autotrophs and serve as primary producers in ecosystems. Autotrophic carbon dioxide fixation in eukaryotes is represented solely by the Calvin cycle, though among the prokaryotes there are several distinct pathways of autotrophic CO_2 fixation: 1) the Calvin cycle; 2) the reverse TCA cycle; 3) acetogenesis; and 4) the hydroxypropionate pathway.

The Calvin cycle, which was discovered initially in the green algae, is also found among many of the photolithotrophic and chemolithotrophic Bacteria, where it serves as the major mechanism for carbon assimilation (Fig. 25). The first step of CO_2 fixation is the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) reaction, resulting in the conversion of one molecule of ribulose-1,5-bisphosphate and CO_2 to two molecules of 3-phosphoglyceric acid. These are then reduced to glyceraldehyde 3-phosphate, followed by several rearrangements to regenerate ribulose-1,5-bisphosphate. Rubisco from the Bacteria has been classified into two types. Type I is found in plants, cyanobacteria and several other prokaryotes and has a subunit structure consisting of eight large and eight small subunits. Type II Rubisco is found in certain purple nonsulfur bacteria and a few other organisms and has been found to be a dimer of large subunits. The phylogenetic tree for Rubisco shows many branching orders considerably different from that of the 16S rRNA tree, and this has been considered strong evidence for rampant genetic transfer of this gene (Delwiche and Palmer, 1996). Moreover, genes resembling those encoding Rubisco have been found in archaeal genomes, and it has been shown that the corresponding proteins have Rubisco activity (Maeda et al., 1999; Watson et al., 1999). These Rubisco homologues form yet another phylogenetic cluster, and the metabolic role of these enzymes in their host organisms has yet to be determined because most evidence indicates that CO_2 in methanogenic archaea is fixed via the acetogenic pathway (see below).

The Chlorobiaceae, known colloquially as the "green sulfur bacteria," were shown not to use the Calvin cycle for CO_2 fixation. Instead, they run the tricarboxylic acid (TCA) cycle in the reverse, reductive direction, using it to fix CO_2 eventually into acetyl-CoA and pyruvate, rather than in the conventional oxidative direction (Fig. 26). Most, but not all of the enzymes will catalyze the reactions in the reverse direction. Thus, the Chlorobiaceae have replaced succinic dehydrogenase with fumarate reductase, substituted an α-ketoglutarate dehydrogenase-ferredoxin oxidoreductase for the conventional α-ketoglutarate dehydrogenase complex, and replaced the irreversible citrate synthase with an ATP-citrate lyase. Since its discovery in the Chlorobiaceae, the reductive TCA cycle has also been found in Desulfobacter hydrogenophilus in the δ-subphylum of the Proteobacteria, in members of the

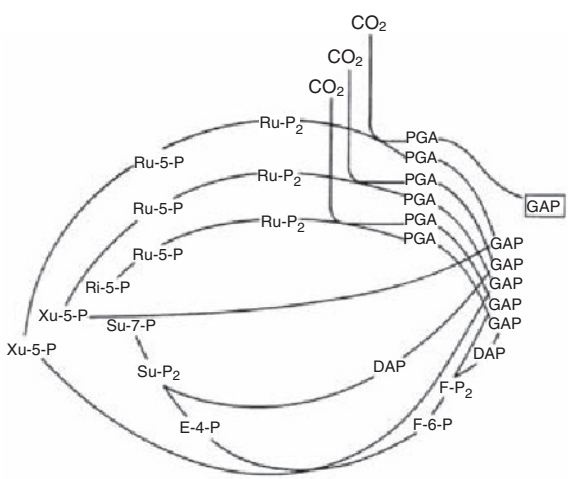


Fig. 25. The Calvin cycle. Abbreviations: Ru-P2, ribulose-1,5-bisphosphate; PGA, 3-phosphoglycerate; GAP, glyceraldehyde-3-phosphate; DAP, dihydroxyacetone phosphate; F-P2, fructose-1,6-bisphosphate; F-6-P, fructose-6-phosphate; E-4-P, erythrose-4-phosphate; Su-P2, sedoheptulose-1,7-bisphosphate; Su-7-P, sedoheptulose-7-phosphate; Xu-5-P, xylulose-5-phosphate; Ri-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate. (Adapted from Gottschalk, 1986.)

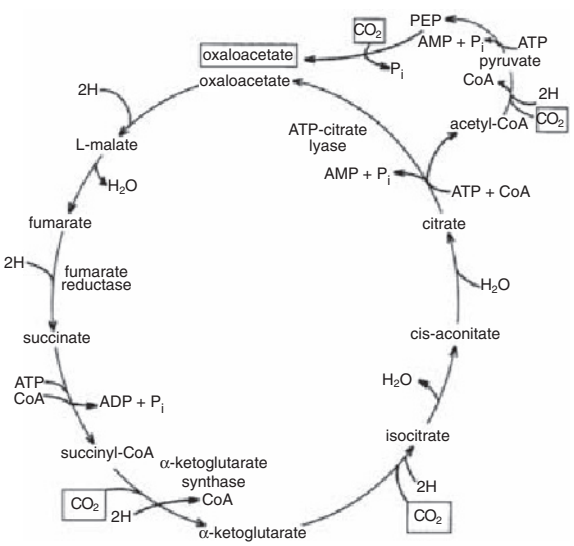


Fig. 26. The reductive or reversed tricarboxylic acid cycle. (Adapted from Gottschalk, 1986.)

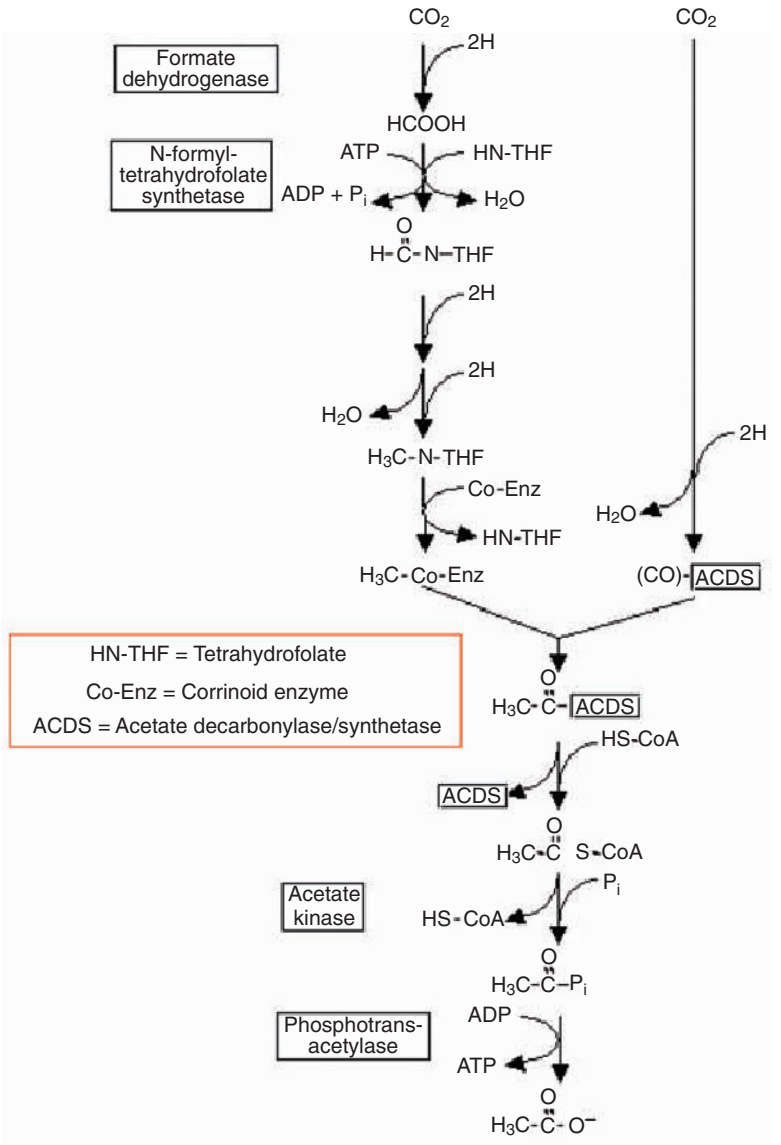


Fig. 27. The acetogenic pathway.

Aquificae, a deeply branching hyperthermophilic phylum in the Bacteria, and in anaerobic members of the Crenarchaeota. It was thought to be present in some aerobic Crenarchaeota, but recent results indicate that a different pathway functions in those organisms (see below).

As mentioned previously in the discussion of anaerobic respiration, acetogenic bacteria can convert H_2 and CO_2 into acetate, the equivalent of fixing CO_2 into organic matter. Indeed, many acetogens can grow in mineral medium using CO_2 as a carbon source and are therefore autotrophs. The pathway, shown in Fig. 27, leads to the fixation of two moles of carbon into acetate. The methyl group of acetate is synthesized by reducing one-carbon units to methyltetrahydrofolate. This methyl group is transferred to a corrinoid-containing iron-nickel sulfur enzyme complex called "carbon monoxide dehydrogenase" or "acetate synthetase/decarbonylase." The methyl group is transferred to the cobalt of a corrinoid and a CO_2 is reduced to the equivalent of carbon monoxide. These are then assembled into an enzyme-bound acetyl group, which is released as acetyl-coenzyme A (CoA), which can be conserved as ATP in catabolism or can be used for biosynthesis. This pathway costs a cell only one ATP per two carbons fixed to acetyl-CoA. This pathway is also found in certain sulfate reducers in the δ-subphylum of the Proteobacteria (Menendez et al., 1999). A variation of this pathway using methanopterin derivatives rather than folates is found in autotrophic methanogenic Archaea as well as in the sulfate-reducing Archaeoglobus (Menendez et al., 1999). Thus, the acetogenic pathway is responsible for much of the CO_2 fixation occurring in anoxic habitats.

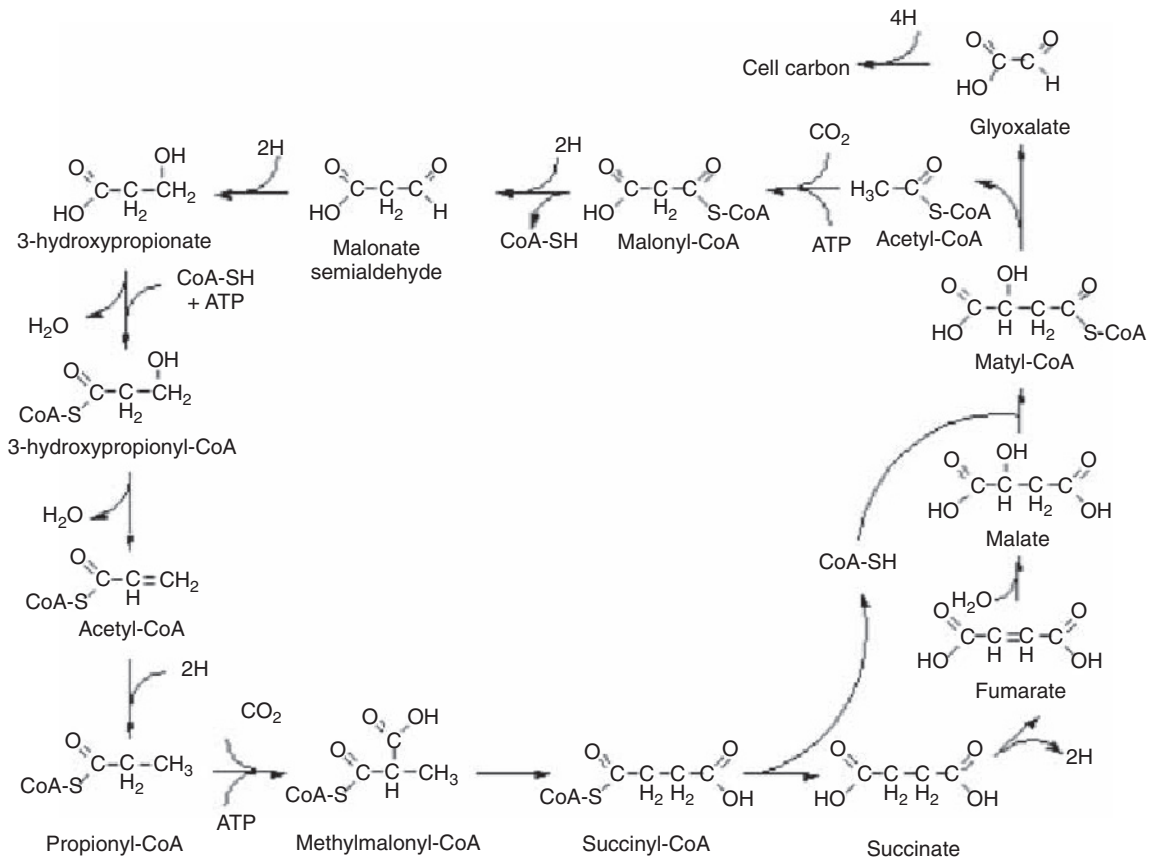


Fig. 28. The 3-hydroxypropionate cycle. (From Menendez et al., 1999.)

Finally, the pathway for CO_2 fixation in the green nonsulfur photosynthetic bacterium *Chloroflexus aurantiacus* was not clear until it was demonstrated (Strauss and Fuchs, 1993) that a novel pathway based on carboxylation of acetyl-CoA and propionyl-CoA (Fig. 28), sometimes called the "3-hydroxypropionate pathway," is responsible for CO_2 fixation to glyoxalate in these organisms. The key enzymes of this pathway are acetyl-CoA carboxylase and propionyl-CoA carboxylase. This pathway has also been found in aerobic lithotrophic Crenarchaeota such as *Sulfolobus* (Menendez et al., 1999). The distribution of these four autotrophic pathways is summarized in Table 4.

Nitrogen Fixation

Fixation of N_2 is carried out solely by prokaryotes. Until the twentieth century, when a large amount of nitrogen was fixed anthropogenically by the Born-Haber process, essentially all nitrogen found in eukaryotes was originally fixed by prokaryotes and transferred to them either indirectly through the food chain or directly in symbiosis, as in the case of the symbioses between legumes and rhizobia found in their root nodules. Nitrogen fixation is found in a variety of physiological types of prokaryotes, occurring in aerobes, microaerophiles, anaerobes, phototrophs and in free-living as well as symbiotic bacteria. Phylogenetically it is widespread in the Bacteria, including members of the phyla Proteobacteria, Firmacutes, Actinobacteria, Chlorobia and Cyanobacteria. In the Archaea, nitrogen fixation has only been found thus far in the methanogens (Lobo and Zinder, 1992).

The enzyme that is responsible for the process, nitrogenase, is a complex of two metalloprotein components. One component usually contains an iron/sulfur/molybdenum cluster that is considered the active site for dinitrogen reduction, as well as an unusual iron/sulfur cluster called the "P cluster" (Dean et al., 1993). The second component is sometimes called the "Fe protein" and contains a single 4Fe-4S cluster per homodimer and transfers electrons to the first component, accompanied by hydrolysis of approximately two ATPs to ADPs per electron transferred. Because this process is so energetically expensive, it is not surprising that most free-living organisms regu-

Table 4. CO_2 fixation pathways found in prokaryotes.

Pathway	Representative organisms	Phylum
Calvin cycle	Plant chloroplasts	Cyanobacteria
	Anabaena cylindrical	Cyanobacteria
	Rhodobacter sphaeroides	Proteobacteria
	Thiobacillus ferrooxidans	Proteobacteria
	Methanococcus jannaschii[a]	Euryarchaeota
Reductive TCA cycle	*Chlorobium limicola*	Chlorobi
	Desulfobacter hydrogenophilus	Proteobacteria
	Aquifex pyrophilus	Aquificae
	Thermoproteus neutrophilus	Crenarchaeota
Reductive acetyl CoA pathway	*Clostridium thermoaceticum*	Firmacutes
	Desulfobacterium autotrophicum	Proteobacteria
	Methanococcus jannaschii[b]	Euryarchaeota
	Ferroglobus placidus	Euryarchaeota
Hydroxypropionate cycle	*Chloroflexus aurantiacus*	Chloroflexi
	Sulfolobus metallicus	Crenarchaeota

Abbreviations: TCA, tricarboxylic acid; and CoA, coenzyme A.
[a]The function of Rubisco found in methanogens and other Euryarchaeota are not presently known.
[b]Pathway uses methanofuran and tetrahydromethanopterin instead of tetrahydrofolate.
Data from Menendez et al. (1999).

late expression of nitrogenase genes (Berger et al., 1994) and activity (Ludden and Roberts, 1988). Alternative nitrogenases exist in which molybdenum (Mo) is replaced by vanadium (V) or in which no metal other than iron has been found (Bishop and Premakumar, 1992). The nitrogenase protein components contain many highly conserved amino acid sequence motifs involved in cofactor binding and subunit interactions. Interestingly, these proteins are homologous to proteins involved in reductive steps of chlorophyll *a* biosynthesis (Fujita and Bauer, 2000), providing a link between the processes of nitrogen fixation and photosynthesis.

Both protein components of nitrogenase are extremely sensitive to oxygen and the bacteria fixing nitrogen aerobically have evolved a variety of strategies to protect the nitrogenase from oxygen poisoning. Among the members of the genus *Azotobacter*, there are three mechanisms for nitrogenase protection. These are respiratory protection, conformational protection, and oxygen regulation of nitrogenase synthesis (Kennedy and Toukdarian, 1987). Respiratory protection occurs because *Azotobacter* can consume oxygen much faster than its rate of entry into the cell. These unusually high rates of respiration thus result in maintaining the nitrogenase in an essentially anoxic environment. Indeed, limiting *Azotobacter* respiration increases their sensitivity to oxygen during nitrogen fixation. Conformational protection is a result of the ability of *Azotobacter* to synthesize another FeS protein that enters into an association with the nitrogenase complex and protects it from O_2 inactivation (Moshiri et al., 1994). During this association, the complex is unable to manifest any nitrogenase activity.

Many organisms are not as adept as Azotobacter at protecting their nitrogenase from O_2 and fix nitrogen only under microaerophilic conditions, even though they otherwise are not microaerophiles. One example of this is the rhizobia (Spaink, 2000). They were not shown to be able to fix nitrogen until 1975, nearly a century after their isolation by Beijerinck. When in the plant root nodule, the rhizobia are protected by the heme protein leghemoglobin, which has an extremely high affinity for O_2 and transports the O_2 necessary for *Rhizobium* growth, simultaneously preventing the access of the O_2 to the nitrogenase (Dilworth and Appleby, 1979).

The nitrogen-fixing cyanobacteria are presented with an even greater challenge than other aerobes because O_2 is one of the main products of their photosynthetic metabolism. Anabaena and other related filamentous, nitrogen-fixing cyanobacteria solve the problem of O_2 poisoning of nitrogenase by segregating the nitrogen-fixing enzymes in a specialized cell called "the heterocyst." The heterocyst insulates the nitrogenase from O_2 in two ways. First, it lacks photosystem II and thus does not generate any O_2; photosystem I is still operative and continues to generate ATP by photophosphorylation. Second, it is surrounded by a laminated structure consisting of a series of unique glycolipids that seem to act as a physical barrier to prevent O_2 from penetrating into the cell. Thus, the cell separates its nitrogenase both from endogenous as well as exogenous O_2. The heterocyst can feed the fixed, reduced nitrogen products to the adjoining vegetative cells, from which it receives the reducing power necessary to convert dinitrogen to amino acids. To add to the elegance of the solution, the heterocysts are interspersed along the filament,

spaced so as to provide an optimum supply of fixed nitrogen to the growing and dividing vegetative cells. A peptide signal, similar to those used for quorum sensing by Gram-positive bacteria, is used to regulate this spacing (Yoon and Golden, 1998). The actinomycete *Frankia*, which fixes nitrogen in nodules in alder trees and several shrubs, utilizes a similar solution, forming specialized cell aggregates called "vesicles" (Benson and Silvester, 1993).

Some of the nonheterocystous cyanobacteria have solved the problem of nitrogen fixation and photosynthetic O_2 evolution by separating the two processes in time rather than in space. Thus, nitrogenase is synthesized and nitrogen fixation takes place in the dark. During the photoperiod, the nitrogenase formed during the previous dark period is presumably destroyed (Stal and Krumbein, 1985). Indeed this was the first evidence for a biological clock in prokaryotes (Johnson and Golden, 1999). It is still not clear, however, how the filamentous nonheterocystous colonial cyanobacterium *Trichodesmium* fixes nitrogen during photosynthesis (Capone et al., 1997).

Finally, it should be mentioned that a thermophilic actinomycete utilizes a nitrogenase enzyme complex in which a modified Mo-Fe protein is coupled to a carbon monoxide dehydrogenase (Ribbe et al., 1997). This system is much more O_2 resistant than the standard nitrogenases, and it is not clear why similar systems have not been found in other aerobes. In summary, the variety of mechanisms devised by the prokaryotes for protecting nitrogenase from O_2 poisoning is an impressive example of the strategic versatility of the prokaryotes.

Adaptation to Environmental Extremes

The prokaryotes not only can tolerate the broadest spectrum of environmental extremes of any group of organisms, but some of their optimum conditions for growth would roast, freeze, acidify or shrivel up most other organisms. Thus, *Pyrolobus fumarii* can grow up to 113°C and survive one hour of autoclaving at 121°C (Blochl et al., 1997), *Halobacterium salinarium* grows optimally at 5.2 M salt (Kushner, 1985), *Ferroplasma* grows at pH 0 (Edwards et al., 2000), and a wide variety of organisms can grow at temperatures below 0°C (Morita, 1975). It should be pointed out that the organisms specifically referred to above are Archaea. While the ability to thrive under extreme conditions is not an exclusive property of the Archaea, they seem to be able to withstand high temperatures, at least partially attributable to their unusual ether-linked lipids.

In most cases, the mechanisms whereby the organisms are able to thrive in an extreme environment have reduced or eliminated the ability of that organism to tolerate the normal or common environment. In other words, the adaptation to the extreme environment has not extended a particular organism's milieu but rather replaced one optimum with another. Thus, the ability to tolerate, indeed the requirement for, a high salt concentration renders *Halobacterium salinarium* fragile in a normal osmotic milieu, *Pyrolobus* cannot grow at below 80°C, and *Ferroplasma* cannot grow at a neutral pH.

The ability to exploit a wide variety of ecological niches and to grow optimally at environmental extremes that are intolerable for higher, more complex organisms is a result of the intense physiological and metabolic specialization among the prokaryotes. In a sense, then, the ability of a complex, multicellular organism to deal with its environment is a compromise among the various specialized and differentiated cells; the extremes that such an organism can tolerate are the lowest common denominator of the properties of its individual cells. That is the price paid for the operational complexity of the multicellular organism. On the other hand, the small size, structural simplicity, and unicellular nature of the prokaryote are admirably suited to permit adaptation to a wide variety of environmental extremes, with each adaptive response narrowly focused on dealing optimally with that particular extreme.

Conclusion

It is appropriate that we end this essay with a quotation from the late mentor of one of us (M.D.), Professor Jackson W. Foster (Foster, 1964):

"The source of the microbiologist's strength, and at the same time, his refuge, is the infinitely large numbers and varieties of microbes known or presumed to be extant. Compounded with those that can be modified artificially, this pool represents an infinitely versatile catalyst. Because, figuratively speaking, it catalyzes innumerable reactions and transformations with virtually any material occurring in nature, the microbe has to be regarded as a prime, natural resource of all men regardless of national boundaries. . . . As a means of emphasizing . . . the diverse, mysterious, and gratifying potential of the microbe, I paraphrase a trenchant saying made famous many years ago by the influential American magazine, the *Ladies Home Journal*. Whereas they were concerned with women, and I with microbes, the small perversion I make does no injustice to either—'Never underestimate the power of the Microbe.' "

Literature Cited

Adams, M. W. W. 1993. Enzymes and proteins from organisms that grow near and above 100 degrees C. Ann. Rev. Microbiol. 47:627–648.

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. Science 211:132–138.

Allen, J. R., D. D. Clark, J. G. Krum, and S. A. Ensign. 1999. A role for coenzyme M (2-mercaptoethanesulfonic acid) in a bacterial pathway of aliphatic epoxide carboxylation. Proc. Natl. Acad. Sci. USA 96:8432–8437.

Angert, E. R., K. D. Clements, and N. R. Pace. 1993. The largest bacterium. Nature (London) 362:239–241.

Bak, F., and N. Pfennig. 1987. Chemolithotrophic growth of Desulfovibrio sulfodismutans sp. nov. by disproportionation of inorganic sulfur compounds. Arch. Microbiol. 147:184–189.

Balkwill, D. L., D. Maratea, and R. P. Blakemore. 1980. Ultrastructure of a magnetotactic spirillum. J. Bacteriol. 141:1399–1408.

Beja, O., L. Aravind, E. V. Koonin, M. T. Suzuki, A. Hadd, L. P. Nguyen, S. B. Jovanovich, C. M. Gates, R. A. Feldman, J. L. Spudich, E. N. Spudich, and E. F. DeLong. 2000. Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902–1906.

Benson, D. R., and W. B. Silvester. 1993. Biology of Frankia strains, actinomycete symbionts of actinorhizal plants. Microbiol. Rev. 57:293–319.

Berg, H. C. 1976. How spirochaetes may move. J. Theoret. Biol. 56:269–273.

Berger, D. K., F. Narberhaus, and S. Kustu. 1994. The isolated catalytic domain of NIFA, bacterial enhancer-binding protein, activates transcription in vitro: Activation is inhibited by NIFL. Proc. Natl. Acad. Sci. USA 91:103–107.

Beveridge, T. J. 1995. The periplasmic space and periplasm in gram-positive and Gram-negative bacteria. ASM News 61:125–130.

Bishop, P. E., and R. Premakumar. 1992. Alternative nitrogen fixation systems. *In:* G. Stacey, R. H. Burris, and H. J. Evans (Eds.) Biological Nitrogen Fixation. Chapman and Hall. New York, NY. 736–762.

Blochl, E., R. Rachel, S. Burggraf, D. Hafenbradl, H. W. Jannasch, and K. O. Stetter. 1997. Pyrolobus fumarii, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113 degrees C. Extremophiles 1:14–21.

Boetius, A., K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Gieseke, R. Amann, B. B. Jorgensen, U. Witte, and O. Pfannkuche. 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407:623–626.

Brun, Y. V., and L. J. Shimkets. 2000. *In:* Y. V. Brun, and L. J. Shimkets (Eds.) Prokaryotic Development. ASM Press. Washington DC, 1–500.

Burchard, R. P. 1984. Gliding motility and taxes. *In:* E. Rosenberg (Ed.) Myxobacteria: Development and Cell Interactions. Springer-Verlag. New York, NY. 139–161.

Canale-Parola, E. 1978. Motility and chemotaxis in spirochaetes. Ann. Rev. Microbiol. 32:69–99.

Capone, D. G., J. P. Zehr, H. W. Paerl, B. Bergman, E. J. Carpenter. 1997. Trichodesmium, a globally significant marine cyanobacterium. Science 276:1221–1229.

Carr, N. G. 1979. Differentiation in filamentous cyanobacteria. *In:* J. H. Parish (Ed.) Developmental Biology of Prokaryotes. University of California Press. Berkeley, CA. 167–201.

Chater, K. E., and D. A. Hopwood. 1973. Differentiation in actinomycetes. *In:* J. M. Ashworth and J. E. Smith (Eds.) Microbial Differentiation. Cambridge University Press. London, 143–160.

Chistoserdova, L., J. A. Vorholt, R. K. Thauer, and M. E. Lidstrom. 1998. C1 transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic Archaea. Science 281:99–102.

Clements, K. D., and S. Bullivant. 1991. An unusual symbiont from the gut of surgeonfishes may be the largest known prokaryote. J. Bacteriol. 173:5359–5362.

Coates, J. D., U. Michaelidou, R. A. Bruce, S. M. O'Connor, J. N. Crespi, and L. A. Achenbach. 1999. Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl. Environ. Microbiol. 65:5234–5241.

Cogdell, R. J., N. W. Isaacs, T. D. Howard, K. McLuskey, N. J. Fraser, and S. M. Prince. 1999. How photosynthetic bacteria harvest solar energy. J. Bacteriol. 181:3869–3879.

Cohn, F. 1875. Untersuchungen über Bacterien. Beiträge zur Biologie der Pflanzen 1:127–222.

Conway, T. 1992. The Entner-Doudoroff pathway: History, physiology and molecular biology. FEMS Microbiol. Rev. 9:1–27.

Cook, K. E., and J. R. Colvin. 1980. Evidence for a beneficial influence of cellulose production on growth of Acetobacter xylinum in liquid medium. Curr. Microbiol. 3:203–205.

Cord-Ruwisch, R., H.-J. Steitz, and R. Conrad. 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Arch. Microbiol. 149:350–357.

Costerton, J. W., J. C. Nickel, and T. I. Ladd. 1986. Suitable methods for the comparative study of free-living and surface-associated bacterial populations. *In:* J. S. Poindexter and E. L. Leadbetter (Eds.) Bacteria in Nature. Plenum Press. New York, NY. 2:49–84.

Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. Science 284:1318–1322.

Cross, T. 1981. The monosporic actinomycetes. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel, eds. The Prokaryotes. Springer, Berlin, 2091–2102.

Dean, D. R., J. T. Bolin, and L. M. Zheng. 1993. Nitrogenase metalloclusters—structures, organization, and synthesis. J. Bacteriol. 175:6737–6744.

De Gier, J. W., M. Lubben, W. N. Reijnders, C. A. Tipker, D. J. Slotboom, R. J. van Spanning, A. H. Stouthamer, and J. van der Oost. 1994. The terminal oxidases of Paracoccus denitrificans. Molec. Microbiol. 13:183–196.

Delwiche, C. F., and J. D. Palmer. 1996. Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. Molec. Biol. Evol. 13:873–882.

DeWeerd, K. A., L. Mandelco, R. S. Tanner, C. R. Woese, and J. M. Suflita. 1990. Desulfomonile tiedjei gen. nov. and sp. nov., a novel anaerobic dehalogenating, sulfate-reducing bacterium. Arch. Microbiol. 154:23–30.

Dilworth, M. J., and C. A. Appleby. 1979. A treatise on dinitrogen fixation. *In:* R. W. F. Hardy, F. Bottomly, and R. C. Burns (Eds.) John Wiley. New York, NY. sec. I, II:691–764.

DiMarco, A. A., T. A. Bobik, and R. S. Wolfe. 1990. Unusual coenzymes of methanogenesis. Ann. Rev. Biochem. 59:355–394.

Dimroth, P., and B. Schink. 1998. Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria. Arch. Microbiol. 170:69–77.

Distel, D. L., and C. M. Cavanaugh. 1994. Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbioses in marine bivalves. J. Bacteriol. 176:1932–1938.

Dow, C. S., and R. Whittenbury. 1979. Prosthecate bacteria. *In:* J. H. Parish (Eds.) Developmental Biology of Prokaryotes. University of California Press. Berkeley, CA.

Dunny, C. M., B. L. Brown, and D. B. Clewell. 1978. Induced cell aggregation and mating in Streptococcus faecalis: Evidence for a bacterial sex hormone. Proc. Nat. Acad. Sci. USA 75:3479–3483.

Dworkin, M., and J. W. Foster. 1956. Studies on Pseudomonas methanica (Sohngen) Nov. Comb. J. Bacteriol. 72:646–659.

Dworkin, M. 1973. Cell-cell interactions in the myxobacteria. *In:* J. M. Ashworth and J. E. Smith (Eds.) Microbial Differentiation. Cambridge University Press. London, 125–142.

Edwards, K. J., P. L. Bond, T. M. Gihring, and J. F. Banfield. 2000. An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. Science 287:1796–1799.

Eichler, B., and N. Pfennig. 1986. Characterization of a new platelet-forming purple sulfur bacterium, Amoebobacter pedioformis sp. nov. Arch. Microbiol. 146:295–300.

Emerson, D., and C. Moyer. 1997. Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. Appl. Environ. Microbiol. 63:4784–4792.

Fosnaugh, K., and E. P. Greenberg. 1988. Motility and chemotaxis of Spirochaeta aurantia: Computer-assisted motion analysis. J. Bacteriol. 170:1768–1774.

Fossing, H., V. A. Gallardo, B. B. Jorgensen, M. Huttel, L. P. Nielsen, H. Schulz, D. E. Canfield, S. Forster, R. N. Glud, J. K. Gundersen, J. Kuver, N. B. Ramsing, A. Teske, B. Thamdrup, and O. Ulloa. 1995. Concentration and transport of nitrate by the mat-forming sulphur bacterium Thioploca. Nature (London) 374:713–715.

Foster, J. W. 1964. Speculative discourse on where and how microbiological science as such may advance with application. *In:* M. P. Starr (Ed.) Global Impacts of Applied Microbiology. John Wiley. New York, NY. 61–73.

Fujita, Y., and C. E. Bauer. 2000. Reconstitution of light-independent protochlorophyllide reductase from purified bchl and BchN-BchB subunits: In vitro confirmation of nitrogenase-like features of a bacteriochlorophyll biosynthesis enzyme. J. Biol. Chem. 275:23583–23588.

Garcia-Horsman, J. A., B. Barquera, J. Rumbley, J. Ma, and R. B. Gennis. 1994. The superfamily of heme-copper respiratory oxidases. J. Bacteriol. 176:5587–5600.

Garrity, G. M., and J. G. Holt. 2001. A road map to the manual. *In:* G. A. Garrity, D. R. Boone, and R. W. Castenholz (ed.), Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:119–166.

Giovannoni, S. J., E. Schabatach, and R. Castenholz. 1987. Isosphaera pallida, gen. and comb. nov., a gliding, budding eubacterium from hot springs. Arch. Microbiol. 147:276–284.

Gottschalk, G., J. R. Andreeson, and H. Hippe. 1981. The genus Clostridium (non-medical aspects). *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel, eds. The Prokaryotes. Springer, Berlin, 1176–1803.

Gottschalk, G. 1986. Bacterial Metabolism, Springer-Verlag. New York, NY. 2nd ed.:1–380.

Gould, S. J. 1996. The power of the modal bacter, or why the tail can't wag the dog. Full House. Three Rivers Press. New York, NY. 167–216.

Gray, M. W., G. Burger, and B. F. Lang. 1999. Mitochondrial evolution. Science 283:1476–1481.

Grimm, A. C., and C. S. Harwood. 1997. Chemotaxis of Pseudomonas spp. to the polyaromatic hydrocarbon naphthalene. Appl. Environ. Microbiol. 63:4111–4115.

Gupta, R. S. 1998. Protein phylogenies and signature sequences: A reappraisal of evolutionary relationships among archaebacteria, eubacteria, and eukaryotes. Microbiol. Molec. Biol. Rev. 62:1435–1491.

Haugland, R. A., D. J. Schlemm, R. P. Lyons, P. R. Sferra, and A. M. Chakrabarty. 1990. Degradation of the chloriated phenoxyacetate herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5, thrichlorophenoxyacetic acid in pure and mixed bacterial cultures. Appl. Environ. Microbiol. 56:1357–1362.

Henrichsen, J. 1983. Twitching motility. Ann. Rev. Microbiol. 37:81–93.

Hoeniger, J. F. M. 1965. Development of flagella by Proteus mirabilis. J. Gen. Microbiol. 40:29–42.

Holliger, C., G. Wohlfarth, and G. Diekert. 1998. Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Rev. 22:383–398.

Holt, S. C., and E. Canale-Parola. 1967. Fine structure of Sarcina maxima and Sarcina ventriculi. J. Bacteriol. 93:399–410.

Huebner, A., C. E. Danganan, L. Xun, and A. M. Chakrabarty. 1998. Genes for 2,4,5-trichlorophenoxyacetic acid metabolism in Burkholderia cepacia AC1100: Characterization of the tftC and tftD genes and locations of the tft operons on multiple replicons. Appl. Environ. Microbiol. 64:2086–2093.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180:4765–4774.

Ingraham, J. L., O. Maaløe, and F. C. Neidhardt. 1983. Growth of the Bacterial Cell. Sinauer Associates, Inc. Sunderland, MA.

Jarrell, K. F., D. P. Bayley, and A. S. Kostyukova. 1996. The archaeal flagellum: A unique motility structure. J. Bacteriol. 178:5057–5064.

Johnson, C. H., and S. S. Golden. 1999. Circadian programs in cyanobacteria: Adaptiveness and mechanism. Ann. Rev. Microbiol. 53:389–409.

Judson, H. F. 1979. Afterward: Always the same impasse. The Eighth Day of Creation. Simon and Schuster. New York, NY. 605–616.

Kandler, O., and N. Weiss. 1986. Regular, non-sporing Gram-positive rods. *In:* P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 2:1208–1260.

Kandler, O. 1994. Cell wall biochemistry and Three-Domain concept of life. Syst. Appl. Microbiol. 16:501–509.

Katz, J., and D. Weihs. 1979. Large amplitude on steady motion of a flexible slender propulsor. J. Fluid Mech. 90:713–723.

Keller, K. H., M. Grady, and M. Dworkin. 1983. Surface tension gradients: Feasible model for gliding motility of Myxococcus xanthus. J. Bacteriol. 155:1358–1366.

Kengen, S. W. M., A. J. M. Stams, and W. M. de Vos. 1996. Sugar metabolism in hyperthermophiles. FEMS Microbiol. Rev. 18:119–137.

Kennedy, C., and A. Toukdarian. 1987. Genetics of azotobacters: Applications to nitrogen fixation and related aspects of metabolism. Ann. Rev. Microbiol. 41:227–258.

Kreig, N. 1984. Aerobic, microaerophilic, motile, helical/vibroid Gram-negative bacteria. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Determinative Bacteriology. Williams and Wilkins. Baltimore, MD. 1:71–124.

Kutzner, H. J. 1981. The family Streptomycetaceae. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes. Springer-Verlag. Berlin, 2028–2090.

Lacy, J. 1971. Thermoactinomyces sacchari sp. nov., a thermophilic actinomycete causing bagassosis. J. Gen. Microbiol. 66:327–338.

Lapidus, I. R., and H. C. Berg. 1982. Gliding motility of Cytophaga strain U67. J. Bacteriol. 151:384–398.

Lechevalier, H., and P. E. Holbert. 1965. Electron microscopic observations of the sporangial structure of a strain of Actinoplanes. J. Bacteriol 89:217–222.

Lobo, A. L., and S. H. Zinder. 1992. Nitrogen fixation by methanogenic bacteria. *In:* H. Evans, Stacey, G. and R. Burris (Eds.) Biological Nitrogen Fixation Routledge, Chapman and Hall. New York, NY. 191–211.

Lovley, D. R., and E. J. Phillips. 1992. Reduction of uranium by Desulfovibrio desulfuricans. Appl. Environ. Microbiol. 58:850–856.

Lovley, D. R., and J. D. Coates. 2000. Novel forms of anaerobic respiration of environmental relevance. Curr. Opin. Microbiol. 3:252–256.

Ludden, P. W., and E. A. Roberts. 1988. Regulation of nitrogenase activity by reversible ADP-ribosylation of dinitrogenase reductase. *In:* H. Boethe, F. J. deBruijn, and W. E. Newton (Eds.) Nitrogen fixation: Hundred Years After. Gustav Fischer Verlag. Stuttgart, 157–162.

Lünsdorf, H., and H. Reichenbach. 1989. Ultrastructural details of the apparatus of gliding motility of Myxococcus fulvus (Myxobacterales). J. Gen. Microbiol 135:1633–1641.

Macnab, R. M. 1999. The bacterial flagellum: reversible rotary propellor and type III export apparatus. J. Bacteriol. 181:7149–7153.

Maeda, N., K. Kitano, T. Fukui, S. Ezaki, H. Atomi, K. Miki, and T. Imanaka. 1999. Ribulose bisphosphate carboxylase/oxygenase from the hyperthermophilic archaeon Pyrococcus kodakaraensis KOD1 is composed solely of large subunits and forms a pentagonal structure. J. Molec. Biol. 293:57–66.

Mägli, A., M. Wendt, and T. Leisinger. 1996. Isolation and characterization of Dehlobacterium formicoaceticum gen. nov. sp. nov., a strictly anaearobic bacterium utilizing dichloromehtane as a source of carbon and energy. Arch. Microbiol. 166:101–108.

Malmquist, A., T. Welander, E. Moore, A. Ternstrom, G. Molin, and I. M. Stentrom. 1994. Edeonella dechloratans, gen. nov., sp. nov., a new bacterium capable of growing anaerobically with chlorate as an electron acceptor. Syst. Appl. Microbiol. 17:58–64.

Matsuyama, T., Y. Takagi, Y. Nakagawa, H. Itoh, J. Wakita, and M. Matsushita. 2000. Dynamic aspects of the structured cell population in a swarming colony of Proteus mirabilis. J. Bacteriol. 182:385–393.

Maymó-Gatell, X., Y. T. Chien, J. M. Gossett, and S. H. Zinder. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276:1568–1571.

Mayr, E. 1998. Two empires or three? Proc. Natl. Acad. Sci. USA 95:9720–9723.

Menendez, C., Z. Bauer, H. Huber, N. Gad'on, K. O. Stetter, and G. Fuchs. 1999. Presence of acetyl coenzyme A (CoA) carboxylase and propionyl-CoA carboxylase in autotrophic Crenarchaeota and indication for operation of a 3-hydroxypropionate cycle in autotrophic carbon fixation. J. Bacteriol. 181:1088–1098.

Merz, A. J., M. So, and M. P. Sheetz. 2000. Pilus retraction powers bacterial twitching motility. Nature 407:98–102.

Messmer, M., G. Wohlfarth, and G. Diekert. 1993. Methyl chloride metabolism of the strictly anaerobic, methyl chloride-utilizing homoacetogen strain MC. Arch. Microbiol. 160:383–387.

Mohn, W. W., and J. M. Tiedje. 1992. Microbial reductive dehalogenation. Microbiol. Rev. 56:482–507.

Moran, N. A., and P. Baumann. 2000. Bacterial endosymbionts in animals. Curr. Opin. Microbiol. 3:270–275.

Moreira, D., H. Le Guyader, and H. Phillippe. 2000. The origin of red algae and the evolution of chloroplasts. Nature 405:69–72.

Morita, R. Y. 1975. Psychrophilic bacteria. Bacteriol. Rev. 39:144–167.

Moshiri, F., J. W. Kim, C. Fu, and R. J. Maier. 1994. The FeSII protein of Azotobacter vinelandii is not essential for aerobic nitrogen fixation, but confers significant protection to oxygen-mediated inactivation of nitrogenase in vitro and in vivo. Molec. Microbiol. 14:101–114.

Nichols, J. M., and D. G. Adams. 1982. Akinetes. *In:* N. G. Carr and B. A. Whitton (Eds.) The Biology of Cyanobacteria. University of California Press. Berkeley, CA. 387–412.

Palleroni, N. J. 1984. Gram-Negative aerobic rods and cocci. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Determinative Bacteriology. Williams and Wilkins. Baltimore, MD. 1:140–406.

Pangborn, J., Kuhn, D. A., and J. R. Woods. 1977. Dorsal-ventral differentiation in Simonsiella and other aspects of its morphology and ultrastructure. Arch. Microbiol. 113:197–204.

Pate, J. L., and E. J. Ordal. 1965. The fine structure of two unusual stalked bacteria. J. Cell Biol. 27:133–150.

Pate, J. L., and L. Y. E. Chang. 1979. Evidence that gliding motility in prokaryotic cells is driven by rotary assemblies in the cell envelope. Curr. Microbiol. 2:257–262.

Peekhaus, N., and T. Conway. 1998. What's for dinner?: Entner-Doudoroff metabolism in Escherichia coli. J. Bacteriol. 180:3495–3502.

Perazzona, B., and J. L. Spudich. 1999. Identification of methylation sites and effects of phototaxis stimuli on transducer methylation in Halobacterium salinarum. J. Bacteriol. 181:5676–5683.

Poindexter, J. S. 1964. Biological properties and classification of the Caulobacter group. Bacteriol. Rev. 28:231–295.

Ragsdale, S. W. 1997. The eastern and western branches of the Wood/Ljungdahl pathway: How the east and west were won. Biofactors 6:3–11.

Reeve, J. N., K. Sandman, and C. J. Daniels. 1997. Archaeal histones, nucleosomes, and transcription initiation. Cell 89:999–1002.

Reichenbach, H., and M. Dworkin. 1981a. Introduction to the gliding bacteria. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes. Springer-Verlag. Berlin, 316–327.

Reichenbach, H., and M. Dworkin. 1981b. The order myxobacterales. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes. Springer-Verlag. Berlin, 328–355.

Reichenbach, H. 1984. Myxobacteria: A most peculiar group of social prokaryotes. *In:* E. Rosenberg (Ed.) Myxobacteria: Development and Cell Interactions. Springer-Verlag. Berlin, 1–50.

Ribbe, M., D. Gadkari, and O. Meyer. 1997. N_2 fixation by Streptomyces thermoautotrophicus involves a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase that couple N_2 reduction to the oxidation of superoxide produced from O_2 by a molybdenum-CO dehydrogenase. J. Biol. Chem. 272: 26627–26633.

Robinson, J. J., J. L. Stein, and C. M. Cavanaugh. 1998. Cloning and sequencing of a form II ribulose-1,5-biphosphate carboxylase/oxygenase from the bacterial symbiont of the hydrothermal vent tubeworm Riftia pachyptila. J. Bacteriol. 180:1596–1599.

Rosenberg, E., K. H. Keller, and M. Dworkin. 1977. Cell density-dependent growth of Myxococcus xanthus on casein. J. Bacteriol. 129:770–779.

Rudolph, J., and D. Oesterhelt. 1996. Deletion analysis of the che operon in the archaeon Halobacterium salinarium. J. Molec. Biol. 258:548–554.

Sadoff, H. L. 1976. Encystment and germination in Azotobacter vinelandii. Bacteriol. Rev. 39:516–539.

Saraste, M. 1999. Oxidative phosphorylation at the fin de siecle. Science 283:1488–1493.

Schäfer, G., W. Purschke, and C. L. Schmidt. 1996. On the origin of respiration: Electron transport proteins from archaea to man. FEMS Microbiol. Rev. 18:173–188.

Schink, B. 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Rev. 61:262–280.

Schleifer, K. H. 1986. Gram-positive cocci. *In:* P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 2:999–1002.

Schulz, H. N., T. Brinkhoff, T. G. Ferdelman, M. H. Marine, A. Teske, and B. B. Jorgensen. 1999. Dense populations of a giant sulfur bacterium in Namibian shelf sediments. Science 284:493–495.

Selig, M., K. B. Xavier, H. Santos, and P. Schonheit. 1997. Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium Thermotoga. Arch. Microbiol. 167:217–232.

Singleton, R. 2000. Historical roots. ASM News 66:510–511.

Sleytr, U. B., P. Messner, D. Pum, and M. Sara. 1993. Crystalline bacterial cell surface layers. Molec. Microbiol. 10:911–916.

Sneath, P. H. A. 1986. Endospore forming Gram-positive rods and cocci. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Determinative Bacteriology. Williams and Wilkins. Baltimore, MD. 2:1104–1207.

Sogin, M. L., and J. D. Silberman. 1998. Evolution of the protists and protistan parasites from the perspective of molecular systematics. Int. J. Parasitol. 28:11–20.

Sowers, K. R., J. E. Boone, and R. P. Gunsalus. 1993. Disaggregation of Methanosarcina spp and growth as single cells at elevated osmolarity. Appl. Environ. Microbiol. 59:3832–3839.

Spaink, H. P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. Ann. Rev. Microbiol. 54:257–288.

Spormann, A. M. 1999. Gliding motility in bacteria: insights from studies of Myxococcus xanthus. Microbiol. Molec. Biol. Rev. 63:621–641.

Spudich, J. L. 1993. Color sensing in the archaea—a eukaryotic-like receptor coupled to a prokaryotic transducer. J. Bacteriol. 175:7755–7761.

Stackebrandt, E., W. Ludwig, M. Weizenegger, S. Dorn, T. J. McGill, G. E. Fox, C. W. Woese, W. Schubert, and K. H. Schleifer. 1987. Comparative 16S rRNA oligonucleotide analyses and murein types of round-spore-forming bacilli and nonsporeforming relatives. J. Gen.Microbiol. 133:2523–2529.

Stackebrandt, E., F. A. Rainey, and N. Ward-Rainey. 1996. Anoxygenic phototrophy across the phylogenetic spectrum: Current understanding and future perspectives. Arch. Microbiol. 166:211–223.

Stal, L. J., and W. E. Krumbein. 1985. Nitrogenase activity in the non-heterocystous cyanobacterium Oscillatoria sp. grown under alternatinglight-dark cycles. Arch. Microbiol. 143:67–71.

Staley, J. T. 1968. Prosthecomicrobium and Ancalomicrobium: new prosthecate freshwater bacteria. J. Bacteriol. 95:1921–1942.

Stanier, R. Y., and C. B. van Niel. 1962. The concept of a bacterium. Arch. Mikrobiol. 42:17–35.

Stanier, R. Y., E. A. Adelberg, J. L. Ingraham, and M. L. Wheelis. 1979. Microbial metabolism: the generation of ATP. Introduction to the Microbial World. Prentice-Hall. New York, NY. 154–186.

Stanier, R. Y., N. Pfennig, and H. G. Trüper. 1981. Introduction to the phototrophic prokaryotes. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes. Springer-Verlag. Berlin, 197–211.

Stephenson, M. 1949. Bacterial Metabolism, 3rd. ed. Longmans, Green. London, v.

Stewart, V. 1994. Regulation of nitrate and nitrite reductase synthesis in enterobacteria. Ant. v. Leeuwenhoek 66:37–45.

Stokes, J. 1954. Studies on the filamentous sheathed iron bacterium Sphaerotilus natans. J. Bacteriol. 67:278–291.

Strauss, G., and G. Fuchs. 1993. Enzymes of a novel autotrophic CO_2 fixation pathway in the phototrophic bacterium Chloroflexus aurantiacus, the 3-hydroxypropionate cycle. Eur. J. Biochem. 215:633–643.

Strous, M., J. A. Fuerst, E. H. Kramer, S. Logemann, G. Muyzer, K. T. van de Pas-Schoonen, R. Webb, J. G. Kuenen, and M. S. Jetten. 1999. Missing lithotroph identified as new planctomycete. Nature 400:446–449.

Sudo, S. Z., and M. Dworkin. 1973. Comparative biology of prokaryotic resting cells. Adv. Microbiol. Physiol. 9:153–224.

Suflita, J. M., A. Horowitz, D. R. Shelton, and J. M. Tiedje. 1982. Dehalogenation: A novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science 218:1115–1117.

Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100–180.

Tomitani, A., K. Okada, H. Miyashita, H. C. Matthijs, T. Ohno, and A. Tanaka. 1999. Chlorophyll b and phycobilins in the common ancestor of cyanobacteria and chloroplasts. Nature 400:159–162.

van Niel, C. B. 1949. The comparative biochemistry of photosynthesis. In: J. Franck and W. E. Loomis (ed.). Photosynthesis in plants. Iowa State College Press. Ames, IA. 400:437–495.

Vargas, M., K. Kashefi, E. L. Blunt-Harris, D. R. Lovley. 1998. Microbiological evidence for Fe(III) reduction on early Earth. Nature (London) 395:65–67.

Walsby, A. E. 1980. A square bacterium. Nature 283:69–71.

Watson, G. M., J. P. Yu, and F. R. Tabita. 1999. Unusual ribulose 1,5-bisphosphate carboxylase/oxygenase of anoxic Archaea. J. Bacteriol. 181:1569–1575.

Weiner, J. H., D. P. MacIsaac, R. E. Bishop, and P. T. Bilous. 1988. Purification and properties of Escherichia coli dimethyl sulfoxide reductase, an iron-sulfur molybdoenzyme with broad substrate specificity. J. Bacteriol. 170:1505–1510.

Weisburg, W. G., T. P. Hatch, and C. R. Woese. 1986. Eubacterial origin of chlamydiae. J. Bacteriol. 167:570–574.

White, D. 1984. Structure and function of myxobacteria cells and fruiting bodies. *In:* E. Rosenberg (Ed.) Myxobacteria: Development and Cell Interactions. Springer-Verlag. Berlin, 51–67.

Whittenbury, R., S. L. Davies, and J. F. Davy. 1970. Exospores and cysts formed by methane-utilizing bacteria. J. Gen. Microbiol. 61:219–226.

Whittenbury, R., and C. S. Dow. 1977. Morphogenesis and differentiation in Rhodomicrobium vannielii and other budding and prosthecatebacteria. Bacteriol. Rev. 41: 754–808.

Widdel, F., S. Schnell, S. Heising, A. Ehrenreich, B. Assmus, and B. Schink. 1993. Ferrous iron oxidation by anoxygenic phtotrophic bacteria. Nature (London) 362:834–836.

Williams, F. D. 1978. Nature of the swarming phenomenon in Proteus. Ann. Rev. Microbiol. 32:101–122.

Wilson, E. O. 1995. Naturalist. Warner Books. New York, NY. 101–122.

Woese, C. R., O. Kandler, and M. L. Wheelis. 1990. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA 87:4576–4579.

Woese, C. R. 1998. Default taxonomy: Ernst Mayr's view of the microbial world. Proc. Natl. Acad. Sci. USA 95:11043–11046.

Wolin, M. J., and T. L. Miller. 1982. Interspecies hydrogen transfer: 15 years later. ASM News 48:561–565.

Xiong, J., W. M. Fischer, K. Inoue, M. Nakahara, and C. E. Bauer. 2000. Molecular evidence for the early evolution of photosynthesis. Science 289:1724–1730.

Yoon, H. S., and J. W. Golden. 1998. Heterocyst pattern formation controlled by a diffusible peptide. Science 282:935–938.

Zengler, K., H. H. Richnow, R. Rossello-Mora, W. Michaelis, and F. Widdel. 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401:266–269.

Zumft, W. G. 1997. Cell biology and molecular basis of denitrification. Microbiol. Molec. Biol. Rev. 61:533–616.

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CHAPTER 1.8

Cell-Cell Interactions

DALE KAISER

Discovery and Distribution

Roland Thaxter published a time bomb in December 1892. He reported that *Chondromyces crocatus*, before then considered an imperfect fungus because of the morphological complexity of its fruiting body, was actually a bacterium. Thaxter had discovered the unicellular vegetative stage of *C. crocatus*; the cells he found were relatively short and they divided by binary fission, unlike the mycelium of a fungus. *C. crocatus* was, he concluded, a "communal bacterium." Thaxter described the locomotion, swarming, aggregation and process of fruiting body formation of *C. crocatus* and its relatives, which are collectively called myxobacteria, with an accuracy that has survived 100 years of scrutiny. He described the behavior of myxobacteria in fructification in terms of a "course of development" because it was "a definitely recurring aggregation of individuals capable of concerted action toward a definite end" (Thaxter, 1892). These qualities of an integrated multicellular unit clearly implied positive interaction between the cells. Existence of favorable cell-cell interactions is one hallmark of multicellular life and the theme of this essay.

Thaxter's discovery called attention to the transition from single, apparently independent cells to an integrated multicellular unit. There is general agreement that this step has been taken many times in the course of the organic evolution of plants and animals. For example, the sponges are thought to have arisen from solitary cells separately from all other animals; moreover the seed plants, the fungi, and the algae are all believed to have gained their multicellular condition independently (Whittaker, 1969). Given the apparently strong natural selection for multicellular life, it would be surprising if prokaryotes also had not adopted the multicellular condition many times, and perhaps even earlier in time.

One current estimate suggests that more than 99% of the bacteria on earth live as cell masses (Costerton et al., 1995), a condition conducive to cell interactions. As the broader significance of multicellular microbial life has been recognized, the cell interactions that facilitate multicellularity have been revealed.

Evolution of Cell Interactions

To imagine how two partners to a cell interaction could coordinately evolve high, yet complementary, specificity seems difficult. To start with a weak and poorly specific signal would seem to have little, if any, selective advantage. But what other way is there to start? The relative structural simplicity of bacteria belies their metabolic versatility and their sophistication in responding to the environment by their sensory systems. Given current knowledge of bacteria and given some advantage for an interaction, at least two evolutionary scenarios are plausible. 1) The progressive evolution of a cell-cell interaction between dissimilar cells could start with cells of one type feeding on the metabolic products of cells of another type. Mutation and selection on both partners would then optimize syntrophic growth. While this cooperative growth was taking place, the release by one cell type of a metabolic regulator for the other type would constitute a primitive signal, subject to improvement by selection. 2) Instead of metabolic cross-feeding, a cell-cell signaling interaction might evolve from an existing sensory system. The wide variety of two-component sensory, regulatory systems would be ground for such evolution (Hoch and Silhavy, 1995). Bacterial sensory systems are robust, having sensitivity and reliability required for an effective interaction (Barkai and Leibler, 1997). Release or display of a signaling factor by cells could trigger one of the sensory systems. Immediately after this event, selection would need to act only on signal production. Then the specificity of matching between trigger and sensor could be improved, or a higher level of sensitivity could evolve. 3) Bacteria have stimulons. If the output of a sensory system as in 2) or a metabolic regulator as in 1) becomes the input to a stimulon, responses would govern many genes. From cell-cell interactions, the evo-

lutionary path to timed gene expression and a circuit that "develops" in Thaxter's terms is simple. Starting with signal 1 and its response, including expression of a set of genes, let one of those genes encode production of signal 2. Iteration of that process would give something like the signaling pattern of fruiting body development in Myxobacteria Fruiting Body Development signaling. 4) Because haploid bacteria are less insulated from their environment than diploid organisms, natural selection can constantly play a role in shaping and improving the interactions between cells, speeding such evolution. Loss of cell-cell interaction in less than one thousand generations after relaxing social selection indicates experimentally the force of social selection (Velicer et al., 1998).

Types of Cell Interactions

Four kinds of cell interactions can be distinguished: 1) Transfer of a chemical signal from one cell to another. The variety of such transfers is presented in several examples below. 2) Signaling by direct physical contact between two cell bodies, which may involve their surfaces or cell appendages, such as fibrils, pili, or flagella. Direct physical contact is often involved in cell swarming. 3) Syntrophic metabolism. Because syntrophic interactions are the subject of another chapter by Schink (Syntrophism Among Prokaryotes in Volume 2), this essay will focus on the other three types. 4) Gene transfer from one cell to another. Eubacterial gene transfer interactions are widespread. Transfer within the Archaea has recently been observed, and their genetics is being developed (Stedman et al., 1999; Whitman et al., 1999). Prokaryotes have three mechanisms for unidirectional gene transfer from a donor to a recipient. These mechanisms are *transformation* in which naked DNA from the donor is taken up by the recipient, generalized *transduction* in which a phage has packaged a head-full of donor DNA and injects that DNA into the recipient, and *conjugation* in which a specialized apparatus in the donor transfers a long DNA segment directly into a conjugating recipient. The three mechanisms have different qualities: transforming DNA fragments may be 10 to 50 kb in length and are vulnerable to extracellular nucleases. Competence of the recipient to accept DNA may be restricted to certain nutritional states: *Bacillus subtilis* growing in minimal salts with glucose and amino acids develops competence postexponentially (Dubnau, 1991), but growing without amino acids, exponential cells are competent when two peptide pheromones are added (Lazazzera et al., 1999). In *Haemophilus influenzae*, competence is restricted to exponential cells after nutritional downshift in a medium that supports protein synthesis but not cell division (Herriott et al., 1970; Kahn et al., 1983), and *Streptococcus* cells are competent in the presence of a secreted competence peptide (Havarstein and Morrison, 1999). The *Bacillus* and *Streptococcus* peptides may be quorum sensors.

Transduced DNA fragments may range up to 100 kb, a limit imposed by the capsid volume of the transducing phage. Although the DNA is shielded from nuclease attack by the capsid proteins, the host range of the phage limits transduction. That typically narrow range is set by the distribution of the adsorption receptor for the phage among bacteria. Phage P1 is an exception because its receptor is the common lipopolysaccharide (LPS) core lipid (Lindberg, 1973), which suggests that practically all Gram-negative bacteria could be P1 recipients (Murooka and Harada, 1979). Transfer is by syringe-like injection that is triggered by phage adsorption (of P22 or P1, for example), or the virus may enter the cell intact and release its DNA once inside (M13 is an example).

In conjugation, most or all of the donor chromosome may be transferred. Conjugal DNA transfer is mediated by physical contact and a multi-molecular machine, consisting of many proteins. Conjugation mediated by F-plasmids begins when the F-pilus binds to the ompA protein on the surface of an *Escherichia coli* recipient (Manoil and Rosenbusch, 1982). A mating signal is sent back to the donor cell (Kingsman and Willetts, 1978), the donor and recipient are then drawn closely together, and an electron dense junction forms at the cell-cell interface (Durrenberger et al., 1991). DNA is then transferred with synthesis in both cells. After transfer and chromosome segregation, the cells actively separate (Achtman et al., 1978; Durrenberger et al., 1991). More than 15 proteins may be in a conjugation machine, including the pilus proteins. This degree of structural complexity suggests a strong selective advantage for conjugation.

Gene transfer by any of these mechanisms may be lateral (in which transfer is from one cell species to another) as well as vertical (in which the donor and recipient belong to the same species). Whereas transduction is restricted to certain members of particular species, transformation and conjugation may have very wide ranges that facilitate lateral gene transfer. Broad host-range plasmids such as RSF 1010 (Frey and Bagdasarian, 1989) can transfer between a wide variety of bacterial species, and RP4 can even transfer to *Saccharomyces cerevisiae* (Heineman and Sprague, 1989). The transkingdom transfer by RP4 requires its five *tra* genes (*tra FGIJK*)

and ten *trb* genes (*trb BCDEFGHIJ* and *L*; Bates et al., 1998), which include the pilin gene, *trb C*. Moreover, in nature, a sector of the pTi plasmid laterally transfers from *Agrobacterium* to a variety of plant cell species, thereby producing crown gall tumors.

Widespread infectious transfer of drug resistance between bacterial species is circumstantial evidence for lateral transfer (Mazodier and Davies, 1991). But recently, as a consequence of whole genome studies and of the construction of phylogenetic trees based on particular proteins, discrepancies between trees have suggested that lateral transfer may be more general. A strong case for the transfer of the gene for glucose-phosphate isomerase from a plant to *E. coli* has been made (Smith et al., 1992). Pathogenicity islands in, for example, *Salmonella*, are large clusters of virulence genes not present in related but benign organisms, and they appear to have been transferred from another organism(s) (Baumler, 1997; Groisman and Ochman, 1997). Ongoing lateral transfer of the retrons that encode production of multicopy single-stranded DNA is evident in *E. coli* and other enteric bacteria. Ten percent of *E. coli* clinical isolates are found to have them, and most retron isolates in these strains are different (Herzer et al., 1990). The retron Ec107 may be spreading from one strain of *E. coli* to others (Lampson, 1993). By contrast, retrons are ubiquitous in the Myxobacteria, and all members of the myxococcus subgroup have a version of Mx162, while members of the other subgroup lack it (Rice and Lampson, 1995). The retron element Mx162 apparently transferred laterally into the common myxococcus ancestor about 100 million years ago and has subsequently been inherited vertically (Rice and Lampson, 1995).

Lateral transfer requires a mechanism for DNA integration that does not depend on perfectly homologous recombination. Transposons such as Mu, Tn*5*, or Mariner insert within short target DNA sequences, and their transposases are thus present in bacteria. The Mariner target, for example, may simply be the AT dinucleotide (Hartl et al., 1997). Site-specific and other nonhomologous recombination mechanisms are also common in bacteria where they are associated with repair of damage to DNA and help preserve genome integrity.

Chemical Signals and Direct Physical Contact

Some themes recur frequently. Certain small molecules like amino acids and their derivatives are frequently used as signal molecules. Examples are homoserine lactones and peptides shorter than ten amino acids. Multicellular sensing is more accurate than unicellular and may be able to compensate for cell-to-cell variations in metabolism, transcription and translation. One example is the A-factor of *M. xanthus*, which is a mixture of 6 amino acids and is a cell-density signal (Kuspa et al., 1992a). The amount of A-factor released is proportional to the number of cells per unit volume, and a certain minimum quantity of A-factor is required to continue development. Thus the A-signal ensures a cell density sufficient to complete a proper fruiting body (Kuspa et al., 1992b). A-factor, which is released about 2 hours after the beginning of starvation, is also a way for cells to vote their individual assessment of nutritional conditions. Since new proteins must be made during aggregation and sporulation, some protein synthetic capacity must be retained, and the cells must begin to aggregate before they have exhausted all their sources of amino acids and energy. To initiate development or to grow slowly is an important choice on which long-term survival depends. An optimal choice is one that anticipates the future. A decision jointly made by a population of cells rather than by one cell is likely to be more reliable. Multicellular, rather than unicellular, feeding on polymeric substrates is more efficient when extracellular hydrolases are employed for digestion.

One example is the social feeding in myxobacteria. The selective advantage for the evolution of multicellularity in myxobacteria is likely to have been cooperative feeding. Myxobacteria feed on particulate organic matter in the soil by means of extracellular bacteriolytic, proteolytic, cellulolytic, and other digestive enzymes (Reichenbach, 1984). Based on their secretion of lytic enzymes, Dworkin (1973) likened myxobacteria to "packs of microbial wolves." Rosenberg et al. (1977) measured the growth rate when the only source of carbon and nitrogen for *M. xanthus* cells in liquid culture was the polymeric substrate casein and found that proteolysis was required for growth. A two-fold increase in growth rate was observed as the cell density was raised above 104 cells/ml. When intact casein was replaced with hydrolyzed casein, the cells grew independent of cell density at the more rapid rate. Evidently extracellular digestion of protein is enhanced by cooperation between cells, akin to syntrophism.

A swarm may be the unit of efficient cooperative feeding. Reichenbach has shown that a single germinating sporangiole of a *C. apiculatus* fruiting body forms an active swarm that behaves much like a swarm of bees (Bonner, 1952; Kuhlwein and Reichenbach, 1968; Quinlan and Raper, 1965). Forming a multicellular fruiting

body ensures that, when conditions favorable for growth are restored, the myxospores can germinate, and the new phase of growth can start as a pre-formed community of efficiently feeding cells. The success of the myxobacterial design is evident in their distribution; they are common inhabitants of soils drawn from all over the world regardless of climate (Reichenbach, 1984).

Multicellularity and cell density effects are prominent in starvation or nutrition-limiting states. Starvation requires management of scarce resources, and for that reason, requires sharper, more accurate perceptions. Examples include sporulation, biofilms, and fruiting bodies.

Possible Strategies for Detecting Signals

Common themes suggest the following possible strategies for detecting signals: 1) Look for cell-density effects (Lazazzera et al., 1999; Rosenberg et al., 1977). Test the response of low-density cells to high-density conditioned medium. 2) Look for signal-production defective mutants that can be rescued by co-cultivation with wild type cells. Such mutants can be divided into classes by mixing mutants with each other, possibly identifying different signal specificities (Hagen et al., 1978). 3) Isolate signal-response-defective mutants (Gorski and Kaiser, 1998). For this purpose, reporter gene constructs are likely to be useful. 4) Signal molecule bioassays can be built upon the provision of signal molecules in crude form (Hastings and Greenberg, 1999). 5) Starvation survival strategies often involve cell interactions. 6) Interactions are common among cells in biofilm communities (Davies et al., 1998). 7) Two-component sensory systems may perceive intercellular signals.

Examples of Interactions

These include: 1) Autochemotaxis; 2) Vibrio, Squid, Lux, autoinducer; 3) Microbial Biofilms in this Volume; 4) Agrobacterium and crown gall; 5) Rhizobium and nodules; 6) Myxobacteria and fruiting bodies: A-signaling and C-signaling; 7) Pheromone-inducible plasmid transfer in *E. faecalis*; 8) Swarming, type IV pili, twitching and gliding; 9) Heterocysts in filamentous cyanobacteria (Anabaena); 10) Sporulation in *B. subtilis*; 11) Syntrophism Among Prokaryotes in Volume 2.

Aggregation by Autochemotaxis

A striking illustration of the power of a single cell-cell interaction to produce complex cellular patterns is shown in Figure 1. This pattern of aggregates of *E. coli* cells developed in 3 days following a single inoculation made in the center of a plate containing succinate and a small amount of methylaspartate.

Fig. 1. Organized *E. coli* cells in a pattern generated by autochemotaxis. This pattern of cell aggregates developed in 3 days from a single inoculation made in the center of a plate. Color added for contrast. From Budrene and Berg, 1995.

When chemotactic *E. coli* cells are inoculated near the center of a Petri plate containing semi-solid agar, they swarm outward in concentric bands (Adler, 1966; Adler, 1969). The cells swim in semisolid (0.3%) agar almost as if it were water. Concentric bands form because of chemotactic responses to spatial gradients generated by transport and metabolism. As the cells take up metabolizable attractants from a complex medium, they grow, divide, and generate spatial gradients of attractants, which they subsequently chase. This leads them to swarm outward in concentric bands.

In medium rich in amino acids, a leading band of *E. coli* cells consumes most of the aspartate aerobically. The Tar methyl-accepting chemotaxis protein detects aspartate (Macnab, 1987), which binds to the extracellular domain. This binding then triggers the phosphorelay cascade of chemotaxis (che) proteins. The cascade signals the proteins at the base of the flagellum, which cause the cell either to continue swimming in the same direction or to tumble and swim in an arbitrary new direction. That the aspartate ring of a swarm depends on chemotaxis has been shown with a series of mutant strains made deficient in one of four chemotaxis receptors or in one of the cytoplasmic chemotaxis proteins (Wolfe and Berg, 1989). The spreading of chemotactic bands has been shown to require the entire regulatory network for chemotaxis, including desensitization and resensitization of the receptor by methylation and demethylation of its highly conserved carboxy tail located in the cytoplasm.

Formation of concentric bands and spots requires a particular set of metabolic conditions, unlikely to be found in nature, but instructive nonetheless. Growing in minimal medium on one of the more highly oxidized dicarboxylic acids of the tricarboxylic acid (TCA) cycle (e.g., 5 mM succinate), *E. coli* forms into a pattern of small, compact, focal aggregates, like those in the center of Fig. 1. If the concentration of succinate is doubled, the pattern becomes one of concentric rings. The pattern begins to form in the central part of the colony, then spreads sequentially outward.

The pattern depends on chemotaxis toward aspartate: the Tar receptor and all the downstream components in the chemotaxis phosphorelay are necessary (Wolfe and Berg, 1989). Ring formation can be suppressed by adding saturating amounts of chemicals that are sensed by the aspartate receptor (e.g., the nonmetabolizable aspartate analog, a-methyl-aspartate), implying in this case that a (self-generated) gradient of aspartate is involved. Formation of a focal pattern requires a high initial cell density in a ring; these cells then redistribute into foci (Budrene and Berg, 1995). Addition of succinate causes the cells to excrete aspartate. (A secretion of 4×10^{-17} moles of aspartate per cell has been measured.) Succinate would be oxidized by the TCA cycle to fumarate that in turn would be aminated to aspartate, some of which is released from the cell. A swarm ring forms when an aspartate gradient is established at the periphery of the spreading colony. The process of focal aggregate formation from a ring has been observed to continue in both directions around a ring until a complete set of aggregates has formed. Cells in the aggregates can be seen moving around, like a swarm of bees that are held together by their mutual attraction. While appearing to be colonies, they are swarms. By changing substrate concentrations and strains, a series of related patterns have been obtained. The formation of swarm rings and focal aggregates has been subjected to a thorough physical-mathematical analysis (Brenner et al., 1998). Thus, remarkable cellular patterns can be generated by chemotaxis to a single diffusible substance released by the cells themselves.

Autoinducer and Quorum Sensing

One of the earliest cell-cell interactions to be investigated is found in the symbiotic luminous vibrios. In nature they inhabit the light organs of certain marine bony (monocentrid) fishes and the bobtail squids (Nealson and Hastings, 1979). The bacterial light is crucial to the survival of these squid in their coastal water habitat. The squid's usual black ink trick is not very effective at night when the squid forages. Instead they emit light from their cultivated bacteria. This light helps camouflage the squid from their predators, which live below them on the floor of the relatively shallow coastal waters. This emitted light erases the shadow cast by the squid from the moonlight and starlight shining down on them from the night sky. A sophisticated "stealth" technology this, in which the squid by means of an iris and lens adjusts the intensity of light to match the light from above.

Luminous *Vibrio fischeri* release energy when they jointly oxidize $FMNH_2$ and tetradecanal with molecular O_2 (Meighen, 1994). The oxidation energy is emitted as fluorescent light, and luciferase catalyzes the oxidation coupled to light emission.

Light production by *Vibrio* is unusual in that the intensity of light produced per cell increases with cell density (Hastings and Greenberg, 1999). In dilute cultures, each cell is very dim. As their cell density is increased, however, the amount of light produced per cell rises as much as 100-fold. During this density-dependent induction, luciferase protein as well as the enzymes that synthesize the aldehyde substrate for luciferase increases. This augmentation is associated with release into the medium of a small molecule, β-ketocaproyl homoserine lactone, the *V. fischeri* autoinducer, or VAI (Eberhard et al., 1981).

An amide bond links homoserine lactone to an acyl chain, which arises from fatty acid biosynthesis.

Why should cells become bright when they have grown to high density and stay dim at low cell density? Light production requires a large amount of chemical energy to synthesize the fatty aldehyde and to keep FMN reduced. The idea is that the luminescent vibrios live two different lives: one when they are growing in the open sea, another when they are populating the light organ of an animal.

Since luminescence is energetically expensive, energy that could be used for growth in the free-living state would be squandered by emission of light. Life in the ocean is hard; carbon, fixed nitrogen, and energy sources are all difficult to find. Thus, the typical cell density of *V. fischeri* in seawater is low, perhaps 10 cells per ml.

A different economy prevails within the light organ of a squid or fish. There, *V. fischeri* reaches very high cell densities of 10^{10}–10^{11} cells per ml;

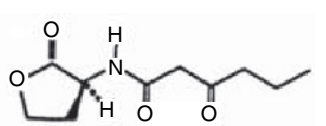

Fig. 2. The structure of *V. fischeri* autoinducer β-ketocaproyl homoserine lactone, or VAI. From Eberhard et al., 1981.

there it is luminescent. The animal pays the energetic cost by feeding its vibrios carbon and fixed nitrogen, allowing them to grow to very high density. The animal pays willingly because in return for food, *V. fischeri* makes the light which allows the animal to escape its predators.

Because of the low density of *V. fischeri* cells in sea water where they are nutrient limited, the extracellular autoinducer concentration never builds up to a significant level, and thus *V. fischeri* is not luminescent in its free-living state. Autoinduction may be viewed as an intercellular signaling system that allows *V. fischeri* to discriminate between a free living, low-density state and a host-associated, high-density state. Accordingly, the luminescence genes are activated only when a host is there to pay the high energy price of light production (Ruby and McFall-Ngai, 1992). The minimum number of bacteria necessary for high level luminescence in the light organ is thought of as a "quorum." This mechanism of regulatory discrimination between low density in the sea and high density in the light organ exemplifies the concept of quorum-sensing (Fuqua et al., 1994).

The autoinducer (VAI) and the regulatory response to its presence in the medium discriminate between the low- and high-density states. Two divergent lux operons, as shown here, have this purpose in *V. fischeri*.

The structural genes responsible for the synthesis and activity of luciferase (*luxA* and *B*), of tetradecanal (*luxC*, *D*, and *E*; Engebrecht and Silverman, 1986), and of the autoinducer synthase (*luxI*) make up one operon in *V. fischeri*. The substrates for autoinducer synthase are S-adenosylmethionine, which donates its methionyl portion, and the appropriate fatty acid conjugated to acyl carrier protein, which is an intermediate in fatty acid biosynthesis. The transcription unit is regulated by *luxR* (Figure 3), which encodes a DNA-binding protein that mediates the effect of autoinducer (Kaplan and Greenberg, 1987; Shadel et al., 1990). Gene *luxR* is divergently transcribed and separated by 155 bp from the *luxI* operon. Between these two transcription units lies a 20 bp inverted repeat, the "lux box," a sequence consensus element which has been found ahead of lux promoters in several organisms. The box is required for activation of both leftward and rightward transcripts and is thought to be the site of LuxR protein binding.

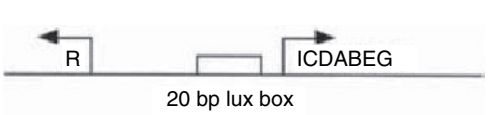

Fig. 3. Map of the two divergently transcribed lux operons. From Stevens and Greenberg, 1999.

The autoinducer is produced at a low constitutive rate during the early stages of *V. fischeri* growth. Once the cell density rises above a critical level, and the concentration of VAI in the medium rises to such a level that its intracellular concentration is high enough to bind LuxR, expression of the lux/CDABEG rises and there is luminescence. Because *luxI* is part of this transcription unit, a positive feedback loop is established which induces higher levels of VAI and locks in the luminescent state (Engebrecht et al., 1983).

Lux R protein is cytoplasmic and has no hydrophobic α-helical regions typical of membrane-spanning proteins. *V. fischeri* cells are freely permeable to acylated homoserine lactone, which is hydrophobic (Stevens et al., 1994). The acylated homoserine lactone (autoinducer) apparently enters and exits cells by passive diffusion through the membrane. Hydrophobic signal molecules have a double advantage: they require neither a specialized secretor nor a specialized transporter for uptake. Then why doesn't a cell signal itself with its own autoinducer? The cell does but the signal is too weak—the cell has no way to retain its "own" autoinducer. A hydrophobic signal molecule that is membrane diffusible in both the signal donor and the signal receiver is a starting point from which a plausible step-by-step Darwinian path could lead to highly specific signal molecules and to cognate and highly specific transmembrane receptors. In addition to the autoinducers of luminescent marine *V. fischeri* and *V. harveyi*, other acylhomoserine lactones are produced by a diverse and growing number (25 could be listed in 1997) of different terrestrial Gram-negative bacteria.

The LuxI homologs in all these organisms direct the synthesis of acylhomoserine lactones (HSL) with saturated or partly unsaturated acyl chains of 4 to 14 carbons, having substitutions at C-3 of either a hydroxyl group or a carbonyl group. Species specificity is provided by the acyl chain—its length, the oxidation state of C-3, and the degree of unsaturation of the hydrocarbon chain. All such acylated HSL compounds are expected to be membrane permeable, as has been demonstrated for β-ketocaproyl homoserine lactone (Kaplan and Greenberg, 1985). Various HSLs regulate diverse physiological processes including bioluminescence, swarming, antibiotic biosynthesis, plasmid conjugal transfer, and the production of exoenzyme virulence determinants in human, animal, and plant pathogens.

Biofilms

In natural aquatic environments, most bacteria are to be found within biofilms, which is the sub-

ject of the chapter by de Beer (Microbial Biofilms in this Volume). Biofilms are polysaccharide-enclosed bacterial masses adhering to a surface, the polysaccharide having been secreted by the bacteria themselves. As the bacteria grow and divide following their initial colonizing adhesion to a surface, the cells may cluster in pillar- and mushroom-like structures (Costerton et al., 1995). A three-dimensional structure, penetrated by anastomosing channels, is believed to provide a circulatory system that brings oxygen and other nutrients in while it flushes metabolic wastes out. Under favorable conditions, a biofilm can achieve a relatively high local density of cells, and the point here is that those cells can interact with each other. Cells within a biofilm are found in different regulatory states from their planktonic siblings, in part due to cell-cell interactions. Cells in the biofilm may be more antibiotic resistant and more exuberant in polysaccharide production and in enzymes for exopolysaccharide biosynthesis, such as alginate (Costerton et al., 1995).

Biofilms form on human epithelia bathed in nutrient-rich fluids. Many human bacterial infections occur in biofilms, such as common dental plaque leading to tooth decay and periodontal disease. *Pseudomonas aeruginosa* can form a biofilm on the skin of burn victims. Such a film tends to shield the bacteria from otherwise protective antibodies. At least one cell-to-cell signal molecule is necessary for the normal development of *P. aeruginosa* biofilms (Davies et al., 1998). The small molecule, *N*-(3-oxododecanoyl)-L-homoserine lactone, is essential for exopolysaccharide production by the Pseudomonad. Initial attachment of cells to a fresh surface requires motility and type IV pili (O'Toole and Kolter, 1998) but not the homoserine lactone. The lactone signal is needed in the subsequent step of biofilm differentiation. Two different acyl-homoserine lactones are produced by *P. aeruginosa*, which are specified by the *lasI* and *rhlI* genes. Only the first is required for biofilm differentiation as shown by specific mutant knockout and by rescue of the mutant with pure *N*-(3-oxododecanoyl)-L-homoserine lactone (see Autoinducer). There is no evidence for chemotaxis; the circumstances of rescue tend to rule out a gradient of the acyl-homoserine lactone. Wild type cells but not *lasI* mutants build a biofilm in which the cells are resistant to the detergent sodium dodecyl-sulfate (SDS). Resistance to SDS can be rescued in the *lasI* mutant by adding pure *N*-(3-oxododecanoyl)-L-homoserine lactone. Thus, cells within a biofilm can be functionally integrated with each other as well as physically attached to a surface. At least one cell-cell signal is required for the multicellular regulatory state of *P. aeruginosa* in a biofilm. The biofilm also integrates metabolic interactions between the cells through the polysaccharide matrix, growth substrates, and waste elimination.

Agrobacterium and Crown Gall

Agrobacterium tumefaciens, a plant pathogen, produces crown gall tumors. Agrobacteria are able to grow within the tumor as a consequence of both genetic and physiologic interactions with plant cells and of genetic interactions among the bacteria.

Virulent agrobacteria harbor a large plasmid called Ti (for tumor inducing). Responding to signal molecules released by wounded plants, a set of virulence (vir) genes on pTi are expressed. The proteins encoded by these genes process and transfer a segment of the Ti plasmid (the T-DNA) from the bacterium to the susceptible plant during infections (Kado, 1998). Following transfer, the T-DNA integrates into the nuclear genome of the plant cell. Expression of some genes on the integrated DNA results in an oncogenic transformation of the plant cell by means of phytohormone induction. The tumor cells also produce novel low-molecular-weight compounds called opines, whose synthesis from normal intermediary metabolites is directed by genes expressed from the T-DNA. Although the genes for opine biosynthesis are present on a bacterial replicon, they are expressed properly only in plant cells, a remarkable evolutionary adaptation (Hong et al., 1997). The genes for opine biosynthesis lack identifiable bacterial promoters but contain 5′ and 3′ sequence motifs characteristic of plant transcriptional signals. In turn, the causative bacteria can utilize the opines produced by the tumors as a source of carbon and energy, and nitrogen in some cases. Thus, *A. tumefaciens* redirects plant cell metabolism to produce specific metabolites which the bacterium can use as growth substrates.

The opine released by the host plant induces production of 3-oxo-octanoyl-HSL (Zhang et al., 1993). In turn, the HSL enhances the transfer of Ti plasmid from *A. tumefaciens* cells that carry the Ti plasmid to any non-carrier *Agrobacteria* within the tumor. The plasmid carries genes for the breakdown of a particular opine; Ti plasmid T-DNA, which encodes plant proteins involved in the biosynthesis of opines in crown gall tumors, is matched by Ti plasmid genes, which confer on the bacterium the ability to take up and utilize the particular set of opines produced by the tumor. For example, tumors that produce octopine, an arginine derivative, contain bacteria that specifically degrade octopine, while tumors that produce agropine contain bacteria that specifically degrade that agropine. Conjugation

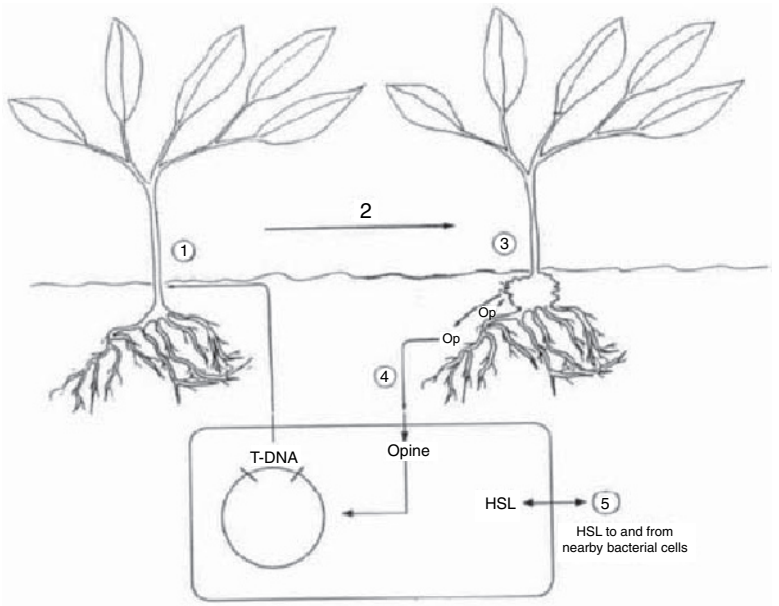

Fig. 4. Diagram showing infection of a plant by *Agrobacterium tumefaciens* and production of a tumor. The pTi plasmid is shown within the bacterium from which T-DNA is transferred to the plant and opine induces homoserine lactone (HSL) production. From Farrand, 1993. Five steps are indicated: 1. Agrobacterial cells enter a susceptible plant. 2. The T-DNA is transferred from the bacteria to plant cell nuclei. 3. Tumor grows, secreting opine (Op). 4. Opine taken up by the bacteria induces production of HSL. 5. The HSL induces conjugation between bacteria and plasmid transfer.

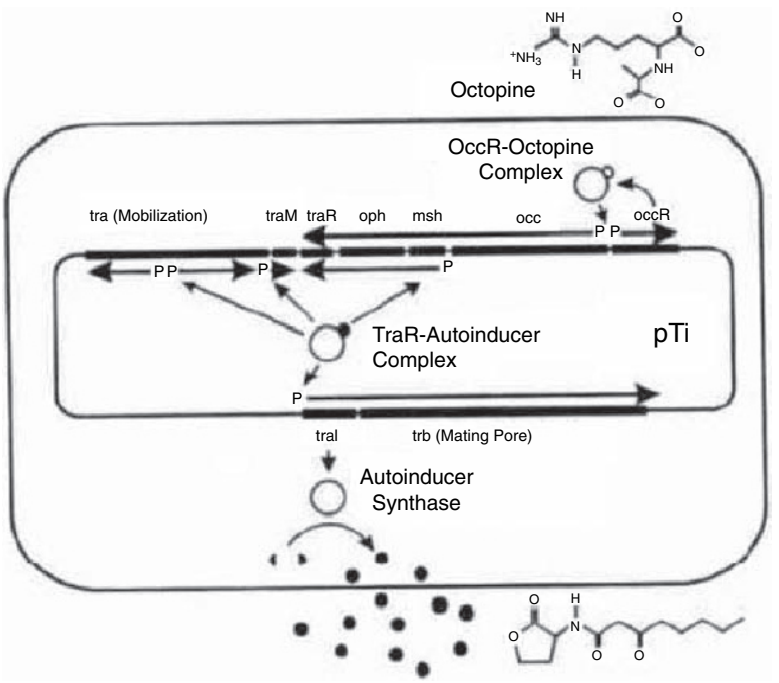

Fig. 5. Quorum-dependent regulation of octopine-type Ti plasmid *tra* and *trb* genes. The octopine-OccR complex elevates transcription of *traR*. The *traR*-autoinducer complex causes a further elevation in *traR* and *traI* expression as well as induces other *tra* and *trb* genes. Modified from Winans et al., 1999.

and plasmid transfer enhance the spread of the appropriate catabolic activity among the bacteria. A high density of donor cells enhances conjugation, and the role of this HSL is to assess the cell density, activate conjugation if appropriate, and spread the plasmid so that the specificity of opine produced by the plant tumor matches the ability of the *Agrobacterium* within the tumor to metabolize it.

Conjugation and plasmid transfer is controlled by a pair of LuxI-LuxR homologs designated *traI* and *traR* (see Fig. 5; Hwang et al., 1994; Piper et al., 1993). An HSL-TraR complex activates expression of transfer (*tra* and *trb*) genes (Piper et al., 1993). One lux box in the *tra* region is located between two divergently transcribed plasmid transfer genes, *traA* and *traC*. In sum, *Agrobacterium* receives chemical signals from its plant host and sends genetic signals to the plant and also to other agrobacterial cells.

Rhizobium

Bacterial signaling is needed to promote nitrogen-fixation within leguminous plants. Members of the genus *Rhizobium* engage in a symbiotic

relationship with peas, soybeans and other legumes to assimilate gaseous nitrogen and incorporate it into cellular material. Fixation is carried out only by a few specialized kinds of bacteria, which include the symbiotic Rhizobia, and these are highly specific for their plant host. Their specificity arises in part through the nature of their cell-to-cell signals.

Rhizobium in the soil invades tiny hairs on the roots of the legume, penetrating into the root tissues. There, the bacteria change their shape and size, becoming rounder and bigger, as they differentiate into bacteroids. Meanwhile, the root cells multiply and give rise to specialized nodules that house and feed the bacteroids. In this mutually beneficial association, bacteroids supply their host with a readily assimilated form of nitrogen (ammonia) and the plant supplies the bacteria with food (carbohydrates).

These developmental changes in both microbe and host result from a reciprocal molecular conversation. Legumes secrete flavonoids into the rhizosphere. When an appropriate flavonoid is recognized by the bacterium, it responds by synthesizing a lipochitooligosaccharide (LCO) signal, known as a Nod factor. Nod factors are major determinants of host specificity. Nod factors have a similar basic structure composed of a chitooligosaccharide (a linear chain of β-1,4-linked *N*-acetylglucosamines) linked to an acyl chain.

This core is modified in a way that is characteristic for each Rhizobium species. Variations include the degree of chitooligosaccharide polymerization (n in the figure), the nature of the fatty acid at R1, and modifications located at one or both ends of the oligosaccharide (R2–R7; Long and Staskawicz, 1993; Mergaert et al., 1997).

The appropriate Nod factor initiates the nodulation program in the plant, triggering the cell divisions that form a nodule, within which the *Rhizobium* differentiates into bacteroids that fix N_2. Thus, a two-way conversation between the bacterium and its plant host instructs them both in the successive steps that build a nodule.

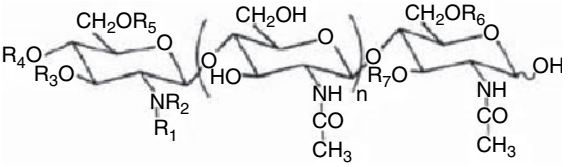

Fig. 6. Generic *Agrobacterium* Nod factor, a modified lipochitooligosaccharide that is synthesized by the bacterium. R1 is a fatty acid; R2 is hydrogen or methyl; R3, R4, or R5 are hydrogen or carbamoyl; R6 or R7 are various glycosyl modifications (Mergaert et al., 1997). The R6 branch may be sulfated (Schwedock et al., 1994). The degree of polymerization, n, may be 3, 4, 5, or 6.

Fruiting Body Development

Myxobacteria have adopted multicellularity as their strategy for survival. When they begin to exhaust their available food supply, they construct fruiting bodies (Reichenbach, 1993). Each fruiting body contains about 100,000 cells differentiated as asexual spores. Multicellular sporulation is thought to improve their long-term survival by enhancing spore dispersion and by providing a high cell density for cooperative feeding when the spores germinate (Reichenbach, 1984). In their vegetative phase, they also feed cooperatively. The figure (7) shows *Myxococcus xanthus*, the "golden" myxobacterium, one of many species of myxobacteria, all of which are phylogenetically related. *Myxococcus* and *Stigmatella*, which has a stalk and multiple sporangioles, have the same size circular genome of 9.5 Mb and common initial steps of their morphological development (Reichenbach and Dworkin, 1981; Shimkets and Woese, 1992).

Using a capacity to move on the surface of other cells, *M. xanthus* builds a fruiting body as shown in Figure 7.

Starting from a uniform sheet of cells (Figure 7, panel 1) a punctate distribution of small asymmetric aggregates appears within a few hours (panel 2). More and more cells enter some of these early aggregates, and after about 10^5 cells (panel 4) have entered, a mound becomes a steep-sided hemisphere. Then the cells differentiate from long rods into spherical spores. Spores have thick walls, are metabolically dormant, resistant to radiation and dessication, and long-lived (White, 1993). A mature fruiting body is entirely filled with close-packed spores (panel 6).

To carry out their program of morphological development, the cells communicate with each other by emitting and responding to extracellular chemical signals. Three signals have been chemically identified: A, E, and C, which are passed between genetically identical cells to coordinate their development. Mutants that are unable to produce the A, C or E signals prematurely arrest the assembly of fruiting bodies (Hagen et al., 1978; Toal et al., 1995); each stops at a stage that corresponds to the time at which the signal is needed (see Figure 8).

These morphologic stages are linked with the developmental expression of sets of genes. Transcriptional fusions of developmentally regulated promoters to *lacZ*, the structural gene for β-galactosidase, report the activity of each promoter as expression of the enzyme. More than nine-tenths of the transcriptional *lacZ* fusion mutants modify development in a limited way, but they are not necessary for its completion

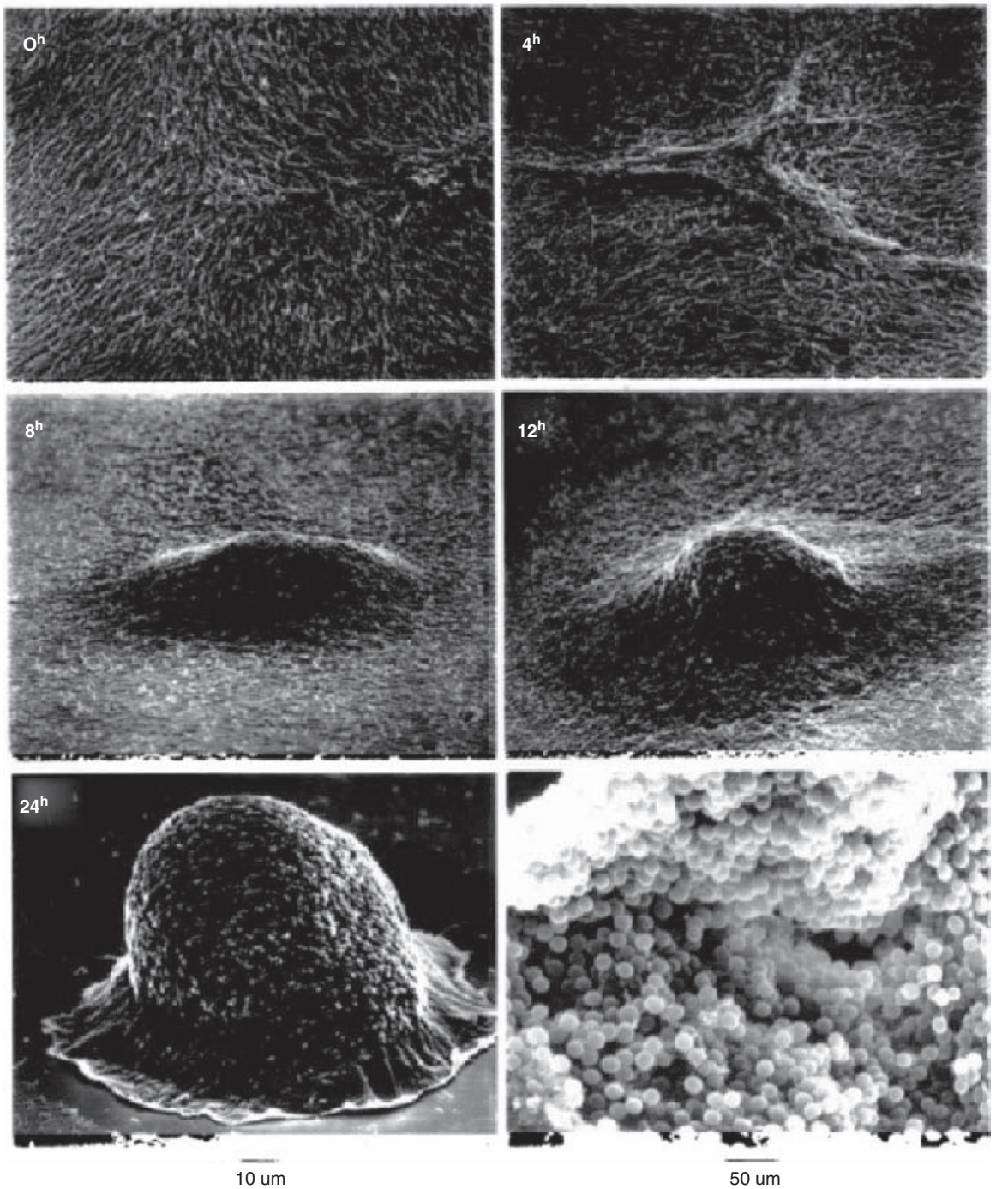

Fig. 7. Fruiting body development in *M. xanthus*. Development was initiated at 0 hours by replacing nutrient medium with a buffer devoid of a usable carbon or nitrogen source. The lower right frame shows a fruiting body that has split open, revealing spores inside. This frame is three times the magnification of the others. Scanning electron microscopy by J. Kuner. From Kuner and Kaiser, 1982.

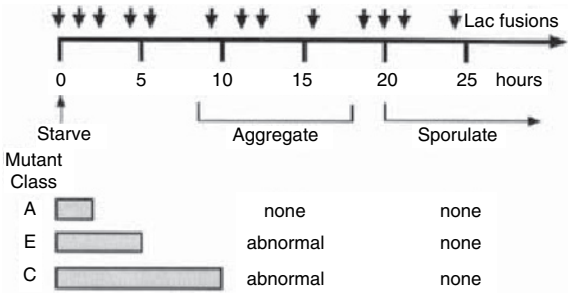

Fig. 8. Three classes of signal-defective developmental mutants of *M. xanthus*. Vertical arrowheads point to the time at which one of the lacZ fusions to a developmentally regulated promoter begins to be expressed. These fusions are reporters of normal development. The horizontal bars indicate the period of normal expression of reporters for each of the indicated mutants. Rightward from the end of the bar, development is defective; expression of the reporters is greatly reduced. The columns to the right show the morphological phenotype with respect to aggregation and sporulation of the indicated mutants.

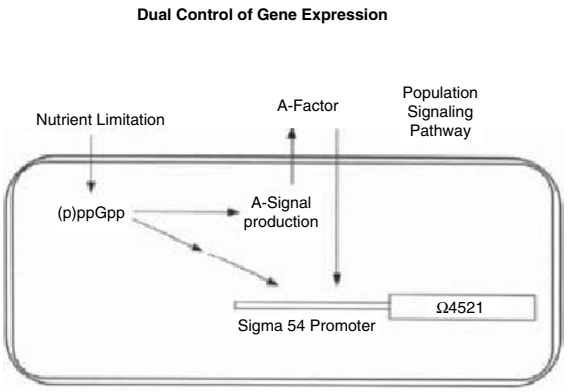

Fig. 9. Dual control of gene expression. As nutrient levels decrease and the protein synthetic capacity of the cell falls, the (p)ppGpp levels increase. Production of A-factor is induced by that increase, and it is released into the medium. Each starved cell releases a fixed amount of the set of A-factor amino acids, which pool in the fluid surrounding the cells. In responding to A-factor each cell perceives the pooled concentration of these amino acids in its vicinity. Promoters of genes that are A-factor and starvation dependent, like Tn5 lac *Ω4521*, receive one input from the cell's (p)ppGpp level and a second input from A-factor; both inputs are necessary for *Ω4521* expression.

(Kroos et al., 1986). Hence the fusions serve as reporters for the various regulatory stages of development without interrupting the overall flow of that program.

Mutants defective in producing extracellular signal A arrest at 1 to 2 hours of development, as a flat film of cells with no sign of focal aggregation (much like panel 1 of Figure 7). A-signal mutants are capable of sensing starvation, however (Singer and Kaiser, 1995), and express several early reporters (Figure 8). Mutants defective in producing extracellular signal E are blocked at 3 to 5 hours, and C mutants arrest after about 6 hours, partially aggregated and having expressed more genes.

A-Signaling

A-signal-production-defective mutants have been found in three genes. One, *asgA*, encodes a protein with a two-component receiver domain followed by a histidine protein kinase domain (Davis et al., 1995; Li and Plamann, 1996; Plamann et al., 1994) and is thought to function as a phosphorelay that, in response to starvation, is required for generation of extracellular A-factor. A-factor is a set of 6 amino acids (trp, pro, phe, tyr, leu and ile), peptides containing these 6 amino acids, or proteases capable of releasing these amino acids from *M. xanthus* cells (Kuspa et al., 1992a; Plamann and Kaplan, 1999; Plamann et al., 1992).

Myxococcus releases small quantities of these amino acids about 2 hours into development, then proceeds to take them back, Figure 9. This release and uptake helps *Myxococcus* choose between two alternative responses to nutrient limitation—on the one hand, entering stationary phase with very slow growth or on the other, fruiting body development with differentiation of spores. At least 30 new proteins are made during fruiting body development (Inouye et al., 1979), so a capacity to synthesize protein must be retained well into the sporulation phase. Accordingly, the cells must make their choice before any nutrient essential for protein synthesis has been totally depleted.

Starvation for any amino acid or starvation for carbon, energy, or phosphorous (Manoil and Kaiser, 1980) but neither starvation for purine or pyrimidine induces fruiting body development (Kimsey and Kaiser, 1991). The set of effective inducing conditions implicates the availability of a complete set of amino-acylated tRNAs. In *Myxococcus*, as in other bacteria, the absence or shortage of any one of the charged tRNAs leads a ribosome, sensing with a codon "hungry" for its cognate amino-acylated tRNA, to synthesize guanosine tetra- (and penta-) phosphate [(p)ppGpp] by condensing ATP and GTP. A rise in this highly phosphorylated nucleotide sets off a stringent response that stops the synthesis of new ribosomes and of the other major polymers of the cell, including DNA, phospholipids, and peptidoglycan (Cashel et al., 1996). Stringent conditions do allow certain genes to be expressed and selected proteins to be synthesized, insofar as activated amino acids are available. In this way, the genes for new fruiting body proteins are expressed and translated.

Accumulation of (p)ppGpp is both necessary and sufficient for fruiting body development. On

the one hand, ectopic production of (p)ppGpp in *M. xanthus* initiates early developmentally specific gene expression (Singer and Kaiser, 1995). The *E. coli relA* gene was introduced into *M. xanthus* for this purpose; its introduction was followed by production of the *E. coli relA* protein and (p)ppGpp accumulation without any prior starvation. Moreover, the rise in (p)ppGpp also induces production of A-factor (Singer and Kaiser, 1995). On the other hand, *M. xanthus* has its own *relA* gene, and either a point mutation or a deletion mutation in that gene blocks starvation-initiated development at the flat biofilm stage of Figure 7 frame 1 (Harris et al., 1998). In fact, these *relA* mutants arrest before expression of any of the developmentally regulated reporters.

A-SIGNALING AND DUAL CONTROL Each starved cell that has accumulated ppGpp releases a fixed amount of the set of A-factor amino acids. Being soluble, these amino acids then pool in the fluid surrounding the cells. In responding to A-factor, each cell perceives the pooled concentration of these amino acids in its vicinity (Kuspa et al., 1992b). Promoters of genes that are A-factor and starvation dependent, like Tn5 lac *Ω4521*, receive one input from the cell's (p)ppGpp level and a second input from A-factor. Both inputs are necessary for *Ω4521* expression (Kuspa et al., 1986). The promoter for *Ω4521* recognizes σ 54 rather than the more common σ 70 (Keseler and Kaiser, 1995). All known σ 54 promoters require an upstream activator protein to initiate open-complex formation and then transcription (Wedel and Kustu, 1995). An activator protein (Gorski and Kaiser, 1998) and the σ-54 holo enzyme are two spatially distinct input sites for controlling transcription of *Ω4521* (see Figure 9). The (p)ppGpp level indicates the level of nutrient currently available, since this nucleotide tracks nutrient changes. A-factor recalls the level of starvation 2 hours previously. The expression of *Ω4521* requires both inputs; very low expression occurs when either input is absent. In this way, the A-factor pool summarizes the votes of all the cells. Thus A-factor belongs to the class of extracellular signals called quorum sensors (Kaiser, 1996). Given the concentration window for an A-factor response, the reliability of the judgment whether to enter stationary phase or to initiate fruiting body development is increased when the whole population of cells has made it.

C-Signal

Primary functions of C-signaling are to bring cells into selected asymmetric foci until they have mounded into hemispheres of about 10^5 cells and to trigger the cells inside a completed hemisphere to differentiate into spores, by transforming the individual motile rod cells into nonmotile spherical dormant cells with tough coats. Null mutants of *csgA*, the gene encoding C-factor protein, form small irregular aggregates (Figure 7, frame 2) and stop developing at this stage. They also do not sporulate; the sporulation frequency of cells with csgA$^+$ is 10^5 fold higher than the mutant. Sporulation requires C-factor to initiate the cell-shape change and for expression of spore genes.

C-factor was purified from whole cells, and a 17-kDa protein was obtained (Kim and Kaiser, 1990d). Antibodies to C-factor reveal its exposure on the cell surface (Shimkets and Rafiee, 1990). Neither an aggregation reporter like the Tn5lac insertion *Ω4499* nor a sporulation reporter like *Ω4435* is expressed in the absence of C-factor. However, both are expressed if purified C-factor is added to *csgA* mutant cells (Kim and Kaiser, 1991). Higher levels of C-factor are needed for sporulation than for aggregation. Both the aggregation and the sporulation reporter were expressed when high levels of C-factor were added; neither was expressed when low levels were added, and only the aggregation reporter was expressed when intermediate levels were added. Whether the amount of C-factor is limited by promoter deletions or by adding variable amounts of purified C-factor, the same gradation of aggregation and sporulation was observed (Kim and Kaiser, 1991; Li et al., 1992).

The amount of C-factor in cell extracts rises between 8 and 18 hours of development, naturally staging aggregation before sporulation (Kim and Kaiser, 1990a). The rise in the concentration of C-factor per cell is a consequence of a positive feedback loop in the C-signaling circuit. Expression of the *csgA* gene is controlled by C-factor itself (Kim and Kaiser, 1991). Once C-signaling has started, *csgA* gene expression begins to rise. Because C-signaling depends on cell contact (Kim and Kaiser, 1991), that rise in C-factor concentration speeds up as aggregation proceeds.

C-Signal Transduction

The C-signal transduction circuit (Figure 10) branches twice, one branch for expression of *csgA* and another for aggregation and sporulation, so that one signal performs 3 tasks (Søgaard-Anderson et al., 1996b). C-factor on the surface of one cell interacts by contact with another cell (Kim and Kaiser, 1990b; Kim and Kaiser, 1990c; Kroos et al., 1988). End-to-end contact is believed to be necessary (Kim and Kaiser, 1990b; Sager and Kaiser, 1994; Wall and Kaiser, 1998). Whether C-factor signals as an enzyme or as a ligand has not been established as yet (Baker, 1994; Lee et al., 1995; Lee and Shimkets, 1994).

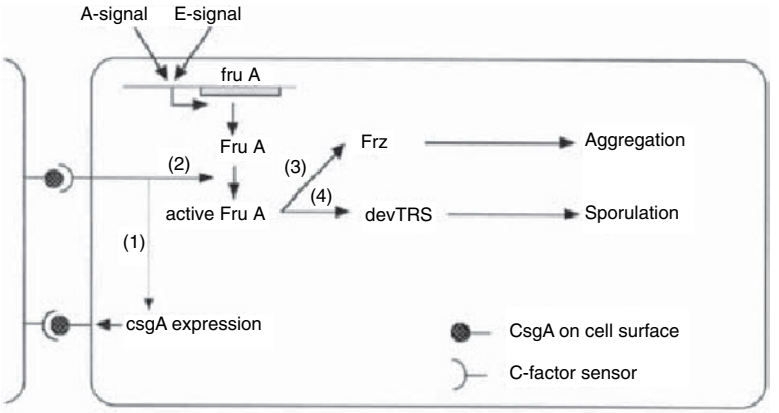

Fig. 10. C-signal transduction pathway. The structural gene *fruA* encodes a basic helix-turn-helix transcription factor. Steps (1), (2), (3), (4) are described in the text. The set of proteins Frz makes up a phosphorelay pathway and *dev* is an autoregulatory operon. The figure shows a pair of rod cells that are C-signaling to each other through their ends.

Following C-signal transmission, step (1) in Figure 10 causes the expression of *csgA* to rise; this is the positive feedback that increases the intensity of signaling once it has started. In step (2) of Figure 10, C-signaling activates FruA protein by a post-translational modification that is evident by its electrophoretic mobility (Ellehauge et al., 1998). The protein FruA is a DNA-binding response regulator with a helix-turn-helix (HTH) motif (Ellehauge et al., 1998; Ogawa et al., 1996). Activated FruA is necessary for aggregation and sporulation; there is no evidence that *csgA* expression is FruA-dependent. Synthesis of FruA protein depends on A-factor and E-factor, but not on C-factor (Figure 10).

The *frz* target of FruA is a phosphorelay, Figure 10 step (3), which modulates the frequency of reversal of direction (Blackhart and Zusman, 1985). In that relay, the FrzCD protein has a domain that resembles the carboxy-end of a methyl-accepting chemotaxis protein (McBride et al., 1989). However, FrzCD protein is not a membrane receptor; it is found in the cytoplasm and has no transmembrane or extracellular domain. Activated FruA sends a signal along the *frz* phosphorelay, as detected by the methylation of the FrzCD protein (Søgaard-Anderson and Kaiser, 1996a), and by a C-factor-induced increase in gliding speed and duration of gliding interval (Jelsbak and Søgaard-Andersen, 1999).

The methylation of FrzCD shifts during fruiting body development (Søgaard-Anderson and Kaiser, 1996a). Starting from a fully nonmethylated state early in development, the FrzCD protein gradually shifts to methylated states, and by 9 hours, the time of symmetrical mound building, all the FrzCD protein is methylated. Extracellular addition of purified C-factor to C-factor-less mutant cells directly and specifically induces the full methylation of their FrzCD protein, paralleling wild-type development (Søgaard-Anderson and Kaiser, 1996a). The cellular mechanism of aggregation, however, has not yet been established.

Both *fruA* and *frz* mutants were found in a screen of Tn5 insertion strains that arrested development in a state of partial aggregation, at the same stage as *csgA* mutants (Søgaard-Anderson et al., 1996b). Unlike *csgA* mutants, however, the targeted mutants are cell-autonomous; they are not rescued by addition of wild-type cells, or by addition of purified C-factor. Comparison of the properties of the *fruA* and *frz* mutants immediately showed that FruA is needed for aggregation and sporulation, but *frz* is only needed for aggregation.

The second target of activated FruA is the *dev* operon, Figure 10 step (4). Operon expression, as measured by the extent and time course of β-galactosidase expression from a Tn5 *Lac* transcriptional fusion to *devR*, depends on FruA in the same manner as it depends on C-factor (Ellehauge et al., 1998). The operon, in turn, is necessary for fruiting body sporulation; null mutants sporulate at 0.1% or less the frequency of wild type cells (Thony-Meyer and Kaiser, 1993). The morphological differentiation of myxospores occurs after aggregation is complete. In *Sigmatella aurantiaca*, another myxobacterium whose fruiting bodies have a more complex morphology (a stalk, branches and multiple cysts containing the spores), the aggregates pass through different intermediate shapes, including a myxococcus-like mound. Nevertheless myxospores do not form until the fruiting bodies are assuming their final branched shape (Qualls et al., 1978).

Activated FruA elevates the transcription of the *dev* operon. This operon has a switch-like quality, evident in the bimodality of operon expression in populations of developing cells (Russo-Marie et al., 1993). The two states may be consequences of the intense C-signaling in the densely packed cells that circulate inside nascent fruiting body aggregates and of little or no C-signaling in cells less densely arrayed in the periphery. Spores are found differentiated inside the fruiting body after its morphogenesis is complete, following intense C-signaling. Hence, the

chemical properties of A- and C-factors match the cell density at which they signal. For A-signaling, the cells are separate, the signal must diffuse between them in a generally aqueous environment, and the A-factor amino acids are water soluble. Quorum sensing A-factor counts the number of votes cast by those cells recommending fruiting body development. For transmitting the cell-bound C-signal, the cells must touch each other. This condition and the requirement for a high level of C-signaling to switch *dev* on test whether the cells have reached the high density characteristic of a nascent fruiting body, ensuring that it has the proper morphology.

Pheromone-Inducible Plasmid Transfer in *Enterococcus faecalis*

Enterococcus faecalis, a nonmotile Gram-positive species, exhibits a highly specific cell interaction to initiate plasmid transfer. Potential recipient cells excrete short peptides that stimulate a mating response by donor cells, which carry the corresponding plasmid. The peptides signal donor cells to synthesize surface adhesins, and mating mixtures form large aggregates of donors and recipients. The cells specifically adhere to one another. The interactions are summarized in the scheme of Figure 11.

At the center of the scheme, a plasmid-free recipient synthesizes two different pheromones, called cA and cB and shown as triangles and squares. The cell carrying plasmid pA (on the left) responds to cA and the cell carrying plasmid pB (on the right) responds to cB. Upon exposure to pheromone, a signal is transduced to the resident plasmid that results in expression of a plasmid gene encoding a surface adhesin (AS) on the donor cells. The adhesin has the capacity to bind to a normal constituent of the cell walls, probably lipoteichoic acid (LTA), the major wall antigen of Gram-positive cells. Once triggered by pheromone, donor cells will bind recipients and other donor cells as well. Chemotaxis toward the pheromones by donor cells is not evident. Within the resulting aggregates, which may be large, plasmid is transferred from donors to recipients by conjugation (see Conjugation in the Introduction).

Several pheromones have been isolated, purified, sequenced and synthesized (Table 1). Typically they are hydrophobic octapeptides. These pheromones are active at concentrations below 5×10^{-11} M, and as few as two molecules per donor cell may be sufficient to induce a mating response (Mori et al., 1988). Their specificity is high: pheromone cAD1 is unable to induce the clumping of cells which carry plasmid pPD1 even at 10^{-6} M, ten thousand times the threshold concentration for pheromone, and pheromone cPD1 is unable to induce the clumping of cells which carry plasmid pAD1. The plasmid also encodes formation and secretion of a competitively inhibitory peptide, which blocks the action of a corresponding pheromone. Several inhibitors have been purified, and they have the same length as their corresponding pheromones, as well as some identical residues. Inhibitors and their sequences also are shown in Table 1.

The plasmid-encoded adhesins, or AS, for pAD1 and pPD1 are large proteins with a proline-rich C-terminal region for cell-wall association, followed by a membrane anchor. The adhesins have been visualized by electron microscopy as dense, hair-like structures on the bacterial cell wall, protruding about 20 nm from

Table 1. *E. faecalis* pheromone amino acid sequences.

Pheromone or inhibitor	Peptide structure
cAD1	LFSLVLAG
iAD1	LFVVTLVG
cPD1	FLVMFLSG
iPD1	ALILTLVS
cCF10	LVTLVFV
iCF10	AITLIFI
cAM373	AIFILAS
iAM373	SIFTLVA
cOB1	VAVLVLGA

Structure of *Enterococcus faecalis* pheromones. The structures are based on work from the laboratories of M. Mori, J. Nakayama, A. Suzucki, and D. Clewell (Clewell, 1999).
L = leucine; F = phenylalanine; S = serine; V = valine; A = alanine; G = glycine; T = threonine; M = methionine; I = isoleucine.

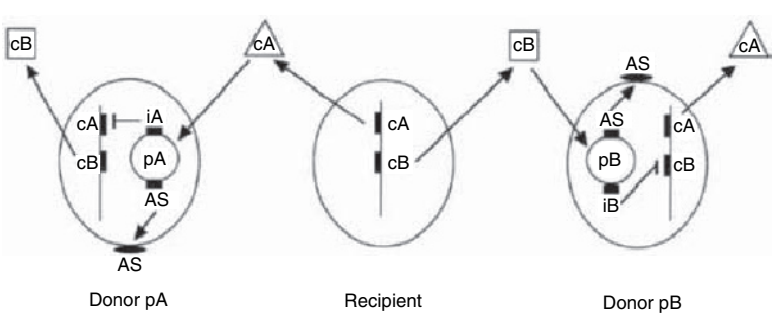

Fig. 11. Conjugal pheromones used by *Enterococcus faecalis*. The cell in the middle is plasmid free, while the cells at either side carry conjugative plasmids pA and pB, respectively. The cA and cB are two different pheromones; iA and iB are their corresponding inhibitor peptides, and AS is aggregation substance.

its surface (Galli et al., 1989). The adhesin molecules are unevenly distributed over the surface of cells, apparently attached only to the cell wall that was present when AS induction by the pheromone began. Such behavior suggests a convenient way for donor cells to dissociate from mating aggregates after plasmid transfer is complete. Growth and cell division would give rise to new donor cells free of the adhesin. These new donors would tend to dissociate from the clumps.

The target for the adhesin on *E. faecalis* cells is thought to be lipoteichoic acid (LTA) because LTA-deficient mutants lose the ability to conjugate with active donor strains. Moreover, a reversal of the normal donor-recipient orientation of adhesin-LTA allowed practically normal conjugation and plasmid transfer (Dunny, 1990).

In the genome sequence of *E. faecalis*, which has been completely determined, three of the pheromones appear in one or another of the recipient protein signal sequences (Clewell, 1999), and a single cell produces at least five different pheromones. The use of signal peptides, which are often released from cells, suggests that the entire pheromone-induced conjugation response may have evolved when potential donors gained a capacity to detect potential recipients by the signal peptides they release. At least 11 different plasmids encode pheromone responses, and the genes that specify their aggregation substances are homologous for 10 of them. Moreover, at least two of these plasmids, pAD1 and pCF10, have a similar organization of pheromone-induced genes and an identity of more than 85% for their structural genes encoding aggregation substance (Galli and Wirth, 1991). The whole set of plasmids may have evolved from a common ancestor plasmid. In conclusion, *E. faecalis* uses sequence-specific peptides to induce the agglutination of plasmid donors and recipients. The plasmid is transferred from donor to recipient within the aggregate; the aggregate then disperses.

Swarming

Swarming cells spread from a point of inoculation radially outward over a solid surface such as agar. Swarming motility, which is cooperative, organized and relatively rapid, is observed in many Gram-negative and some Gram-positive bacteria. Motility depends on interactions between cells that have appendages. However, both flagella and type IV pili promote swarming, whereas other pili do not. Flagella and pili are thought to propel a cell by different means; nevertheless the two cause remarkably similar spreading patterns. Comparison of the two modes may offer insight into the cell-cell interactions that generate swarm behavior. Swarming is inducible in flagellated cells but constitutive in cells with type IV pili.

Swarming Based on Flagella

Among Gram-negative bacteria, swarming with flagella has been found in the genera *Aeromonas*, *Escherichia*, *Proteus*, *Pseudomonas*, *Rhodospirillum*, *Salmonella*, *Serratia*, *Vibrio*, and *Yersinnia* (Harshey, 1994a). Swarming also has been observed in *Bacillus* and *Clostridium*, which are Gram-positive (Harshey, 1994a). All these swarming bacteria possess flagella, which they use for swimming, but swarming requires that the swimming cells change size and physiology. The shape modifications adapt cells to life on a surface, instead of swimming in a liquid environment. The differentiation involves flagella and other gene products that control cell division. In *Vibrio parahaemolyticus*, 40 genes or more are expressed for the swarming state. The differentiation inhibits septation and produces an elongated multinucleated cell (up to 80 μm long in *Proteus*) with a two to fifty-fold higher surface density of flagella, depending on the organism (Harshey, 1994a). *E. coli*, *Proteus*, *Salmonella typhimurium*, and *Serratia marcesens* use the same type of flagellar organelle for swimming and swarming. The structures of the flagella of *E. coli* and *S. typhimurium* are well known, and they are essentially identical (Macnab, 1996).

V. parahaemolyticus is the only swarmer to have distinct organelles for swimming and swarming (Harshey, 1994a). It possesses hundreds of lateral flagella, while undifferentiated swimming cells have but a single polar flagellum. A proton-motive force powers the lateral flagella, while a sodium-motive force drives the polar flagellum (Atsumi et al., 1992). These distinctions have been turned to experimental advantage in the study of swarming.

Signaling (using the chemotaxis phosphorelay) but not chemotactic behavior has been shown to be essential for the differentiation of swarm cells in *E. coli* (Burkart et al., 1998). A quadruple mutant that lacks all four inner membrane chemoreceptor-transducers (Tar, Tsr, Tap, and Trg) fails to swarm, but mutants lacking any one of the four continue to swarm (Burkart et al., 1998). Swarming is rescued in the quadruple mutant by restoring either Tsr or Tar, which happen to be the most abundant chemoreceptors in *E. coli*. However, neither the presence of saturating chemoeffector concentrations or mutations that destroy ligand binding to Tsr or Tar interferes with swarming or with hyperflagellation. In addition, mutants with defects in *cheA*, *B*, *R*, *W*, *Y*, or *Z* elongate, but none

hyperflagellate or swarm (Harshey and Matsuyama, 1994b). Apparently communication of the receptors with the *cheA* kinase is essential. For swarming, it appears that the chemoreceptors signal through the chemotaxis pathway and induce swarmer-cell differentiation, but the receptors are responding to other unknown signals rather than their well-known chemoeffectors (Burkart et al., 1998).

Interference with either the *fla* genes or the functioning of the polar motor results in constitutive expression of lateral flagella in *V. parahaemolyticus* (Kawagishi et al., 1996; McCarter et al., 1988). Transposon insertion within the *lonS* gene of *V. parahaemolyticus*, which encodes an ATP-dependent protease homologous to the *E. coli lon* gene, causes expression of *lafX*, one of the genes high in the pathway of differentiation and cell filamentation—even in the absence of UV irradiation (Stewart et al., 1997). Targets of *lonS* might include a transcriptional activator of *laf* genes and an inhibitor of cell division (Stewart et al., 1997). FlhDC in *P. mirabilis*, an operon known to regulate transcription of the genes of the flagellar hierarchy, has been suggested to control swarmmer differentiation as well since it plays a role in the inhibition of cell division (Furness et al., 1997). The operon also affects cell division in *E. coli* (Pruss and Matsumura, 1996). Transcription of the *flhDC* operon rises during differentiation and decays as the cells dedifferentiate back into swimmers (Furness et al., 1997).

The natural trigger to differentiate *V. parahaemolyticus* swarm cells may be the high medium viscosity which is found on a surface and places a mechanical load on the polar flagellum, thereby signaling and inducing the cell to differentiate (McCarter et al., 1988). A high viscosity medium also triggers hyperflagellation in *Se. marcesans* (Alberti and Harshey, 1990) and *P. mirabilis* (Allison et al., 1993). Swarmer cells of *P. mirabilis* transferred to liquid medium return rapidly to normal vegetative morphology; cells in the center of a swarm colony on a plate also return. Both suggest that swarmer cells must be continuously signaled to maintain the differentiated state (Harshey, 1994a; Shinoda and Okamoto, 1977).

Isolated swarmer cells barely move, suggesting that cells must be close together to swarm, and that flagella from neighboring cells coalesce into shared bundles. Swarm cells of *P. mirabilis* do align closely along their long axis, forming two-dimensional rafts that migrate by coordinate flagellar action. A mutant, *ccmA*, that has an abnormal curved shape loses the capacity to swarm but not to swim, as if, due to its shape, it could not align with other *ccmA* cells (Hay et al., 1997).

Swarming Based on Type IV Pili

Pili have been implicated in the swarming of a wide variety of Gram-negative bacteria, many of which are pathogenic (Henrichsen, 1975). These include *Acinetobacter calcoaceticus*, *Dichelobacter nodosus*, *Eikenella corrodens*, *Kingella denitrificans*, *Moraxella bovis*, *Myxococcus xanthus*, *Neisseria gonorrhoeae*, *N. meningitidis*, *Pseudomonas aeruginosa*, Synechocystis PCC6803 and enteropathogenic *Escherichia coli* (Henrichsen, 1983; Kaiser, 1979; Manning and Meyer, 1997; Strom and Lory, 1993). Remarkably, the swarming pili of all these organisms belong to the structural class known as type IV. Type IV pili (abbreviated Tfp) are distinguished from other pili by their strictly polar origins on cells, by N-terminal sequence conservation of the pilin monomer units, by sequence conservation of a core set of proteins for pilus assembly, and by supporting bacterial motility (Ottow, 1975; Strom and Lory, 1993). A broad collection of reviews on Tfp can be found in Manning and Meyer, 1997, and a recent review on Tfp and motility in Wall and Kaiser, 1999a.

Pilus-Dependent Swarming in Myxobacteria

Myxobacteria move by gliding, which occurs at a solid-liquid, solid-air, or solid-solid interface. Gliding consists of smooth motions in the interfacial plane that are directed along the long axis of the cell, with occasional reversals of direction (Hodgkin and Kaiser, 1979a).

Myxococcus xanthus has two independent gene systems named A and S, which control swarming behavior based on gliding (Hodgkin and Kaiser, 1979b). Mutations in any gene of either system only inactivate that system alone, but cells can still swarm by means of their remaining system, albeit less efficiently. However, no A^- S^- double mutant cell can move more than its length, nor can it swarm. More than fifty A^- S^- double mutants have been constructed which are defective in different A and S genes, and all fail to swarm. While the wild type cells (A^+ S^+) produce large, flat, spreading colonies with a serrate edge, the A^- S^- mutants produce small domed-shaped colonies with sharp, non-serrate edges. Ten mutants have been mapped to the *mglA* (mutual gliding) gene, which encodes a small Ras-like G-protein. This gene is required to complement both A^- and S^- motility defects (Hartzell, 1997; Hodgkin and Kaiser, 1979a).

Pili are essential for S-motility, but not for A-motility (Kaiser, 1979; Wu and Kaiser, 1995). Although wild type cells (A^+ S^+) swarm by combining the A and S swarm patterns, any A^-

S^+ mutant cells swarm by the S swarm pattern, and the role of pili is thereby revealed. Pili are usually found in tufts of 2 to 8 fibers, and always at the poles. The S-motility is lost when pili are shaved off cells by violent shearing action, but reappears as the pili grow back (Rosenbluh and Eisenbach, 1992). The S-motile cells cannot move at all when they are far apart. Nevertheless, individual cells that are separated from their nearest neighbor by less than a cell length do move (Kaiser and Crosby, 1983). Because pili tend to break when cells are washed, it is difficult to determine the length of pili on cells isolated from colonies; nevertheless many pili are roughly the length of cells. The rate at which an S-motile swarm expands depends on the cell density, showing strong dependence on cell interactions (Kaiser and Crosby, 1983). The swarming rate of S-motile cells is highest when the average cell-cell distance is less than about 1.5 μm. The bodies of cells do not have to touch to interact (Kaiser and Crosby, 1983). Spreading is evident in the change in morphology of a young S-motile colony after 2 hours (Figure 12). The colony has spearhead-like clusters of 50 or more cells but almost no single cells.

Non-swarming *M. xanthus* strains, either A^- S^- or *mglA* mutants, are unable to form fruiting bodies (see *M. xanthus* A- and C-signaling). Almost all A^- S^+ and A^+ S^- single mutants delay fruiting body aggregation, reflecting a need for the type IV pilus system (Cheng, Wee, Wu and Kaiser, unpublished). Abnormal fruiting body development is a consequence of the defective swarming when either system is absent (Hodgkin and Kaiser, 1979b; Wu et al., 1998).

UV-irradiation, chemical mutagenesis, or transposon insertion was used to create a library of about 160 independent S-motility mutants to explore the role of TfP in swarming. The S-motile (A^- S^+) colonies are flat and their spreading edges are smoothly serrated, whereas A^- S^- mutants of an A^- S^+ strain have smooth, dome-shaped colonies with sharp non-serrate edges. This library of S-motility mutants is divided into four groups named *pil*, *tgl*, *dsp*, and *frz*.

The largest group of S-motility genes is the *pil* genes. Over 100 mutations have been mapped within a contiguous *pil* gene cluster that contains 17 genes and is shown in Figure 13 (Wall et al., 1999b; Wu and Kaiser, 1995; Wu et al., 1998; Wu et al., 1997b). Products of fourteen of these genes

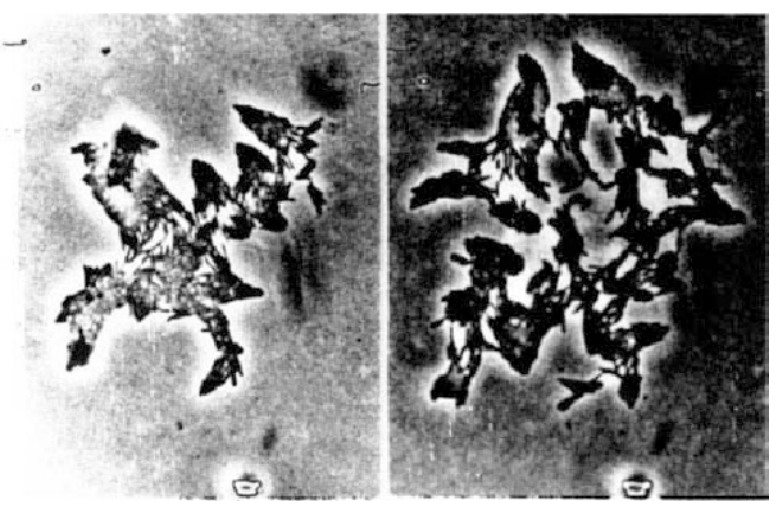

Fig. 12. Expansion of an S-motile microcolony. The two panels show the same colony at 0 (left) and 2 hours (right) at 33°C. Magnification scale: 1 cm is 15 μm. From Kaiser and Crosby, 1983.

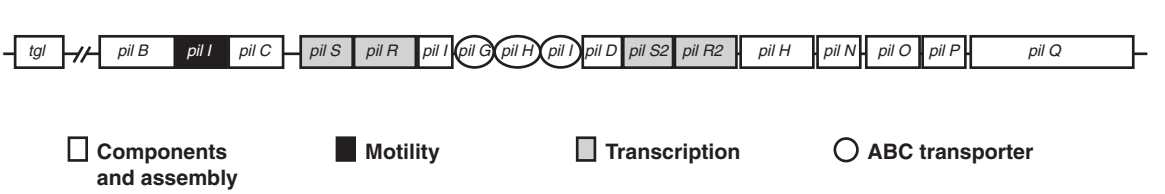

Fig. 13. *M. xanthus* Pil gene cluster and the function of each gene in type IV pilus assembly, motility, and control of gene expression. From Wall and Kaiser, 1999a.

are about 30% identical and 50% similar to proteins in *P. aeruginosa* and *N. gonorrhoeae* that encode their type IV pilin and related assembly proteins. The *Myxococcus pilG*, *pilH* and *pilI* genes have no homologs among other Tfp systems. The *pilH* gene encodes a member of the ABC transporter family with an ATP-binding cassette (Wu et al., 1998). In-frame deletions, transposon insertions and other null mutants reveal the functions of all 17 *pil* genes except *pilD*, for which deletion appears to be lethal. Null mutation in 14 of the 17 genes, including *pilG*, *pilH* and *pilI*, abolishes S-motility. These mutations, with the exception of *pilT*, also abolish the production of pili. Pili remain in a *pilT* mutant; this gene appears to be necessary for pilus function as opposed to pilus assembly. Mutant screening and sequencing left and right of the *pil* cluster shown in Figure 13 revealed no other *pil* homologs or S-motility genes in that vicinity. Clearly, Tfp is essential for S-motile swarming.

The *tgl* mutants lack S-motility and pili, like the *pil* mutants. They are distinguished from the *pil* mutants by their map position (distant from the *pil* cluster) and by their capacity to be stimulated, a process of phenotypic rescue (Hodgkin and Kaiser, 1977; Rodriguez-Soto and Kaiser, 1997a; Rodriguez-Soto and Kaiser, 1997b). It is the only stimulatable gene in the S system, although five stimulatable genes are known in the A system (Hodgkin and Kaiser, 1977; Rodriguez and Spormann, 1999). The *tgl* gene contains a type II signal sequence, suggesting it encodes a lipoprotein. Six tandem but degenerate tetratrico peptide repeats (TPR) follow the signal sequence (Rodriguez-Soto and Kaiser, 1997a). Tetratrico peptide repeats motifs are associated with proteins that interact with other proteins (Das et al., 1998). The Tgl protein may be an assembly factor for the pili, since it is not part of the pilus fiber (Rodriguez-Soto and Kaiser, 1997b).

The third group of S^- mutants is the *dsp*. All 21 *dsp* mutants map in one cluster. Transductional linkage within this group suggests an approximately 10 kb *dsp* region. Unlike wild type cells, the *dsp* mutants grow as *disp*ersed cultures in liquid medium; they neither clump nor aggregate, even though they have pili. Autoaggregation of cells in suspension is common for bacteria that have Tfp (Bieber et al., 1998; Knutton et al., 1999; Manning and Meyer, 1997; Wu et al., 1997b). Like *pilT* mutants, all *dsp* mutants retain pili, yet lack S-motility. The *dsp* mutants are depleted in extracellular fibrils, but do not totally lack them (Chang and Dworkin, 1994; Dana and Shimkets, 1993). Fibrils are peritrichous filaments composed of approximately equal amounts of polysaccharide and protein, and help to hold cells together. In addition to a cohesive function, it has been suggested that fibrils transfer signals, perhaps by ADP-ribosylation (Dworkin, 1999). A chaperone of the HSP70 family encoded by the *sglK* gene is necessary for production of one of the protein components of fibrils, and *sglK* mutants are S^- (Weimer et al., 1998). However, the relationship between S-motility and fibrils could be indirect, because *esg* mutants, which are also defective in fibril production, retain S-motility (Ramaswamy et al., 1997).

The final group of S-motility genes is called *frizzy*. These genes control the frequency of reversal of gliding direction (Blackhart and Zusman, 1985), and possibly speed (Spormann and Kaiser, 1999). The *frz* genes are homologous to the enteric bacterial chemotaxis genes for swimming (McBride et al., 1989). When a *frz* mutation is combined with an A^- mutation, the *frz* genes behave like S-genes (Fontes and Kaiser, 1999). Swarming of A^- *frz* double mutants is greatly diminished. However, it is not eliminated, resembling A^- *dsp* mutants.

It may be useful to think of S-motile swarming as the product of a pathway shown in Figure 14. Blocks at any stage in the pathway would create an S^- mutant. The first stage (pilin synthesis) includes control of *pilA* expression by PilS/PilR, a two-component regulatory system (Wu and Kaiser, 1997a), membrane insertion of a pilin monomer, and finally processing by the bifunctional PilD peptidase/N-terminal methylase. Processed subunits are then polymerized into a pilus fiber. The pili contact other cells and other pili, providing cell interactions that may also involve the fibrils and proteins bound to fibrils. In the last step, pilT and frz proteins may help to control movement behavior. The pili, and possibly the fibrils, are thus essential for social swarming, but their role is not yet clear (Kaiser, 1979; Wu et al., 1998).

Twitching Motility Twitching describes a type of surface translocation that, like gliding, does not depend on flagella, and in which the movements of individual cells appear as small, intermittent jerks (Henrichsen, 1972). Like gliding, twitching depends on humid agar but unlike gliding, twitching is jerky, not smooth (Henrichsen, 1972; Henrichsen, 1975; Henrichsen, 1983). Henrichsen surveyed some 1,000 bacterial

Fig. 14. Steps on the dependent pathway to social swarming in *M. xanthus*.

strains representing more than 50 species, classifying their surface translocations as twitching or gliding. About 20 of his species were found to twitch, and some he demonstrated to have polar pili. All strains of the same species with polar pili exhibited twitching. Moreover, twitching was not found in variants of the strains that had lost their polar pili by mutation, or in strains with pili that were not type IV. Henrichsen concluded from this study that twitching depends on polar pili. Subsequent genetic studies of *P. aeruginosa* and *N. gonorrhoeae* showed that their twitching depends on their Tfp (Strom and Lory, 1993). Behavior, sequence conservation of Tfp genes among *P. aeruginosa*, *N. gonorrhoeae*, and *M. xanthus*, and strong similarities of mutant phenotype clearly connect twitching and social gliding (Mattick et al., 1996; Wall et al., 1999b; Wu and Kaiser, 1995).

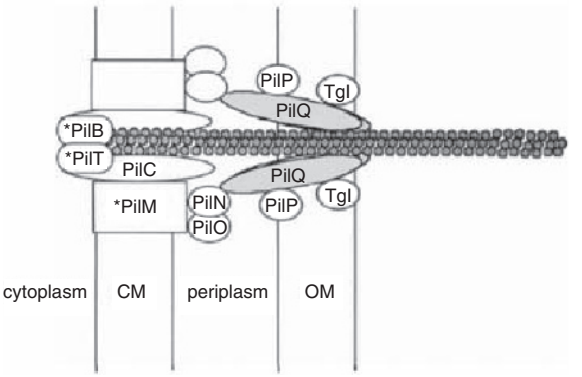

Fig. 15. Model of the type IV pilus in *M. xanthus*, based on genetic studies and on location of the individual proteins. From Wall and Kaiser, 1999a.

Type IV Pilus Fiber Structure

The structure of the pilus fiber and its mode of assembly (and possibly disassembly) are relevant to pilus function in twitching and gliding, as well as to the cell interactions necessary for swarming. The Tfp fiber has a three-layered helical structure of coiled α-helices surrounded by a β-sheet. These two inner layers are covered with the C-terminal regions of adjacent monomers, based on a 2.6 Å resolution X-ray crystal structure of dimers of pilin protein from *N. gonorrhoeae* (Forest and Tainer, 1997; Parge et al., 1995). The *N. gonorrhoeae* fiber has 5 pilin monomers per helical turn, a rise of about 4 nm per monomer, and an outer diameter of about 6 nm. The helix parameters and diameter agree with fiber diffraction data from *P. aeruginosa* Tfp, which suggests that the proposed structure is representative of the Tfp in many organisms. The N-terminal amino acid sequence, which is highly conserved from one bacterial species to another, forms the innermost coil of staggered, hydrophobically packed, parallel α-helices. Hydrophobic packing and the flexibility of α-helices may allow pili to bend and to adopt twisted or bundled conformations. The middle layer of β-sheet is continuous from one pilin monomer to the next in the sense that any crosssection of the fiber would cut through 25 β-strands. The β-sheet hydrogen bonding may provide much of the mechanical stability required for a fiber whose length of up to 4 microns approaches 600 times its diameter of 6 nm. It is generally believed that the pilus is assembled from its base, since there is no channel in the center of the fiber and one would be needed for assembly from the tip. Moreover, a pool of pilin is found in the cytoplasmic membrane, ready to drive assembly. No pool of pilin in the cytoplasm would be required for assembly from the tip, as found in the assembly of flagella from flagellin.

Based on the structure of the pilus fiber, on the genetic studies, and on the localization of pilus proteins, a model of the pilus and its assembly system is presented in Figure 15. This structural model is neutral with respect to theories of pilus retraction by disassembly and reassembly of the fiber as suggested by Bradley and others (Bradley, 1980).

In *conclusion*, swarming is observed only when the cell density is high. The similarities in spreading behavior between flagellar swarming and type IV pilus swarming could arise because both types of cells move in groups, both involve elongate cells which tend to move parallel to their long axis, and both types of cells maintain close contact with other cells.

Heterocyst Differentiation in Cyanobacteria

Cyanobacteria carry out photosynthesis as well as nitrogen fixation. In photosynthesis, atmospheric CO_2 is reduced using water as the primary electron donor and O_2 is generated. This generation of O_2 is a problem for nitrogen fixation because nitrogenase, the enzyme that catalyzes reduction of N_2, is sensitive to O_2. Perhaps, to solve this problem, cyanobacteria of the genus *Anabaena* differentiate specialized cells called heterocysts to house their nitrogenase. The heterocysts have thick walls, which prevent O_2 from penetrating; nitrogenase is thereby permitted to function inside. *Anabaena* forms chains of photosynthesizing vegetative cells, punctuated with an occasional heterocyst.

If *Anabaena* is grown in a medium that provides ample fixed nitrogen as NH_4^+, nitrogen

fixation is not required and no heterocysts are formed, only vegetative cells. But when cells that have been grown with ample NH_4^+ are washed, then resuspended in medium free of fixed nitrogen, they develop heterocysts during the next 24 hours. Since the vegetative generation time under these conditions is also about 24 hours, heterocyst differentiation can be thought of as growing a new cell with a different wall and a somewhat different set of enzymes. The RNA hybridization data suggest as many as 1,000 protein differences between vegetative cells and heterocysts (Buikema and Haselkorn, 1993).

When nitrogen fixation is needed, about one cell in 10 becomes a heterocyst. Nitrogen fixation and respiration in the heterocyst require a supply of reductant and of carbon from the adjacent, photosynthesizing, vegetative cells. Reductant and carbon are provided in the form of maltose, sucrose, or other disaccharides. In return, the heterocyst releases fixed nitrogen in the form of glutamine to its vegetative neighbors.

The intercellular exchange of metabolites illustrates the metabolic interdependence of vegetative cells and heterocysts. These cells are also developmentally interdependent in that the heterocysts differentiate at fairly regular spatial intervals (Wilcox et al., 1973). Chain-breaking experiments show that many more cells have the potential to become than normally become heterocysts (Wilcox et al., 1973). Because all the cells in a chain have received the same environmental cue, the implication is that some kind of local cell interaction underlies the regular spatial pattern of heterocysts. To appreciate this pattern, consider that all cells in a chain of vegetative cells grow and divide, causing the chain to elongate internally. To maintain a fixed ratio of heterocysts to vegetative cells, new heterocysts must differentiate in proportion to the new vegetative cells that form. Moreover, a regulatory system that appropriately selects particular vegetative cells to become heterocysts would seem to be necessary so that fixed nitrogen (glutamine) will be equally available to all the growing cells. Since the levels of hundreds of proteins differ between heterocysts and vegetative cells, the process needs coordination. It is hard to escape the inference that a cell interaction triggers one daughter cell to become a proheterocyst and its sister to remain a vegetative cell.

Each new heterocyst forms very near the center of a segment of vegetative cells. This maintains a stable 1/10 ratio and is clearly the most efficient way to exchange fixed carbon and fixed nitrogen between vegetative cells and heterocysts. But what are the cell interactions and how do they generate the pattern? A promising start to answering this question comes with the discovery of the gene *patS* (Yoon and Golden, 1998).

Overexpression of *patS* blocks heterocyst differentiation, while a *patS* null mutant has an increased frequency of heterocysts clustered abnormally along the chain of cells. This contrast of phenotypes suggests that *patS* encodes an inhibitor of heterocyst differentiation. Moreover, *patS* is expressed in proheterocysts but not in vegetative cells. The *patS* gene can encode a peptide of 17 amino acids, and a synthetic peptide corresponding to its C-terminal pentapeptide has the capacity to inhibit heterocyst development. Yoon and Golden propose that heterocysts synthesize and secrete a *patS* peptide that prevents neighboring vegetative cells from becoming heterocysts.

Sporulation of *Bacillus* Subtilis

Bacillus subtilis usually divides by binary fission, but when the cells are starved for carbon, nitrogen, or phosphorous they differentiate a new type of cell, an endospore. The spore can survive for years without food and retains an ability to germinate and to produce rapidly growing and dividing vegetative cells when food is again available. The developing cell undergoes a rigid sequence of morphological and biochemical changes over a period of about 8 hours as it makes the spore. A program involving the expression of more than 100 genes governs progression through the morphological and biochemical stages. The transcriptional program is controlled by the sequential activation of different subunits of RNA polymerase.

The earliest morphological event in that developmental program is a redirection of septum formation from its usual midcell position to a sub-polar one. An asymmetric septum partitions one copy of the chromosome to the larger mother cell and one copy to the smaller forespore. However, the two cells are held together by a common cell wall. These two cells, each with its own nucleus and cytoplasm, are in a position to interact with each other, and they do. Their interactions can be considered to couple gene expression to morphology. This example will focus on the best understood interaction between the two daughters as they codevelop. There is evidence for more interactions in the sporulation program as well (Kroos et al., 1999; Sonenshein, 1999; Stragier and Losick, 1996).

A different cascade of σ factors is active within each compartment; in the mother cell σ^E is replaced by σ^K, while in the developing spore σ^F is replaced by σ^G. Moreover all four of these sporulation-specific σ factors are inactive at the moment of their synthesis. The σ^E and σ^K factors are synthesized in the mother cell as inactive precursor proteins, while in the developing spore σ^F and σ^G are held in an inactive state by associ-

ation with inhibitory proteins. In particular, σ^K is synthesized as a larger but inactive precursor, pro-σ^K. Activation of σ^K in the mother cell occurs by proteolytic removal of its 20 amino acid N-terminal (Kroos et al., 1989). During sporulation, the activation of σ factors is carefully regulated. Pro-σ^K associates with both the mother cell membrane and the outermost membrane surrounding the forespore after engulfment. The processing enzyme, thought to be the product of *spoIVFB*, is also made in the mother cell and is located in the same membrane as pro-σ^K (Kroos et al., 1999). However, cleavage in the mother cell is not constitutive, but depends on a protein located in the inner membrane of the forespore, the spoIVB protein (Cutting et al., 1991). Concentration or timing changes of this protein, which is synthesized under control of σ^G in the forespore, changes the timing of pro-σ^K, which strongly supports a regulatory role for spoIVB in that processing (Stragier and Losick, 1996). Two other membrane proteins, believed to form a complex with it, block the activity of the spoIVFB protease. This complex is thought to be the target of the spoIVB protein from the forespore. This interaction relieves the inhibition of the protease, and pro-σ^K is processed in response to the forespore signal. Thus, during *B. subtilis* sporulation, the mother cell and forespore interact with each other to activate a stage-specific sigma factor.

Literature Cited

Achtman, M., G. Morelli, and S. Schwuchow. 1978. Cell-cell interactions in conjugating Escherichia coli: Role of F pili and fate of mating aggregates. J. Bacteriol. 135:1053–1061.

Adler, J. 1966. Chemotaxis in bacteria. Science 153:708–716.

Adler, J. 1969. Chemoreceptors in bacteria. Science 166:1588–1597.

Alberti, L., and R. M. Harshey. 1990. Differentiation of Serratia marcesens 274 into swimmer and swarmer cells. J. Bacteriol. 172:4322–4328.

Allison, C., H.-C. Lai, D. Gygi, and C. Hughes. 1993. Cell differentiation of Proteus mirabilis is initiated by glutamine, a specific chemoattractant for swarming cells. Molec. Microbiol. 8:53–60.

Atsumi, T., L. McCarter, and Y. Imae. 1992. Polar and lateral flagellar motors of marine Vibrio are driven by different ion-motive forces. Nature 355:182–182.

Baker, M. 1994. Myxococcus xanthus C-factor, a morphogenetic paracrine signal, is homologous to E. coli 3-ketoacyl-acyl carrier protein reductase and human 17 β-hydroxysteroid dehydrogenase. Biochem J. 301:311–312.

Barkai, N., and S. Leibler. 1997. Robustness in simple biochemical networks. Nature 387:913–917.

Bates, S., A. M. Cashmore, and B. M. Wilkins. 1998. IncP plasmids are unusually effective in mediating conjugation of Escherichia coli and Saccharomyces cerevisiae: Involvement of the Tra2 mating system. J. Bacteriol. 180:6538–6543.

Baumler, A. J. 1997. The record of horizontal gene transfer in Salmonella. Trends in Microbiology 5:318–322.

Bieber, D., S. W. Ramer, C.-Y. Wu, W. J. Murray, T. Tobe, and R. Fernandez. 1998. Type IV pili, transient bacterial aggregates and virulence in enteropathogenic Escherichia coli. Science 280:2114–2118.

Blackhart, B. D., and D. Zusman. 1985. The frizzy genes of Myxococcus xanthus control directional movement of gliding motility. Proc. Natl. Acad. Sci. USA 82:8767–8770.

Bonner, J. T. 1952. Morphogenesis, an essay on development. Princeton University Press. Princeton, NJ.

Bradley, D. E. 1980. A function of Pseudomonas aeruginosa PAO pili: Twitching motility. Can. J. Microbiol. 126:146–154.

Brenner, M. P., L. S. Levitov, and E. O. Budrene. 1998. Physical mechanisms for chemotactic pattern formation by bacteria. Biophys. J. 74:1677–1693.

Budrene, E. O., and H. C. Berg. 1995. Dynamics of formation of symmetrical patterns by chemotactic bacteria. Nature 376:49–53.

Buikema, W. J., and R. Haselkorn. 1993. Molecular genetics of cyanobacterial development. Ann. Rev. Plant Physiol. Plant Mol. Biol. 44:33–52.

Burkart, M., A. Toguchi, and R. M. Harshey. 1998. The chemotaxis system, but not chemotaxis, is essential for swarming motility in Escherichia coli. Proc. Natl. Acad. Sci. USA 95:2568–2573.

Cashel, M., D. R. Gentry, V. J. Hernandez, and D. Vinella. 1996. The stringent response. *In:* F. Neidhardt (Ed.) Escherichia coli and Salmonella. ASM Press. Washington DC.

Chang, B.-Y., and M. Dworkin. 1994. Isolated fibrils rescue cohesion and development in the Dsp mutant of Myxococcus xanthus. J. Bacteriol. 176:7190–7196.

Clewell, D. B. 1999. Sex pheromone systems in Enterococci. *In:* G. M. Dunny and S. C. Winans (Eds.) Cell-cell Signaling in Bacteria. ASM Press. Washington, DC. 47–65.

Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott. 1995. Microbial biofilms. Ann. Rev. Microbiol. 49:711–745.

Cutting, S., A. Driks, R. Schmidt, B. Kunkel, and R. Losick. 1991. Forespore-specific transcription of a gene in the signal transduction pathway that governs pro-σ^K processing in Bacillus subtilis. Genes Dev. 5:456–466.

Dana, J. R., and L. J. Shimkets. 1993. Regulation of cohesion-dependent cell interactions in Myxococcus xanthus. J. Bacteriol. 175:3636–3647.

Das, A. K., P. T. W. Cohen, and D. Barford. 1998. The structure of the tetratricopeptide repeats of protein phosphatase 5: Implications for TPR-mediated protein-protein interactions. EMBO Journal 17:1192–1199.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298.

Davis, J. M., J. Mayor, and L. Plamann. 1995. A missense mutation in rpoD results in an A-signaling defect in Myxococcus xanthus. Molec. Microbiol. 18:943–952.

Dubnau, D. 1991. Genetic competence in Bacillus subtilis. Microbiol. Rev. 55:395–424.

Dunny, G. M. 1990. Genetic functions and cell-cell interactions in the pheromone-inducible plasmid transfer system of Enterococcus faecalis. Molec. Microbiol. 4:689–696.

Durrenberger, M. B., W. Villiger, and T. Bachi. 1991. Conjugational junctions: morphology of specific contacts in conjugating Escherichia coli bacteria. J. Struct. Biol. 107:146–156.

Dworkin, M. 1973. Cell-cell interactions in the Myxobacteria. Symp. Soc. Gen. Microbiol. 23:125–147.

Dworkin, M. 1999. Fibrils as extracellular appendages of bacteria: their role in contact-mediated cell-cell interactions in Myxococcus xanthus. BioEssays 21:590–595.

Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheim. 1981. Structural identification of autoinducer of Photobacterium fischeri luciferase. Biochemistry 20:2444–2449.

Ellehauge, E., M. Norregaard-Madsen, and L. Søgaard-Anderson. 1998. The FruA signal transduction protein provides a checkpoint for the temporal coordination of intercellular signals in M. xanthus development. Molec. Microbiol. 30:807–813.

Engebrecht, J., K. H. Nealson, and M. Silverman. 1983. Bacterial bioluminescence: Isolation and genetic analysis of functions from Vibrio fischeri. Cell 32:773–781.

Engebrecht, J., and M. Silverman. 1986. Regulation of expression of bacterial genes for bioluminescence. Genet. Eng. 8:31–44.

Farrand, S. K. 1993. Conjugal transfer of Agrobacterium plasmids. *In:* D. B. Clewell (Ed.) Bacterial Conjugation. Plenum Press. New York, NY.

Fontes, M., and D. Kaiser. 1999. Myxococcus cells respond to elastic forces in their substrate. Proc. Natl. Acad. Sci. USA 96:8052–8057.

Forest, K. T., and J. A. Tainer. 1997. Type IV pilus structure: outside to inside and top to bottom—a minireview. Gene 192:165–169.

Frey, J., and M. Bagdasarian. 1989. The molecular biology of IncQ plasmids. *In:* C. M. Thomas (Ed.) Promiscuous plasmids of Gram-negative bacteria. Academic Press. New York, NY. 79–94.

Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. 176:269–275.

Furness, R. B., G. M. Fraser, N. A. Hay, and C. Hughes. 1997. Negative feedback from a Proteus class II flagellum export defect to the flhDC master operon controlling cell division and flagellum assembly. J. Bacteriol. 179:5585–5588.

Galli, D., R. Wirth, and G. Wanner. 1989. Identification of aggregation substances of Enterococcus faecalis cells after induction by sex pheromones. Arch. Microbiol. 151:486–490.

Galli, D., and R. Wirth. 1991. Comparative analysis of Enterococcus faecalis sex pheromone plasmids identifies a single homologous DNA region which codes for aggregation substance. J. Bacteriol. 173:3029–3033.

Gorski, L., and D. Kaiser. 1998. Targeted mutagenesis of sigma-54 activator proteins in Myxococcus xanthus. J. Bacteriol. 180:5896–5905.

Groisman, E. A., and H. Ochman. 1997. How Salmonella became a pathogen. Trends Microbiol. 5:343–349.

Hagen, D. C., A. P. Bretscher, and D. Kaiser. 1978. Synergism between morphogenetic mutants of Myxococcus xanthus. Dev. Biol. 64:284–296.

Harris, B. Z., D. Kaiser, and M. Singer. 1998. The guanosine nucleotide (p)ppGpp initiates development and A-factor production in Myxococcus xanthus. Genes Dev. 12:1022–1035.

Harshey, R. M. 1994. Bees aren't the only ones: Swarming in Gram-negative bacteria. Molec. Microbiol. 13:389–394.

Harshey, R. M., and T. Matsuyama. 1994. Dimorphic transition in E. coli and S. typhimurium: Surface-induced differentiation into hyperflagellate swarmer cells. Proc. Natl. Acad. Sci. USA 91:8631–8634.

Hartl, D. L., A. R. Lohe, and E. R. Lozovskaya. 1997. Modern thoughts on an ancyent marinere: Function, evolution, regulation. Ann. Rev. Genet. 31:337–358.

Hartzell, P. L. 1997. Complementation of sporulation and motility defects in a prokaryote by a eukaryotic GTPase. Proc. Natl. Acad. Sci. USA 94:9881–9886.

Hastings, J. W., and E. P. Greenberg. 1999. Quorum sensing: The explanation of a curious phenomenon reveals a common characteristic of bacteria. J. Bacteriol. 181:2667–2668.

Havarstein, L. S., and D. A. Morrison. 1999. Quorum sensing and peptide pheromones in streptococcal competence for genetic transformation. *In:* G. M. Dunny, and S. C. Winans (Eds.) Cell-cell Signaling in Bacteria. ASM Press. Washington DC, 9–26.

Hay, N. A., D. J. Tipper, D. Gygi, and C. Hughes. 1997. A nonswarming mutant of Proteus mirabilis lacks the Lrp global transcriptional regulator. J. Bacteriol. 179:4741–4746.

Heineman, J. A., and G. F. Sprague. 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nature 340:205–209.

Henrichsen, J. 1972. Bacterial surface translocation: a survey and a classification. Bacteriol. Rev. 36:478–503.

Henrichsen, J. 1975. Twitching motility and its mechanism. Acta Path. Microbiol. Scand. B83:187–190.

Henrichsen, J. 1983. Twitching motility. Ann. Rev. Microbiol. 37:81–93.

Herriott, R. M., E. M. Meyer, and M. Vogt. 1970. Defined nongrowth media for stage II development of competence in Haemophilus influenzae. J. Bacteriol. 101:517–525.

Herzer, P. J., S. Inouye, M. Inouye, and T. S. Whittam. 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of Escherichia coli. J. Bacteriol. 172:6175–6181.

Hoch, J. A., and T. J. Silhavy. 1995. Two component signal transduction. ASM Press, Washington DC, 1–504.

Hodgkin, J., and D. Kaiser. 1977. Cell-to-cell stimulation of movement in nonmotile mutants of Myxococcus. Proc. Natl. Acad. Sci. USA 74:2938–2942.

Hodgkin, J., and D. Kaiser. 1979. Genetics of Gliding Motility in M. xanthus (Myxobacterales): Genes Controlling Movement of Single Cells. Mol. Gen. Genet. 171:167–176.

Hodgkin, J., and D. Kaiser. 1979. Genetics of gliding motility in M. xanthus (Myxobacterales): Two gene systems control movement. Mol. Gen. Genet. 171:177–191.

Hong, S.-B., I. Hwang, Y. Dessaux, P. Guyon, K.-S. Kim, and S. K. Farra. 1997. A T-DNA gene required for agropine biosynthesis by transformed plants is functionally and evolutionarily related to a Ti plasmid gene required for catabolism of agropine by Agrobacterium strains. J. Bacteriol. 179:4831–4840.

Hwang, I., P. L. Li, L. Zhang, K. R. Piper, D. M. Cook, M. E. Tate, and S. K. Farra. 1994. TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid N-acylhomoserine lactone autoinducer. Proc. Natl. Acad. Sci. USA 91:4639–4643.

Inouye, M., S. Inouye, and D. Zusman. 1979. Gene expression during development of Myxococcus xanthus: Pattern of protein synthesis. Devel. Biol. 68:579–591.

Jelsbak, L., and L. Søgaard-Andersen. 1999. The cell-surface associated C-signal induces behavioral changes in individual M. xanthus cells during fruiting body morphogenesis. Proc. Natl. Acad. Sci. USA 96:5031–5036.

Kado, C. 1998. Agrobacterium-mediated horizontal gene transfer. *In:* J. K. Setlow (Ed.) Genetic Engineering. Plenum Press. New York, NY.

Kahn, M. E., F. Barany, and H. O. Smith. 1983. Transformasomes: Specialized membranous structures that protect DNA during Haemophilus transformation. Proc. Natl. Acad. Sci. USA 80:6927–6931.

Kaiser, D. 1979. Social gliding is correlated with the presence of pili in Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 76:5952–5956.

Kaiser, D., and C. Crosby. 1983. Cell movement and its coordination in swarms of Myxococcus xanthus. Cell Motility 3:227–245.

Kaiser, D. 1996. Bacteria also vote. Science 272:1598–1599.

Kaplan, H. B., and E. P. Greenberg. 1985. Diffusion of autoinducer is involved in regulation of the Vibrio fischeri luminescence system. J. Bacteriol. 163:1210–1214.

Kaplan, H. B., and E. P. Greenberg. 1987. Overproduction and purification of the luxR gene product: The transcriptional activation of the Vibrio fischeri luminescence system. Proc. Natl. Acad. Sci. USA 84:6639–6643.

Kawagishi, I., M. Imagawa, Y. Imae, L. McCarter, and M. Homma. 1996. The sodium-driven polar flagellar motor of marine Vibrio as the mechanosensor that regulates lateral flagellar expression. Molec. Microbiol. 20:693–699.

Keseler, I. M., and D. Kaiser. 1995. An early A-signal-dependent gene in Myxococcus xanthus has a sigma-54-like promoter. J. Bacteriol. 177:4638–4644.

Kim, S. K., and D. Kaiser. 1990. C-factor: A cell-cell signaling protein required for fruiting body morphogenesis of M. xanthus. Cell 61:19–26.

Kim, S. K., and D. Kaiser. 1990. Cell alignment required in differentiation of Myxococcus xanthus. Science 249:926–928.

Kim, S. K., and D. Kaiser. 1990. Cell motility is required for the transmission of C-factor, an intercellular signal that coordinates fruiting body morphogenesis of Myxococcus xanthus. Genes Dev. 4:896–905.

Kim, S. K., and D. Kaiser. 1990. Purification and properties of Myxococcus xanthus C-factor, an intercellular signaling protein. Proc. Natl. Acad. Sci. USA 87:3635–3639.

Kim, S. K., and D. Kaiser. 1991. C-factor has distinct aggregation and sporulation thresholds during Myxococcus development. J. Bacteriol. 173:1722–1728.

Kimsey, H. H., and D. Kaiser. 1991. Targeted disruption of the Myxococcus xanthus orotidine 5'-monophosphate decarboxylase gene: Effects on growth and fruiting-body development. J. Bacteriol. 173:6790–6797.

Kingsman, A., and N. Willetts. 1978. The requirements for conjugal DNA synthesis in the donor strain during Flac transfer. J. Molec. Biol. 122:287–300.

Knutton, S., R. K. Shaw, R. P. Anantha, M. S. Donnenberg, and A. A. Zorgani. 1999. The type IV bundle-forming pilus of enteropathogenic Escherichia coli undergoes dramatic alterations in structure associated with bacterial adherence, aggregation and dispersal. Molec. Microbiol. 33:499–509.

Kroos, L., A. Kuspa, and D. Kaiser. 1986. A global analysis of developmentally regulated genes in Myxococcus xanthus. Dev. Biol. 117:252–266.

Kroos, L., P. Hartzell, K. Stephens, and D. Kaiser. 1988. A link between cell movement and gene expression argues that motility is required for cell-cell signaling during fruiting body development. Genes Dev. 2:1677–1685.

Kroos, L., B. Kunkel, and R. Losick. 1989. Switch protein alters specificity of RNA polymerase containing a compartment-specific sigma factor. Science 243:526–529.

Kroos, L., B. Zhang, H. Ichikawa, and U.-T. N. Yu. 1999. Control of s factor activity during Bacillus subtilis sporulation. Molec. Microbiol. 31:1285–1294.

Kuhlwein, H., and H. Reichenbach. 1968. Swarming and morphogenesis in Myxobacteria. Film C893/1965:Inst. Wiss. Film. Göttingen.

Kuner, J., and D. Kaiser. 1982. Fruiting body morphogenesis in submerged cultures of Myxococcus xanthus. J. Bacteriol. 151:458–461.

Kuspa, A., L. Kroos, and D. Kaiser. 1986. Intercellular signaling is required for developmental gene expression in Myxococcus xanthus. Dev. Biol. 117:267–276.

Kuspa, A., L. Plamann, and D. Kaiser. 1992. Identification of heat-stable A-factor from Myxococcus xanthus. J. Bacteriol. 174:3319–3326.

Kuspa, A., L. Plamann, and D. Kaiser. 1992. A-signaling and the cell density requirement for Myxococcus xanthus development. J. Bacteriol. 174:7360–7369.

Lampson, B. C. 1993. Retron elements of the Myxobacteria. *In:* M. Dworkin, and D. Kaiser (Eds.) Myxobacteria. II:ASM Press. Washington DC, 109–128.

Lazazzera, B. A., T. Palmer, J. Quisel, and A. D. Grossman. 1999. Cell density control of gene expression and development in Bacillus subtilis. *In:* G. M. Dunny, and S. C. Winans (Eds.) Cell-cell Signaling in Bacteria. ASM Press. Washington DC, 27–46.

Lee, K., and L. J. Shimkets. 1994. Cloning and characterization of the socA locus which restores development to Myxococcus xanthus C-signaling mutants. J. Bacteriol. 176:2200–2209.

Lee, B.-U., K. Lee, J. Mendez, and L. J. Shimkets. 1995. A tactile sensory system of Myxococcus xanthus involves an extracellular NAD(P)+-containing protein. Genes Dev. 9:2964–2973.

Li, S., B. U. Lee, and L. Shimkets. 1992. csgA expression entrains Myxococcus xanthus development. Genes and Dev. 6:401–410.

Li, Y., and L. Plamann. 1996. Purification and phosphorylation of Myxococcus xanthus AsgA protein. J. Bacteriol. 178:289–292.

Lindberg, A. A. 1973. Bacteriophage receptors. Ann. Rev. Microbiol. 27:205–241.

Long, S. R., and B. J. Staskawicz. 1993. Prokaryotic plant parasites. Cell 73:921–935.

Macnab, R. M. 1987. Motility and Chemotaxis. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella typhimurium: Cellular and molecular biology. ASM Press. Washington DC, 732–759.

Macnab, R. M. 1996. Flagella and motility. *In:* F. C. Neidhardt (Ed.) Escherichia coli and Salmonella typhimurium: Cellular and molecular biology. ASM Press. Washington DC, 123–145.

Manning, P. A., and T. F. Meyer. 1997. Type-4 pili: biogenesis, adhesins, protein export and DNA import. Gene 192:1–198.

Manoil, C., and D. Kaiser. 1980. Accumulation of guanosine tetraphosphate and guanosine pentaphosphate in Myxococcus xanthus during starvation and myxospore formation. J. Bacteriol. 141:297–304.

Manoil, C., and J. Rosenbusch. 1982. Conjugation-deficient mutants of Escherichia coli distinguish classes of functions of the outer membrane OmpA protein. Mol. Gen. Genet. 187:148–156.

Mattick, J. S., C. B. Whitchurch, and R. A. Alm. 1996. The molecular genetics of type-4 fimbriae in Pseudomonas aeruginosa—a review. Gene 179:147–155.

Mazodier, P., and J. Davies. 1991. Gene transfer between distantly related bacteria. Ann. Rev. Genet. 25:147–171.

McBride, M. J., R. A. Weinberg, and D. R. Zusman. 1989. Frizzy aggregation genes of the gliding bacterium Myxococcus xanthus show sequence similarities to the chemotaxis genes of enteric bacteria. Proc. Natl. Acad. Sci. USA 86:424–428.

McCarter, L., M. Hilmen, and M. Silverman. 1988. Flagellar dynamometer controls swarmer cell differentiation of V. parahaemolyticus. Cell 54:345–351.

Meighen, E. A. 1994. Genetics of bacterial bioluminescence. Ann. Rev. Genet. 28:117–139.

Mergaert, P., M. V. Montagu, and M. Holsters. 1997. Molecular mechanisms of Nod factor diversity. Molec. Microbiol. 25:811–817.

Mori, M., Y. Sakagami, Y. Ishii, A. Isogai, C. Kitada, M. Fujino, J. C. Adsit, G. M. Dunny, and A. Suzu. 1988. Structure of cCF10, a peptide sex pheromone which induces conjugative transfer of the Streptococcus faecalis tetracycline resistance plasmid, pCF10. J. Biol. Chem. 263:14574–14578.

Murooka, Y., and T. Harada. 1979. Expansion of the host range of coliphage P1 and gene transfer from enteric bacteria to other Gram-negative bacteria. J. Appl. Environ. Microbiol. 38:754–757.

Nealson, K. H., and J. W. Hastings. 1979. Bacterial bioluminescence: its control and ecological significance. Microbiol. Rev. 43:496–518.

O'Toole, G. A., and R. Kolter. 1998. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis. Molec. Microbiol. 28:449–461.

Ogawa, M., S. Fujitani, X. Mao, S. Inouye, and T. Komano. 1996. FruA, a putative transcription factor essential for the development of Myxococcus xanthus. Molec. Microbiol. 22:757–767.

Ottow, J. C. G. 1975. Ecology, physiology, and genetics of fimbriae and pili. Ann. Rev. Microbiol. 29:79–108.

Parge, H. E., K. T. Forest, M. J. Hickey, D. A. Christensen, E. D. Getzoff, and J. A. Tainer. 1995. Structure of the fibre-forming protein pilin at 2.6Å resolution. Nature 378:32–38.

Piper, K. R., S. Beck von Bodman, and S. K. Farrand. 1993. Conjugation factor of Agrobacterium tumefaciens regulates Ti plasmid transfer by autoinduction. Nature 362:448–450.

Plamann, L., A. Kuspa, and D. Kaiser. 1992. Proteins that rescue A-signal-defective mutants of Myxococcus xanthus. J. Bacteriol. 174:3311–3318.

Plamann, L., J. M. Davis, B. Cantwell, and J. Mayor. 1994. Evidence that asgB encodes a DNA-binding protein essential for growth and development of Myxococcus xanthus. J. Bacteriol. 176:2013–2020.

Plamann, L., and H. B. Kaplan. 1999. Cell-density sensing during early development in Myxococcus xanthus. *In:* G. M. Dunny, and S. C. Winans (Eds.) Cell-cell Signaling in Bacteria. ASM Press. Washington DC, 67–82.

Pruss, B. M., and P. Matsumura. 1996. A regulator of the flagellar regulon of Escherichia coli, flhD, also affects cell division. J. Bacteriol. 178:668–674.

Qualls, G. T., K. Stephens, and D. White. 1978. Morphogenetic movements and multicellular development in the fruiting Myxobacterium, Stigmatella aurantiaca. Dev. Biol. 66:270–274.

Quinlan, M. S., and K. B. Raper. 1965. Development of the myxobacteria. Hdb. Pflanzenphysiol. 15:596–611.

Ramaswamy, S., M. Dworkin, and J. Downard. 1997. Identification and characterization of Myxococcus xanthus mutants deficient in calcofluor white binding. J. Bacteriol. 179:2863–2871.

Reichenbach, H., and M. Dworkin. 1981. The order Myxobacterales. *In:* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes. Springer-Verlag. Berlin, 328–355.

Reichenbach, H. 1984. Myxobacteria: A most peculiar group of social prokaryotes. *In:* E. Rosenberg (Ed.) Myxobacteria. Springer-Verlag. New York, NY. 1–50.

Reichenbach, H. 1993. Biology of the Myxobacteria: Ecology and taxonomy. *In:* M. Dworkin, and D. Kaiser (Eds.) Myxobacteria. II:ASM Press. Washington DC, 13–62.

Rice, S. A., and B. C. Lampson. 1995. Phylogenetic comparison of retron elements among the myxobacteria: Evidence for vertical inheritance. J. Bacteriol. 177:37–45.

Rodriguez, A., and A. Spormann. 1999. Genetic and molecular analysis of cglB, a gene essential for single cell gliding in Myxococcus xanthus. J. Bacteriol. 181:4381–4390.

Rodriguez-Soto, J. P., and D. Kaiser. 1997. Identification and localization of the tgl protein, which is required for Myxococcus xanthus social motility. J. Bacteriol. 179:4372–4381.

Rodriguez-Soto, J. P., and D. Kaiser. 1997. The tgl gene: social motility and stimulation in Myxococcus xanthus. J. Bacteriol. 179:4361–4371.

Rosenberg, E., K. Keller, and M. Dworkin. 1977. Cell-density dependent growth of Myxococcus xanthus on casein. J. Bacteriol. 129:770–777.

Rosenbluh, A., and M. Eisenbach. 1992. The effect of mechanical removal of pili on gliding motility in Myxococcus xanthus. J. Bacteriol. 174:5406–5413.

Ruby, E. G., and M. J. McFall-Ngai. 1992. A squid that glows in the night: Development of an animal-bacterial mutualism. J. Bacteriol. 174:4865–4870.

Russo-Marie, F., M. Roederer, B. Sager, L. A. Herzenberg, and D. Kaiser. 1993. β-galactosidase activity in single differentiating bacterial cells. Proc. Natl. Acad. Sci. USA 90:8194–8198.

Sager, B., and D. Kaiser. 1994. Intercellular C-signaling and the traveling waves of Myxococcus. Genes Dev. 8:2793–2804.

Schwedock, J. S., C. Liu, and T. S. Leyh. 1994. Rhizobium meliloti nodP and nodQ form a multifunctional sulfate-

activating complex requiring GTP for activity. J. Bacteriol. 176:7055–7064.
Shadel, G., J. H. Devine, and T. O. Baldwin. 1990. Control of the lux regulon of Vibrio fischeri. J. Biolumin. Chemilumin. 5:99–106.
Shimkets, L. J., and H. Rafiee. 1990. CsgA, an extracellular protein essential for Myxococcus xanthus development. J. Bacteriol. 172:5299–5306.
Shimkets, L., and C. R. Woese. 1992. A phylogenetic analysis of the myxobacteria: Basis for their classification. Proc. Natl. Acad. Sci. USA 89:9459–9463.
Shinoda, S., and K. Okamoto. 1977. Formation and function of Vibrio parahaemolyticus lateral flagella. J. Bacteriol. 129:1266–1271.
Singer, M., and D. Kaiser. 1995. Ectopic production of guanosine penta-and tetra-phosphate can initiate early developmental gene expression in Myxococcus xanthus. Genes Dev. 9:1633–1644.
Smith, M. W., D.-F. Feng, and R. F. Doolittle. 1992. Evolution by acquisition: the case for horizontal gene transfers. Trends Biochem. Sci. 17:489–493.
Søgaard-Anderson, L., and D. Kaiser. 1996. C-factor, a cell-surface-associated intercellular signaling protein, stimulates the cytoplasmic Frz signal transduction system in Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 93:2675–2679.
Søgaard-Anderson, L., F. Slack, H. Kimsey, and D. Kaiser. 1996. Intercellular C-signaling in Myxococcus xanthus involves a branched signal transduction pathway. Genes Dev. 10:740–754.
Sonenshein, A. L. 1999. Endospore-forming bacteria: An overview. *In:* Y. Brun, and L. Shimkets (Eds.) Prokaryotic Development. ASM Press, Washington DC, 1–475.
Spormann, A., and D. Kaiser. 1999. Gliding mutants of Myxococcus xanthus with high reversal frequencies and small displacements. J. Bacteriol. 181:2593–2601.
Stedman, K. M., C. Schleper, E. Rumpf, and W. Zillig. 1999. Genetic requirements for the function of the archaeal virus SSV1 in Sulfolobus solfataricus: construction and testing of viral shuttle vectors. Genetics 152:1397–1405.
Stevens, A. M., K. M. Dolan, and E. P. Greenberg. 1994. Synergistic binding of the Vibrio fischeri LuxR transcriptional activator domain and RNA polymerase to the lux promoter region. Proc. Natl. Acad. Sci. USA 91:12619–12623.
Stevens, A. M., and E. P. Greenberg. 1999. Transcriptional activation by LuxR. *In:* G. M. Dunny, and S. C. Winans (Eds.) Cell-cell Signaling in Bacteria. ASM Press. Washington DC, 231–242.
Stewart, B. J., J. L. Enos-Berlage, and L. L. McCarter. 1997. The lonS gene regulates swarmer cell differentiation of Vibrio parahaemolyticus. J. Bacteriol. 179:107–114.
Stragier, P., and R. Losick. 1996. Molecular genetics of sporulation in Bacillus subtilis. Ann. Rev. Genet. 30:297–341.
Strom, M. S., and S. Lory. 1993. Structure-function and biogenesis of the type IV pili. Ann. Rev. Microbiol. 47:565–596.
Thaxter, R. 1892. On the Myxobacteriaceae, a new order of Schizomycetes. Bot. Gaz. 17:389–406.
Thony-Meyer, L., and D. Kaiser. 1993. devRS, an autoregulated and essential genetic locus for fruiting body development in Myxococcus xanthus. J. Bacteriol. 175: 7450–7462.
Toal, D. R., S. W. Clifton, B. A. Roe, and J. Downard. 1995. The esg locus of Myxococcus xanthus encodes the E1a and E1b subunits of a branched-chain keto acid dehydrogenase. Molec. Microbiol. 16:177–189.
Velicer, G., L. Kroos, and R. E. Lenski. 1998. Loss of social behaviors by Myxococcus xanthus during evolution in an unstructured habitat. Proc. Natl. Acad. Sci. USA 95:12376–12380.
Wall, D., and D. Kaiser. 1998. Alignment enhances the cell-to-cell transfer of pilus phenotype. Proc. Natl. Acad. Sci. USA 95:3054–3058.
Wall, D., and D. Kaiser. 1999. Type IV pili and cell motility. Molec. Microbiol. 32:1–10.
Wall, D., P. E. Kolenbrander, and D. Kaiser. 1999. The Myxococcus xanthus pilQ (sglA) gene encodes a secretin homolog required for type IV pili biogenesis, S motility and development. J. Bacteriol. 181:24–33.
Wedel, A., and S. Kustu. 1995. The bacterial enhancer-binding protein NTRC is a molecular machine: ATP hydrolysis is coupled to transcriptional activation. Genes Dev. 9:2042–2052.
Weimer, R. M., C. Creighton, A. Stassinopoulos, P. Youderian, and P. L. Hartze. 1998. A chaperone in the HSP70 family controls production of extracellular fibrils in Myxococcus xanthus. J. Bacteriol. 180:5357–5368.
White, D. 1993. Myxospore and fruiting body morphogenesis. *In:* M. Dworkin, and D. Kaiser (Eds.) Myxobacteria. II:ASM Press. Washington DC, 307–332.
Whitman, W. B., F. Pfeifer, P. Blum, and A. Klein. 1999. What Archaea have to tell biologists. Genetics 152:1245–1248.
Whittaker, R. H. 1969. New concepts of kingdoms of organisms. Science 163:150–160.
Wilcox, M., G. J. Mitchison, and R. J. Smith. 1973. Pattern formation in the blue-green alga, Anabaena. J. Cell. Sci. 12:707–723.
Winans, S. C., J. Zhu, and M. I. More. 1999. Cell density-dependent gene expression by Agrobacterium tumefaciens during colonization of crown gall tumors. *In:* G. Dunny, and S. C. Winans (Eds.) Cell-cell Signaling in Bacteria. ASM Press. Washington DC, 117–128.
Wolfe, A. J., and H. C. Berg. 1989. Migration of bacteria in semisolid agar. Proc. Natl. Acad. Sci. USA 86:6973–6977.
Wu, S. S., and D. Kaiser. 1995. Genetic and functional evidence that Type IV pili are required for social gliding motility in Myxococcus xanthus. Molec. Microbiol. 18:547–558.
Wu, S. S., and D. Kaiser. 1997. Regulation of expression of the pilA gene in Myxococcus xanthus. J. Bacteriol. 179:7748–7758.
Wu, S. S., J. Wu, and D. Kaiser. 1997. The Myxococcus xanthus pilT locus is required for social gliding motility although pili are still produced. Molec. Microbiol. 23:109–121.
Wu, S. S., J. Wu, Y. L. Cheng, and D. Kaiser. 1998. The pilH gene encodes an ABC transporter homologue required for type IV pilus biogenesis and social motility in Myxococcus xanthus. Molec. Microbiol. 29:1249–1261.
Yoon, H. S., and J. W. Golden. 1998. Heterocyst pattern formation controlled by a diffusible peptide. Science 282:935–938.
Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate. 1993. Agrobacterium conjugation and gene regulation by N-acyl-L-homoserine lactones. Nature 362:446–448.

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CHAPTER 1.9

Prokaryotic Genomics

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Introduction

Haemophilus influenzae strain Rd became the first free-living organism to have its genome sequenced (Fleischmann et al., 1995). The floodgates have opened with over 100 prokaryotic genomes completely or partially sequenced. However, the acquisition and analysis of sequence data is not an end in itself; instead it is a starting point for generating hypotheses that can be tested in the laboratory. It is clear that knowledge of the complete genome sequence of an organism does not tell us a great deal about the composition or functional capabilities of the organism. Homology, or sequence similarity, provides clues, but it does not prove gene function. Furthermore, a large percentage of genes have no matches to known genes. For example, at the time of sequence release, up to 62% of predicted protein-coding genes in the *Methanococcus jannaschii* genome had no matches with genes from other organisms (Bult et al., 1996). Elucidating the function of these "ORFan" or "FUN" (function unknown) genes is one of the biggest challenges of the post-genomic era.

The avalanche of genome sequence data has coincided with important technological advances in four research areas: bioinformatics, gene mutagenesis, nucleic acid hybridization technology and protein chemistry. These advances will liberate scientific understanding from the piecemeal study of individual genes or operons towards a comprehensive analysis of the entire gene and protein complement of the prokaryotic cell. This new technology will allow a holistic approach to the functional characterization of prokaryotes at the mutational, transcriptional, and protein expression levels (see Fig. 1). The application of functional genomic approaches in the smaller genomes of prokaryotes is the forerunner for the study of functional genomics in higher organisms, including humans. An important exception is the efforts of the *Saccharomyces cerevisiae* research community, which is a shining example of what can be achieved through functional genomics studies (Lashkari et al., 1997; Winzeler et al., 1998; Winzeler et al., 1999a; Winzeler et al., 1999b; Uetz et al., 2000).

Prokaryotic Genome Projects and the Birth of Comparative Genomics

The availability of genome sequences has spawned the new scientific discipline of comparative genomics, which allows the comparison of genome sequence data between strains, species, genera and even kingdoms. Such studies will provide important taxonomic insights and will have far-reaching implications for the study of evolution. The virtual genome center is a useful web-based site (alces.med.umn.edu/VGC.html) for evolutionary comparisons of proteins, protein families, and genome sequences. In the future, comparative analysis of genome sequence data will be facilitated by high-density array DNA hybridization analysis (see section on Applications of High-Density DNA Arrays and Genomotyping in this Chapter).

The salient features of prokaryotic genome sequences are summarized in chronological order of publication. First, *Haemophilus influenzae* strain KW20 (Rd; 1.83 Mb; Fleischmann et al., 1995) was sequenced using the now widely adopted whole genome, random shotgun, sequencing approach. The full complement of genes enabled the deciphering of the polysaccharide structure of the organism. Several iterative sequences (dinucleotide and tetranucleotide) repeats were identified suggesting *H. influenzae* probably uses recombination and slipped-strand mispairing within repeats as a mechanism for antigenic/phase variation and adaptive evolution (Hood et al., 1996).

Mycoplasma genitalium (0.58 Mb; Fraser et al., 1995) has the smallest known genome content of any free-living organism. As a consequence its physiology and metabolic capacity differ from that of most living organisms. *Mycoplasma genitalium* represents an important system for determination of the minimal number of genes required for host-independent existence (Hutchison et al., 1999).

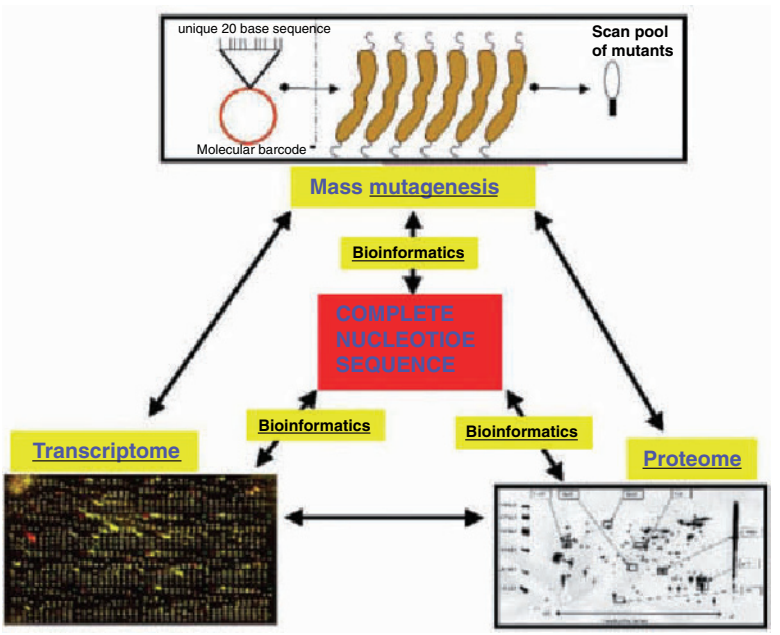

Fig. 1. Schematic showing available complementary functional genomics approaches.

Methanococcus jannaschii (1.66 Mb; Bult et al., 1996) was the first archaeon to be sequenced. In *M. jannaschii* the majority of genes related to cell division, energy production and metabolism are more similar to those found in bacteria; by contrast, most of the genes involved in transcription, translation and replication are more similar to those found in eukaryotes.

Synechocystis sp. strain PCC6803 (3.57 Mb; Kaneko et al., 1996) is a photosynthetic bacterium where 5% of identified open reading frames (ORFs) were dedicated to photosynthetic reactions and 99 ORFs showed similarity to transposase genes, suggesting frequent rearrangement of the genome.

The sequencing of *Mycoplasma pneumoniae* M129 (0.81 Mb; Himmelreich et al., 1996) was the first occasion that two organisms within the same genera were sequenced. A subset of essential genes was identified in both *Mycoplasma* species. Anabolic and metabolic pathways were absent, which is consistent with its obligate parasitic lifestyle.

Sequence analysis of *Helicobacter pylori*, strain 26695 (1.67 Mb; Tomb et al., 1997), demonstrated that a surprisingly large proportion of the genome content was dedicated to DNA restriction modification, motility and sequestration of iron. Similarly, several adhesins and outer-membrane proteins were identified suggesting a complex host-pathogen life style. Surprisingly, few regulatory sequences and σ factors were identified, which is consistent with the restricted ecological niche of the human stomach in which the pathogen resides.

The *Escherichia coli* K-12 (4.64 Mb; Blattner et al., 1997) genome appeared highly organized and contains insertion sequence (IS) elements and phage remnants, indicating genome plasticity through horizontal transfer.

The *Methanobacterium thermoautotrophicum* strain delta H (1.75 Mb; Smith et al., 1997) sequence predicted that most of the proteins involved in DNA metabolism, transcription and translation were of eukaryotic origin, whereas gene structure and organization have features that are typical of bacteria. Comparisons with the *M. jannaschii* genome underline the extensive divergence that has occurred between these two *Methanogens*.

Bacillus subtilis (4.21 Mb; Kunst, 1997) was the first Gram-positive organism to be sequenced. A quarter of the genome corresponds to several gene families that have been greatly expanded by gene duplication, and a significant proportion of the genetic capacity is devoted to the utilization of a variety of carbon sources. The genome contains at least ten prophages suggesting that bacteriophage infection has played an important evolutionary role in horizontal gene transfer.

Archaeoglobus fulgidus VC-16 (2.18 Mb; Klenk et al., 1997) was the first sulfur-metabolizing organism to have its genome sequence determined. A quarter of the genome encodes novel proteins indicating substantial archaeal gene diversity.

Borrelia burgdorferi B31 (1.44 Mb; Fraser et al., 1997) appears unique among prokaryotes as its genome contains a linear chromosome (0.91 Mb) and at least 17 linear and circular plas-

mids (totaling 0.53 Mb). The biological significance of the multiple plasmid-encoded genes is not clear, although it is postulated that they may be involved in antigenic variation or immune evasion.

The complex metabolic machinery needed for *Aquifex aeolicus* (1.55 Mb; Deckert et al., 1998) to function as a chemolithoautotroph is encoded within a genome a third the size of the *E. coli* genome. Metabolic flexibility seems to be reduced as a result of the limited genome size. Although *A. aeolicus* grows at 95°C, the extreme thermal limit of the bacteria, only a few specific indications of thermophily are apparent from the genome.

Pyrococcus horikoshii OT3 (1.74 Mb; Kawarabayasi et al., 1998) is a hyperthermophilic archaebacterium whose genome sequence provided evidence that a considerable number of ORFs were generated by sequence duplication. Eleven ORFs were assumed to contain inteins.

Mycobacterium tuberculosis strain H37Rv, (4.41 Mb; Cole et al., 1998) has a very high G+C content that is reflected in the biased amino acid content of the proteins. *Mycobacterium tuberculosis* differs from other bacteria in that much of its coding capacity is devoted to lipogenesis and lipolysis. Two novel families of glycine-rich proteins are present that have a repetitive structure, which may represent a source of antigenic variation.

In *Treponema pallidum* (1.13 Mb; Fraser et al., 1998), the systems for DNA replication, transcription, translation and repair are intact, but catabolic and biosynthetic activities are minimized. Comparison of the *T. pallidum* genome sequence with that of another pathogenic spirochete, *B. burgdorferi*, identified both unique and common genes and confirms the considerable diversity observed among pathogenic spirochetes.

Although the obligate intracellular pathogen *Chlamydia trachomatis* (1.04 Mb; Stephens et al., 1998) lacks many biosynthetic capabilities, it retains functions for the interconversion of metabolites obtained from their mammalian host cells. The apparent wide origin of chlamydial genes, including a large number of genes of eukaryote origin, implies a complex evolution for adaptation to obligate intracellular parasitic status.

The sequence of *Rickettsia prowazekii* (1.11 Mb; Andersson et al., 1998) revealed surprising similarity to mitochondrial genes. For example, ATP production in *Rickettsia* is the same as that in mitochondria. The *R. prowazekii* genome contains the highest proportion of noncoding DNA (24%) for a prokaryote, and *R. prowazekii* more closely related to mitochondria than any other microbe studied to date.

The sequence of *Helicobacter pylori* strain J99 (Alm et al., 1999) allowed the first genome comparison of two strains from the same species. Prior to the availability of the second sequence, *H. pylori* was thought to exhibit a large degree of genomic and allelic diversity, but the overall genomic organization and gene order appeared quite similar. Between 6 to 7% of the genes are specific to each strain. Almost half of these genes are clustered in a single hypervariable region, termed "a plasticity zone."

Analysis of the *Chlamydia pneumoniae* (1.23 Mb; Kalman et al., 1999) genome revealed 214 protein-coding sequences not found in *C. trachomatis*, many without similarity to other known sequences. Significant comparative findings included conservation of a type-III secretion virulence system, expansion of a novel family of outer-membrane proteins, and three serine/threonine protein kinases.

Aeropyrum pernix K1 (1.70 Mb; Kawarabayasi et al., 1999) grows optimally at 95°C. All genes in the tricarboxylic acid (TCA) cycle were present except for that of α-ketoglutarate dehydrogenase. Sequence comparison among the assigned ORFs suggested that a considerable number of ORFs were generated by sequence duplication.

Of the eubacteria sequenced to date, *Thermotoga maritima* MSB8 (1.86 Mb; Nelson et al., 1999) has the highest percentage (24%) of genes that are most similar to archaeal genes. Conservation of gene order between *T. maritima* and archaea in several clustered regions of the genome suggests that lateral gene transfer may have occurred between thermophilic eubacteria and archaea.

The genome of the radiation-resistant bacterium *Deinococcus radiodurans* R1 (3.28 Mb; White et al., 1999) is, unusually, composed of two chromosomes (2.65 and 0.41 Mb), a large plasmid (0.178 Mb) and a small plasmid (0.045 Mb). Several regions of the genome were identified that allow *D. radiodurans* to survive under conditions of oxidative stress, desiccation, starvation and high amounts of DNA damage.

Campylobacter jejuni (1.64 Mb; Parkhill et al., 2000) was the first foodborne pathogen to be sequenced. The genome is unusual in that there are virtually no IS or phage-associated sequences and very few repeat sequences. A striking feature was the presence of hypervariable sequences commonly found in genes encoding surface structures. The apparently high rate of variation of these homopolymeric tracts may play an important role in the survival strategy of *C. jejuni*. Despite its close phylogenetic relationship to *H. pylori*, strong similarities between these organisms are mainly confined to housekeeping

genes. In most functions related to survival, transmission, and pathogenesis, the organisms have remarkably little in common. This indicates that selective pressures have driven profound evolutionary changes to create two very different pathogens from a close common ancestor.

Neisseria meningitidis serotype B strain MC58 (2.23 Mb; Tettelin et al., 2000) revealed three major regions of horizontal DNA transfer. The sequence revealed insight into the commensal and virulence nature of the organism; in particular, the organism appears to undergo more phase variation than any prokaryote studied to date. In an accompanying paper, over 350 candidate antigens were expressed in *E. coli* and tested for their vaccine efficacy (Pizza et al., 2000). The sequence *Neisseria meningitidis* serotype A strain Z2491 (2.18 Mb) revealed hundreds of repeat elements ranging from short homopolymeric tracts to gene duplications again suggesting extreme genome fluidity, which probably plays a significant role in antigenic variation of this human specific pathogen (Parkhill et al., 2000). Comparison between *N. meningitidis* serotype B and *N. meningitidis* serotype A awaits further analysis.

Bioinformatics

Bioinformatics and the range of new supercomputers are poised to change forever the way in which we tackle prokaryotic research. The unprecedented deluge of sequence data requires processing and rapid access to functional genomics information and is central to the revolution that is taking place in prokaryotic molecular genetics. Bioinformatics is essentially the evolution of computer-based technology dedicated to the analysis of genome sequences. It is a cross-disciplinary activity, including aspects of computer science, software engineering, molecular biology, and mathematics. The past few years have seen vast improvements in the algorithms used to analyze sequence data, and an increasing range of bioinformatics software has been developed and released into the public domain by way of the Internet. Careful and intelligent use of this software can afford important new insights into protein structure and function and allow the generation of testable hypotheses.

Coincident with the availability of genome sequence data, several other factors have meant that it is easier than ever before for scientists to exploit such data. For example, the rise of the Internet and the World Wide Web, coupled with well-supported free software facilities, has made it easier than ever for scientists to use remote computing facilities (see examples on Table 1).

The most common use of bioinformatics is the search for sequence similarity with homologous genes/gene products deposited in the numerous nucleotide and protein databases worldwide. The basic local alignment search tool (BLAST); Altschul et al., 1990) is the most widely used program for such analysis. A simple example of the application of bioinformatics is the rapid identification of iterative nucleotide sequences in a genome sequence. These can act as markers for polymorphic regions important for antigenic/phase variation and in host-pathogen interactions (Hood et al., 1996). Comparative analysis of gene pathways from several complete genome sequences allows more definitive information on components of the pathway. Comparison of the citric acid cycle from numerous genome sequences makes it possible to reason confidently about the absence or presence of the different parts and branches of the cycle and even the overall metabolic scheme of the organisms (Huynen et al., 1999). *The Kyoto Encyclopedia of Genes and Genomes* worldwide (http://www.genome.ad.jp/kegg/kegg2.html) shows many metabolic and regulatory pathways based

Table 1. Bioinformatics-based web sites.

Resource	Links
BLAST searches	
Microbial Genomes at NCBI	
PEDANT	http://pedant.mips.biochem.mpg.de/index.html
ARTEMIS	http://www.sanger.ac.uk/
Genome Annotation Consortium	http://grail.lsd.oml.gov/gac/
A compendium of electronic resources for molecular biology research	http://www.sdsc.edu/ResTools/cmshp.html
Genome browsers for bacterial pathogens	http://www.medmicro.mds.qmw.ac.uk/genomes
General functional genomics software (focus on *E. coli*)	http://arep.med.harvard.edu/
STD Sequence Databases (STD pathogens)	http://www.stdgen.lanl.gov/

Abbreviations: BLAST, basic local alignment search tool; NCBI, National Center for Biotechnology Information; PEDANT, protein extraction, description, and analysis tool; ARTEMIS, a DNA sequence viewer and annotation tool; STD, sexually transmitted disease.

Table 2. General functional genomics web sites.

Resource	Purpose	Links
Virtual genome center	Evolutionary comparison of genomes	http://alces.med.umn.edu/VGC.html
General functional genomics	Pharmaceutical research emphasized	http://www.phrma.org/genomics/index.html
Functional genomic analysis		http://www.doubletwist.com
E. cell project	An environment for modeling and simulating biochemical and genetic processes	http://www.e-cell.org/project
Encyclopedia of genes and genomes	Emphasis on metabolic and regulatory pathways	http://www.genome.ad.jp/kegg/kegg.html
Microbial biodegradation		http://www.labmed.umn.edu/umbbd/index.html
Mass spectrometry		http://base-peak.wiley.com
E. coli proteome		http://web.bham.ac.uk/bcm4ght6/genome.html
DNA microarrays	Construction and application	http://cmgm.stanford.edu/pbrown/index.html
Bacterial pathogen DNA microarrays		http://www.sghms.ac.uk/depts/medmicro/bugs
Affymetrix biochip		http://www.gene-chip.com
Bacterial pathogenesis		http://www.medmicro.mds.qmw.ac.uk/underground/ketbook
Encyclopaedia of *E. coli* genes and metabolism		http://ecocyc.PangeaSystems.com/ecocyc/ecocyc.html
MICADO (*B. subtilis* and *E. coli*)	A network-oriented database for microbial genomes	http://locus.jouy.inra.fr/micado

on orthologous sequences from databases. Observations on chromosomal organization and gene order have proven useful in identifying recently acquired DNA sequences. This approach has been exploited in the *T. maritima* genome project to suggest that lateral gene transfer has occurred between thermophilic eubacteria and archaea (Nelson et al., 1999). The operon structure of prokaryotes makes it possible to identify coregulations, coexpressions, and more generally, gene clusters that might infer a common function (Overbeek et al., 1999).

The published annotated forms of the genome sequences fall short of being definitive. Generally, narrow sets of analysis programs have been employed, and no doubt, several reanalyses of the sequence data will be undertaken. In addition to the benefits of such static analysis, an ongoing dynamic analysis is needed, constantly reevaluating the sequence data in the light of newly published sequences. One example of such re-analysis software is the protein extraction, description, and analysis tool PEDANT, which is a software system that utilizes modern bioinformatics methods to provide complete functional and structural characterization of protein sequence sets from individual sequences to complete genomes. For other examples, see Table 1.

Sustained improvements in computing speed and in hard-drive capacity mean that even very computationally intensive analyses can be performed on readily available hardware. The availability of supercomputers will trigger a revolution in the complexity of problems we tackle to understand the basic biology and evolution of the prokaryotes (Butler, 2000).

Functional Genomics

In the past, geneticists have assigned gene function by specifically disabling or "knocking out" a single gene, usually by transposon mutagenesis (insertion mutagenesis) or allelic replacement (usually deletion mutagenesis), and then comparing the phenotypes of the mutant strain with the parent strain. This approach is still valid, but with the availability of information at the genome-wide level, a global approach to the study of gene function at the mutational, transcriptional and translational levels is now possible.

Mutational Analysis

The construction of defined mutants by transposon mutagenesis or allelic replacement has proven to be a powerful method for determining gene function in numerous prokaryotes. Information about the biological functions can be inferred by monitoring the fitness of the null mutant under a variety of selected growth conditions. However, in conjunction with the construction of mutants is the potential to label each mutant with a unique DNA-signature tag permitting simultaneous analysis of several hundred mutants for phenotypic features (Fig. 2; Hensel et al., 1995).

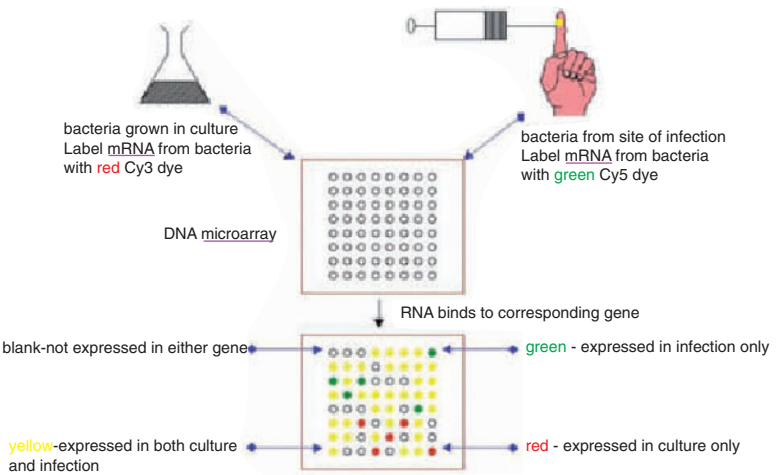

Fig. 2. Schematic showing how differential gene expression of a bacterium at a site of infection or in culture is determined using a DNA microarray.

Signature-Tagged Mutagenesis The use of DNA-signature tags was validated using *Salmonella* and a murine model of typhoid fever (Hensel et al., 1995). In the original design, the tags consisted of a central 40-bp variable region that allows differentiation between tags, flanked by constant 20-bp arms to which primers can bind for DNA amplification. By negative selection, mutants that fail to be recovered from the host following inoculation of a mixed pool of mutants can be identified (Fig. 2). Thus, when the hybridization signals from the tagged input pool of 96 mutants were compared with the respective tagged output pools of mutants, several mutants essential for the in vivo survival of *Salmonella typhimurium* could be identified (see Fig. 2; Hensel et al., 1995). This included the identification, and subsequent characterization, of a novel type III secretion system (SPI 2) in *S. typhimurium* (Shea et al., 1996). Signature-tagged mutagenesis (STM) has since been successfully used in the identification of virulence-associated factors in *Staphylococcus aureus*, *Vibrio cholerae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Legionella pneumophila*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Proteus mirabilis*, *Mycobacterium tuberculosis*, and *Brucella suis* (Mei et al., 1997; Chiang and Mekalanos, 1998; Claus et al., 1998; Polissi, 1998; Camacho et al., 1999; Darwin and Miller, 1999; Edelstein et al., 1999; Foulongne et al., 2000).

The use of wholescale tagging of bacterial mutants will have its greatest potential impact on in vivo studies, insofar as the number of animal experiments required for the assessment of bacterial virulence can be drastically reduced. A review on how STM can help to identify virulence genes and other applications has been published recently (Shea and Holden, 2000). However, the tagging and analysis of prokaryotes also should be useful to measure the survivability of mutants in other complex environments ranging from biofilms to deep-sea ocean beds.

Exploitation of a DNA array of a given organism (see Macroarrays and Microarrays in this Chapter) may obviate the need to tag transposons and could be used to identify essential genes. Assuming that a transposon can insert randomly into a prokaryotic genome, a single primer reading out from the transposon (Karlyshev et al., 2000) could be used to detect all interrupted genes in a single hybridization to an organism-specific DNA array. Potentially by screening populations of input pools and output pools from a selective environment (e.g., stress), some transposon mutants will drop out of the pool, and those genes required for stress survival will be identified. Additionally, because cDNA will only be synthesized from genes with an integrated transposon, by deduction it should be possible to detect genes essential for the survival of the organism throughout the genome.

Signature-Tagged Allele Replacement Because transposons often fail to integrate (or integrate randomly) into the chromosome of many prokaryotes, transposon mutagenesis is not universally applicable. Allelic replacement is often a useful alternative to the construction of defined deletion mutants. Furthermore, the availability of entire genomic sequences means that the large-scale, systematic construction of defined mutants is now possible. Thus, all genes can be tested methodically under a particular condition. The coupling of the incorporation of DNA tags with allelic replacement has been referred to as signature-tagged allele replacement (STAR). The STAR method does not require the use of transposons, but enables a systematic unbiased genetic analysis of the

genome. As the gene target is predetermined, the need to sequence mutation sites is obviated, and by using a systematic approach, the number of mutants required for screening is minimized. Such an attempt has been made in *S. cerevisiae* (Shoemaker et al., 1996; Winzeler et al., 1999), which was coupled to the quantifying mutants with a specifically designed Affymetrix "barcoding" biochip (see Affymetrix Oligonucleotide Arrays in this Chapter) containing all the complementary DNA sequences of the tags used in mutant construction. The unique tags are based on an algorithm to select a set of over 9,000 maximally distinguishable 20mer sequences with similar Tm values (61 ± 5°C; Shoemaker et al., 1996). The optimized DNA tags increase the sensitivity of probes in complex hybridization reactions and enable the semiquantitative determination of viable bacteria. Thus, the barcoding biochip has the capacity to measure the relative abundance of defined mutants and can measure the growth rate of all tagged mutants simultaneously.

Parallel quantitative assessment of multiple strains significantly decreases labor and material required for screening, and it increases the reliability of data obtained. This approach also removes the need for onerous, hazardous, and repetitive filter-based radioactive hybridizations. Finally, the inclusion of DNA tags is an invaluable way of tracking and validating strains distributed to laboratories worldwide.

Transcriptome Analysis

Cellular processes are governed by the repertoire of expressed genes, in particular by the levels and the timing of their expression. The mRNA complement of a cell reflects the state of a cell, uniquely defining growth, division, stress adaptation and apoptosis. Transcriptome analysis offers the potential for the simultaneous measurement of expression levels for all transcripts (mRNAs) from a genome, giving a "snapshot" of the transcriptional activity of all genes in that genome. Analyses can be performed simultaneously at a given time point in growth or in any environment. This versatility is possible because mRNA expressed under a range of environmental conditions can be extracted and hybridized to a high-density gridded array of an organism's DNA content. The availability of ever-cheaper oligonucleotides, 384-well polymerase chain reaction (PCR) technology, robotics, and complete genome sequence data makes possible the highly attractive option of using gridded libraries of PCR products, constituting a defined and complete set of ORFs and intergenic regions. Such high-throughput analysis allows massive parallel gene expression and gene discovery studies to be undertaken.

High-Density DNA Arrays

There are two main high-density DNA array formats. These are generally referred to as microarrays (or macroarrays), which consist of 100- to 1,000-bp stretches of DNA, and Affymetrix biochips that consist of in situ synthesized oligonucleotides (~20 bases).

DNA Macroarrays and Microarrays High-speed robots assemble DNA arrays on nylon membrane (often referred to as DNA macroarrays) or glass-slide solid supports (DNA microarrays). Pat Browne and colleagues, at Stanford University, have pioneered the construction and application DNA microarrays (Brown and Botstein, 1999; DeRisi and Iyer, 1999), which include the building of a robotic microarrayer from component parts. The principles and an example of the application of a DNA microarray for transcriptome analysis (also referred to as differential gene expression) are shown in Fig. 3.

To measure relative differences in gene expression, sample DNA or RNA is labeled (normally by fluorescence) and hybridized to the array (Fig. 3). For example, mRNA from cells grown under standard culture conditions is labeled with the red fluorescent dye Cy3, and sample mRNA from a site of infection is labeled with the green fluorescent dye Cy5. After cohybridization to the microarray, the fluorescent intensities of each fixed DNA sample are read to determine the relative abundance of mRNA from the two test conditions. A red signal indicates gene expression of cells grown in culture, a green signal indicates gene expression only during infection, and a yellow signal indicates genes expressed in both conditions (Fig. 3). To date, most applications of DNA microarrays have been used on eukaryotic systems (Ross et al., 2000; Scherf et al., 2000). A useful web site for the construction and application of bacterial pathogen DNA microarrays is the (http://www.sghms.ac.uk/depts/medmicro/ {BUGAS website}).

Affymetrix Oligonucleotide Arrays Oligonucleotide arrays are constructed by in situ light-directed combinatorial nucleotide synthesis (Chee et al., 1996), a process termed "photolithography" (Fig. 4; see http://www.genechip.com/). Because Affymetrix DNA arrays (biochips) consist of in situ synthesized oligonucleotide sequences, they offer two major advantages over DNA microarrays: 1) greater sample

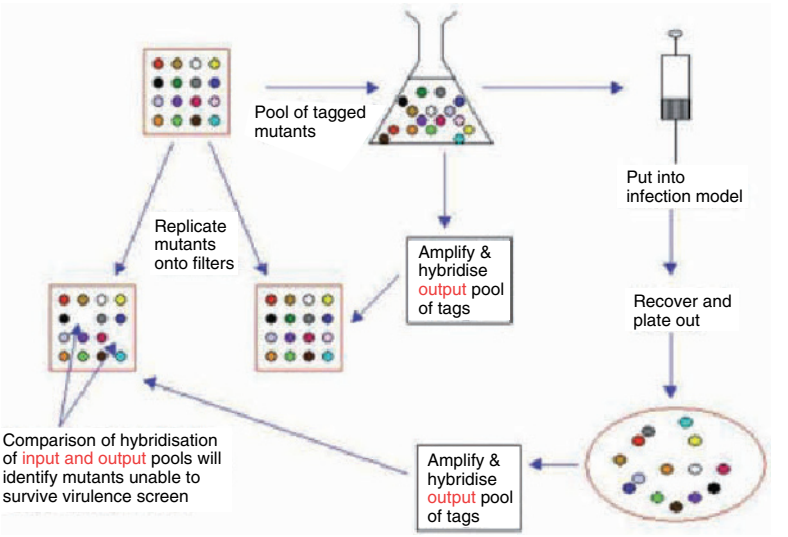

Fig. 3. Schematic illustrating the principles of a signature-tagged mutagenesis screen, which can distinguish virulent from nonvirulent mutants.

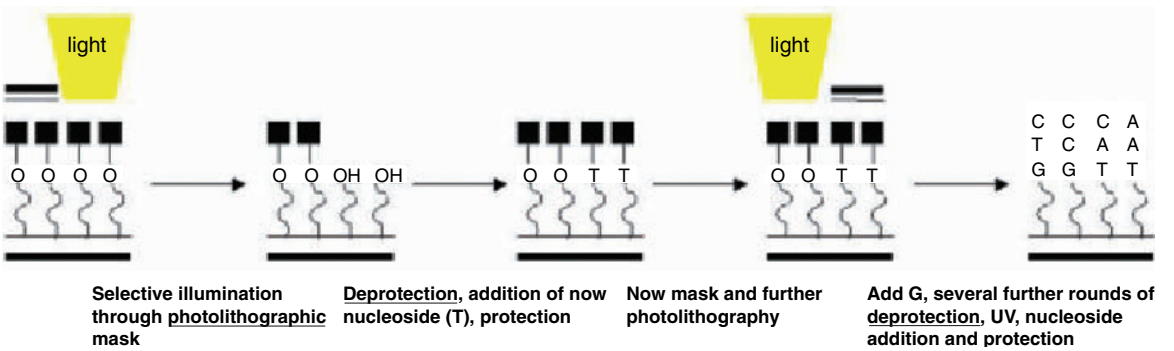

Fig. 4. Schematic showing stages in the in situ synthesis of an Affymetrix oligonucleotide array.

capacity (>50,000 samples per cm^2 compared with 4,000 samples per cm^2 for a typical DNA microarray) and 2) an ability to detect single nucleotide polymorphisms. However, due to cost and selective availability of biochips, Affymetrix technology is beyond the reach of most academic institutes. The large capacity of Affymetrix biochips has meant that their application has been used mainly for eukaryotic systems (Chiang and Mekalanos, 1998; Cho et al., 1998; Cho et al., 1999). However, synthesis of prokaryotic biochips containing several genome sequences (e.g., *E. coli*, *B. subtilis*, *H. pylori*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*) is planned.

Applications of High-Density DNA Arrays and Genomotyping

Perhaps the most straightforward application of DNA microarrays is hybridization test strain DNA (e.g., from clinical or environmental isolates) with the genome of a sequenced strain arrayed on the solid support. Thus, a comprehensive assessment of genome diversity of a large number of strains can be rapidly attained. The use of DNA microarrays to compare the genome complements of several strains has been termed "genomotyping." Among bacterial pathogens, such an approach will be an invaluable molecular epidemiological tool, allowing the sources and routes of transmission of economically and clinically important pathovars to be determined. By assessing correlates of pathogenicity, a basic understanding of the evolution of virulence can be gained. To date, microarrays have been reported in the identification of gene sequences absent in the attenuated *Mycobacterium bovis* strain BCG compared to virulent *M. tuberculosis* strain H37Rv (Behr et al., 1999) and have been applied to *S. cerevisiae* (Winzeler et al., 1999).

Other potential applications of microarrays to prokaryote analysis include: 1) differential gene expression (DGE) by hybridizing mRNA extracted under varying environmental condi-

tions; 2) DGE by comparing mutant to the wild-type strains, particularly to decipher regulatory networks; 3) testing genome plasticity of an individual strain by DNA hybridization; and 4) identifying single nucleotide polymorphisms (SNPs) by use of the Affymetrix DNA array.

Proteome Analysis

The case for global monitoring of mRNA applies equally to proteins, with the added advantage that post-translational modifications, which frequently play key roles in prokaryotic interactions, also can be studied. Proteomics, the study of the complete set of proteins that is expressed and modified by the entire genome in the lifetime of a cell, is an important rapidly evolving discipline, readily applied to prokaryotes.

2-D Gel Electrophoresis Protein Identification

Recent improvements in high-sensitivity biological mass spectrometry have provided a powerful adjunct to traditional 2D-gel electrophoresis (Pappin, 1997; Fernandez et al., 1998). Proteins cut out of a 2D-gel can now be peptide-mass-fingerprinted, and constituent peptides can be sequenced by mass spectrometry. New software takes data from mass spectrometry and uses it to find the best match in a sequence database, allowing one to go from a spot on a polyacrylamide gel electrophoresis (PAGE) gel to protein identification in a matter of hours (Fig. 1). Thus, the entire complement of soluble proteins expressed by a cell (the proteome) can be defined. This kind of approach already has been used to provide insights into the function of an anatomical subset of the proteome (such as the cell envelope; Qi, 1996). Proteome studies are made even more powerful when applied to an organism whose genome has been sequenced. Synergistic interactions between the two approaches maximize information return. For example, ORFan or FUN gene products can be identified as functional proteins. Proteome or partial proteome analysis has been reported for *M. genitalium*, *S. typhimurium*, and *M. tuberculosis* (Wasinger et al. 2000; O'Connor et al., 1997; Sonnenberg and Belisle, 1997; Jungblut et al., 1999; Tekaia et al., 1999). The continued *E. coli* proteome is on the http://web.bham.ac.uk web. A comprehensive web resource for mass spectrometrists is found on Base Peak (basepeak.wiley.com/).

Potential applications of 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE)/mass spectrometry analysis for prokaryotes include: 1) characterization of regulons and stimulons; 2) study of posttranslational modifications; 3) study of protein complexes; 4) identification of immunogenic proteins by immunoblotting; and 5) determination of mechanisms of drug action and identifying drug targets.

Protein-Protein Interaction Maps

Interactions between proteins mediate the majority of biological processes, and various biochemical assays have been developed to measure such interactions. The most widely applied is the yeast two-hybrid system. The two-hybrid system exploits the ability of two interacting proteins to fetch a transcription domain into the locality of a DNA-binding site that regulates the expression of an adjacent reporter gene (Fields and Song, 1989; Chien et al., 1991; Fields and Sternglanz, 1994). The system can be used to identify proteins that bind to a protein of interest or to define domains or residues critical for an interaction (Fields and Song, 1989; Chien et al., 1991; Fields and Sternglanz, 1994).

Protein-protein interaction maps of selected components of prokaryotes should provide invaluable data on structural components of the cell. Some recent examples where this has been applied include outer-membrane proteins, secretion systems, and mutation proteins (Williams et al., 1998; Day and Plano, 1998; Hartland et al., 1999; Hall and Matson, 1999).

Once all DNA, RNA, and proteins are known, it should be possible to compile complete interaction maps of the genome. This already has been accomplished for bacteriophage (55 proteins), and it should be possible to tackle prokaryotes. Indeed, a comprehensive analysis of protein-protein interactions in *S. cerevisiae* has been performed recently using exhaustive yeast two-hybrid screens (Uetz et al., 2000).

Studies and Practical Implications

A multidimensional analysis, looking at genome sequences, mutants, transcripts, and proteins, will result in a quantum leap in our understanding of the biology of prokaryotes. In determining the activity of large sets of genes, proteins, and the interactions between them, an important step towards constructing a functional model of the entire organism will be taken. This basic information will provide the framework for future research; for example, in bacterial pathogens, opportunities for vaccine design are unprece-

dented because the complete inventory of genes encoding every virulence factor and potential immunogen is available for selection. The combination of 2D-gel electrophoresis and immunoblot analysis of the whole organism, or a subset such as the cell envelope, should identify all immunodominant proteins. Indeed, in an attempt to identify a *N. meningitidis* vaccine target, over 350 candidate antigens based on the recently completed *N. meningitidis* type B genome sequence were expressed in *E. coli* and tested for their vaccine efficacy (Pizza et al., 2000). By deduction, approaches for the systematic mutagenesis of all genes in a genome will identify genes essential for the viability of the organism. Such genes/gene products are potential targets for drug design. The availability of the genome content of multiple organisms on a DNA microarray or an Affymetrix biochip should allow more accurate identification of organisms and, in a clinical setting, more rapid diagnosis. Exploitation of a database of nucleotide differences among strains should allow the design of a biochip to differentiate subtle differences between strains. Such a universal prokaryote biochip would have profound implications in studying the epidemiology, population genetics, molecular phylogeny and evolution of prokaryotes.

Genomics Glossary

Adapted from the (http://www.phrma.org/genomics/ website).

Affymetrix DNA chip: High-density array of evenly spaced in-situ synthesized oligonucleotides on a silicon support. The company Affymetrix produces these DNA arrays using in-situ, light-directed, combinatorial nucleotide synthesis. An Affymetrix DNA chip the size of a thumbnail can contain up to 50,000 oligonucleotide probes.

allelic replacement: The exchange of a gene via homologous recombination. This method is often used to specifically exchange a wild-type gene with a mutated gene to construct a rationally defined isogenic mutant.

apoptosis: The process by which cells are programmed to self-destruct at an appropriate moment in an organism's life cycle. If the apoptotic process malfunctions in a cell, uncontrolled cell growth may result and can contribute to the development of cancer.

bioinformatics: The science of informatics as applied to biological research. Informatics is the management and analysis of data using advanced computing techniques. Bioinformatics is particularly important as an adjunct to genomics research because of the large amount of complex data this research generates.

cloning vector: A DNA molecule originating from a virus, a plasmid, or the cell of a higher organism into which another DNA fragment of appropriate size can be integrated without loss of the vector's capacity for self-replication. Vectors introduce foreign DNA into host cells, where it can be reproduced in large quantities; examples are plasmids, cosmids, and yeast artificial chromosomes. Vectors are often recombinant molecules containing DNA sequences from several sources.

combinatorial chemistry: A technique for rapidly and systematically assembling a variety of molecular entities, or building blocks, in many different combinations, to create tens of thousands of diverse compounds that can be tested in drug discovery screening assays.

comparative genomics: The study of the degree of relatedness of complete genome sequences from different strains or organisms. Such studies provide important taxonomic and evolutionary insights.

contigs: Groups of clones representing overlapping regions of a genome.

differential gene expression (DGE): Comparative analysis of mRNA levels of individual genes from cells functioning in different environments. This method is frequently performed at the genome level as transcriptome analysis.

directed mutagenesis: A specific alteration of a cloned gene in vitro before the gene is placed back into the organism.

DNA microarray: High-density array of evenly spaced DNA spots of generally 100 to 1,000 bp gridded onto glass slides. Typically, about 4,000 individual gene fragments can be spotted onto a microscope slide.

DNA macroarray: High-density array of evenly spaced DNA spots of generally 100 to 1,000 bp gridded onto nylon membranes. Generally, arrays have a lower density than Affymetrix chips or microarrays and generally they are not amenable to differential fluorescence hybridizaton analysis.

expressed sequence tag (EST): A short strand of DNA (ca. 200 bps), which is part of a cDNA. Because cDNAs correspond to a particular gene in the genome, and ESTs correspond to particular cDNAs, ESTs can be used to help identify unknown genes and to map their position in the genome.

functional genomics: The process of determining the function of individual genes at a genome-wide scale.

gene library: A collection of cloned DNA fragments, which, taken together, represent the entire genome of a specific organism. Such libraries or "gene banks" are assembled so as to allow the isolation and study of individual genes. Gene libraries are produced by first breaking up

or "fractionating" an entire genome. This fractionation can be accomplished either by sonication or other physical methods.

gene expression: The process by which the information in a gene is used to create proteins.

genetic polymorphism: A difference in DNA sequence among individuals, groups or populations. Genetic polymorphisms may be the result of chance processes or may have been induced by external agents (such as viruses or radiation). If a difference in DNA sequence among individuals has been shown to be associated with disease, it usually will be called a genetic mutation. Changes in DNA sequence, which have been confirmed to be caused by external agents, are also generally called "mutations" rather than "polymorphisms."

genetic map: A map of a genome that shows the relative positions of the genes and/or markers on the chromosomes.

genome: All the genetic material in a particular organism; its size is generally given as its total number of base pairs.

genomic library: A collection of clones made from a set of randomly generated overlapping DNA fragments representing the entire genome of an organism.

genomics: The study of genes and their function. Recent advances in genomics are bringing about a revolution in our understanding of the molecular mechanisms of disease, including the complex interplay of genetic and environmental factors.

homologies: Similarities in DNA or protein sequences between individuals of the same species or between species.

hybridization: The process of joining two complementary strands of DNA or one of DNA or of RNA to form a double-stranded molecule.

messenger RNA (mRNA): The DNA of a gene is transcribed into mRNA molecules, which then serve as templates for protein synthesis.

mutation: A change, deletion, or rearrangement in the DNA sequence that may lead to the synthesis of an altered inactive protein or to the loss of the ability to produce the protein.

oligonucleotide: A molecule made up of a small number of nucleotides, typically fewer than 25. Oligonucleotides are frequently used as DNA synthesis primers in sequencing, in PCR, or in Affymetrix DNA arrays.

orthologues: Homologous genes between species.

paralogues: Homologous genes within the same strain/species.

pharmacogenomics: The science of understanding the correlation between an individual patient's genetic makeup (genotype) and their response to drug treatment. Some drugs work well in some patient populations and not as well in others. Studying the genetic basis of patient response to therapeutics allows drug developers to more effectively design therapeutic treatments.

physical map: A map of the locations of identifiable landmarks on DNA (e.g., restriction enzyme cutting sites, genes), regardless of inheritance. Distance is measured in base pairs. For the human genome, the lowest-resolution physical map is the banding patterns on the 24 different chromosomes; the highest-resolution map would be the complete nucleotide sequence of the chromosomes.

pleiotropy: One gene leading to many different phenotypic expressions. An example of a pleiotropic gene is *phoP* in *Salmonella*, which controls over 40 genes associated with virulence and survival inside and outside the host.

proteome: The complete set of proteins that is expressed and modified by the entire genome in the lifetime of a cell.

proteomics: The study of the proteome using technologies of large-scale protein separation and identification.

rational drug design: A process for designing drugs based upon the structure of the protein target of the drug. This approach has been enhanced recently through use of combinatorial chemistry and high-throughput screening.

restriction fragment length polymorphism (RFLP): Variation between individuals in DNA fragment sizes produced after cutting DNA with specific restriction enzymes. Polymorphic sequences that result in RFLPs are used as markers on both physical maps and genetic linkage maps. Almost all RFLPs are caused by mutations in the restriction enzyme recognition site.

sequencing: Determining the order of nucleotides in a DNA or RNA molecule, or determining the order of amino acids in a protein.

shotgun method: Cloning of DNA fragments randomly generated from a genome.

signature-tagged allele replacement (STAR): A modification of STM where mutants are DNA-tagged systematically prior to allele replacement during their construction.

signature-tagged mutagenesis (STM): Mutants generated by transposon mutagenesis are tagged with 20-base sequences, which act as unique identifiers. The pool of tagged mutants allows one to study en masse the relative abundance and survivability of all mutants in any given environment.

single nucleotide polymorphism (SNP): A single nucleotide alteration in a gene, frequently characteristic of a genetic trait. A SNP can be assayed en masse using an Affymetrix DNA array.

telomere: A series of repeated DNA sequences located at the end of a chromosome.

Telomeres serve to assure that a chromosome is replicated properly each time a cell divides. Each time a cell divides, some of the telomere is lost in the process. Eventually little or no telomere remains and the cell dies.

transcriptome analysis: The simultaneous analysis of all transcripts within a cell by hybridizing mRNA to a DNA micro-, macro- or Affymetrix DNA array.

transposable elements: A class of DNA sequences that can move from one chromosome or plasmid site to another. Transposable elements are frequently referred to as jumping genes.

transposon mutagenesis: Insertion of a transposable element into a genome (usually bacterial) to generate a pool of random mutants.

toxicogenomics: A new scientific subdiscipline that combines the emerging technologies of genomics and bioinformatics to identify and characterize mechanisms of action of known and suspected toxicants. Currently, the premier toxicogenomic tools are the DNA microarray and the DNA chip.

wild type: The form of an organism that occurs most frequently in nature.

Literature Cited

Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen Helicobacter pylori. Nature 397:176–180.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Molec. Biol. 215:403–410.

Andersson, S. G., A. Zomorodipour, J. O. Andersson, T. Sicheritz-Ponten, U. C. Alsmark, R. M. Podowski, A. K. Naslund, A. S. Eriksson, H. H. Winkler, and C. G. Kurland. 1998. The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature 396:133–140.

Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284:1520–1523.

Blattner, F. R., G. Plunkett 3rd, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of Escherichia coli K-12. Science 277:1453–1474.

Brown, P. O., and D. Botstein. 1999. Exploring the new world of the genome with DNA microarrays. Nat. Genet. 21:33–37.

Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J. F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, and J. C. Venter. 1996. Complete genome sequence of the methanogenic archaeon, Methanococcus jannaschii. Science 273:1058–1073.

Butler, D. 2000. Computing 2010: From black holes to biology. Nature 402:67–70.

Camacho, L. R., D. Ensergueix, E. Perez, B. Gicquel, and C. Guilhot. 1999. Identification of a virulence gene cluster of Mycobacterium tuberculosis by signature-tagged transposon mutagenesis. Molec. Microbiol. 34:257–267.

Chee, M., R. Yang, E. Hubbell, A. Berno, X. C. Huang, D. Stern, J. Winkler, D. J. Lockhart, M. S. Morris, and S. P. Fodor. 1996. Accessing genetic information with high-density DNA arrays. Science 274:610–614.

Chiang, S. L., and J. J. Mekalanos. 1998. Use of signature-tagged transposon mutagenesis to identify Vibrio cholerae genes critical for colonization. Molec. Microbiol. 27:797–805.

Chien, C. T., P. L. Bartel, R. Sternglanz, and S. Fields. 1991. The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA 88:9578–7582.

Cho, R. J., M. J. Campbell, E. A. Winzeler, L. Steinmetz, A. Conway, L. Wodicka, T. G. Wolfsberg, A. E. Gabrielian, D. Landsman, D. J. Lockhart, and R. W. Davis. 1998. A genome-wide transcriptional analysis of the mitotic cell cycle. Mol Cell 2:65–73.

Cho, R. J., M. Mindrinos, D. R. Richards, R. J. Sapolsky, M. Anderson, E. Drenkard, J. Dewdney, T. L. Reuber, M. Stammers, N. Federspiel, A. Theologis, W. H. Yang, E. Hubbell, M. Au, E. Y. Chung, D. Lashkari, B. Lemieux, C. Dean, R. J. Lipshutz, F. M. Ausubel, R. W. Davis, and P. J. Oefner. 1999. Genome-wide mapping with biallelic markers in Arabidopsis thaliana. Nat. Genet. 23:203–207.

Claus, H., M. Frosch, and U. Vogel. 1998. Identification of a hotspot for transformation of Neisseria meningitidis by shuttle mutagenesis using signature-tagged transposons. Mol. Gen. Genet. 259:363–371.

Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, et al. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393:537–544.

Darwin, A. J., and V. L. Miller. 1999. Identification of Yersinia enterocolitica genes affecting survival in an animal host using signature-tagged transposon mutagenesis. Molec. Microbiol. 32:51–62.

Day, J. B., and G. V. Plano. 1998. A complex composed of SycN and YscB functions as a specific chaperone for YopN in Yersinia pestis. Molec. Microbiol. 30:777–788.

Deckert, G., P. V. Warren, T. Gaasterland, W. G. Young, A. L. Lenox, D. E. Graham, R. Overbeek, M. A. Snead, M. Keller, M. Aujay, R. Huber, R. A. Feldman, J. M. Short, G. J. Olsen, and R. V. Swanson. 1998. The complete genome of the hyperthermophilic bacterium Aquifex aeolicus. Nature 392:353–358.

DeRisi, J. L., and V. R. Iyer. 1999. Genomics and array technology. Curr. Opin. Oncol. 11:76–79.

Edelstein, P. H., M. A. Edelstein, F. Higa, and S. Falkow. 1999. Discovery of virulence genes of Legionella pneu-

mophila by using signature tagged mutagenesis in a guinea pig pneumonia model. Proc. Natl. Acad. Sci. USA 96:8190–8195.

Fernandez, J., F. Gharahdaghi, and S. M. Mische. 1998. Routine identification of proteins from sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) gels or polyvinyl difluoride membranes using matrix assisted laser desorption/ionization- time of flight-mass spectrometry (MALDI-TOF-MS). Electrophoresis 19: 1036–1045.

Fields, S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. Nature 340:245–246.

Fields, S., and R. Sternglanz. 1994. The two-hybrid system: an assay for protein-protein interactions. Trends Genet. 10:286–292.

Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al. 1995. Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science 269:496–512.

Foulongne, V., G. Bourg, C. Cazevieille, S. Michaux-Charachon, and D. O'Callaghan. 2000. Identification of Brucella suis genes affecting intracellular survival in an in vitro human macrophage infection model by signature-tagged transposon mutagenesis. Infect. Immunol. 68:1297–1303.

Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, et al. 1995. The minimal gene complement of Mycoplasma genitalium. Science 270: 397–403.

Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. C. Venter, et al. 1997. Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. Nature 390:580–586.

Fraser, C. M., S. J. Norris, G. M. Weinstock, O. White, G. G. Sutton, R. Dodson, M. Gwinn, E. K. Hickey, R. Clayton, K. A. Ketchum, E. Sodergren, J. M. Hardham, M. P. McLeod, S. Salzberg, J. Peterson, H. Khalak, D. Richardson, J. K. Howell, M. Chidambaram, T. Utterback, L. McDonald, P. Artiach, C. Bowman, M. D. Cotton, J. C. Venter, et al. 1998. Complete genome sequence of Treponema pallidum, the syphilis spirochete. Science 281:375–388.

Hall, M. C., and S. W. Matson. 1999. The Escherichia coli MutL protein physically interacts with MutH and stimulates the MutH-associated endonuclease activity. J. Biol. Chem. 274:1306–1312.

Hartland, E. L., M. Batchelor, R. M. Delahay, C. Hale, S. Matthews, G. Dougan, S. Knutton, I. Connerton, and G. Frankel. 1999. Binding of intimin from enteropathogenic Escherichia coli to Tir and to host cells. Molec. Microbiol. 32:151–158.

Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science 269:400–403.

Himmelreich, R., H. Hilbert, H. Plagens, E. Pirkl, B. C. Li, and R. Herrmann. 1996. Complete sequence analysis of the genome of the bacterium Mycoplasma pneumoniae. Nucleic Acids Res. 24:4420–4449.

Hood, D. W., M. E. Deadman, M. P. Jennings, M. Bisercic, R. D. Fleischmann, J. C. Venter, and E. R. Moxon. 1996. DNA repeats identify novel virulence genes in Haemophilus influenzae. Proc. Natl. Acad. Sci. USA 93:11121–11125.

Hutchison, C. A., S. N. Peterson, S. R. Gill, R. T. Cline, O. White, C. M. Fraser, H. O. Smith, and J. C. Venter. 1999. Global transposon mutagenesis and a minimal Mycoplasma genome. Science 286:2165–2169.

Huynen, M. A., T. Dandekar, and P. Bork. 1999. Variation and evolution of the citric-acid cycle: A genomic perspective. Trends Microbiol. 7:281–291.

Jungblut, P. R., U. E. Schaible, H. J. Mollenkopf, U. Zimny-Arndt, B. Raupach, J. Mattow, P. Halada, S. Lamer, K. Hagens, and S. H. Kaufmann. 1999. Comparative proteome analysis of Mycobacterium tuberculosis and Mycobacterium bovis BCG strains: Towards functional genomics of microbial pathogens. Molec. Microbiol. 33:1103–1117.

Kalman, S., W. Mitchell, R. Marathe, C. Lammel, J. Fan, R. W. Hyman, L. Olinger, J. Grimwood, R. W. Davis, and R. S. Stephens. 1999. Comparative genomes of Chlamydia pneumoniae and C. trachomatis. Nat. Genet. 21:385–389.

Kaneko, T., S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, and S. Tabata. 1996. Sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II: Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. 3:109–136.

Karlyshev, A. V., M. Pallen, and B. W. Wren. in press. A single primer PCR (SP-PCR) procedure for rapid identification of transposon insertion sites. *In:* Biotechniques.

Kawarabayasi, Y., M. Sawada, H. Horikawa, Y. Haikawa, Y. Hino, S. Yamamoto, M. Sekine, S. Baba, H. Kosugi, A. Hosoyama, Y. Nagai, M. Sakai, K. Ogura, R. Otsuka, H. Nakazawa, M. Takamiya, Y. Ohfuku, T. Funahashi, T. Tanaka, Y. Kudoh, J. Yamazaki, N. Kushida, A. Oguchi, K. Aoki, and H. Kikuchi. 1998. Complete sequence and gene organization of the genome of a hyperthermophilic archaebacterium, Pyrococcus horikoshii OT3. DNA Res. 5:55–76.

Kawarabayasi, Y., Y. Hino, H. Horikawa, S. Yamazaki, Y. Haikawa, K. Jin-no, M. Takahashi, M. Sekine, S. Baba, A. Ankai, H. Kosugi, A. Hosoyama, S. Fukui, Y. Nagai, K. Nishijima, H. Nakazawa, M. Takamiya, S. Masuda, T. Funahashi, T. Tanaka, Y. Kudoh, J. Yamazaki, N. Kushida, A. Oguchi, H. Kikuchi, et al. 1999. Complete genome sequence of an aerobic hyper-thermophilic crenarchaeon, Aeropyrum pernix K1. DNA Res. 6:83–101, 145–152.

Klenk, H. P., R. A. Clayton, J. F. Tomb, O. White, K. E. Nelson, K. A. Ketchum, R. J. Dodson, M. Gwinn, E. K. Hickey, J. D. Peterson, D. L. Richardson, A. R. Kerlavage, D. E. Graham, N. C. Kyrpides, R. D. Fleischmann, J. Quackenbush, N. H. Lee, G. G. Sutton, S. Gill, E. F. Kirkness, B. A. Dougherty, K. McKenney, M. D. Adams, B. Loftus, J. C. Venter, et al. 1997. The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon Archaeoglobus fulgidus. Nature 390:364–370.

Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolo-

tin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al. 1997. The complete genome sequence of the Gram-positive bacterium Bacillus subtilis. Nature 390:249–256.

Lashkari, D. A., J. L. DeRisi, J. H. McCusker, A. F. Namath, C. Gentile, S. Y. Hwang, P. O. Brown, and R. W. Davis. 1997. Yeast microarrays for genome wide parallel genetic and gene expression analysis. Proc. Natl. Acad. Sci. USA 94:13057–13062.

Mei, J. M., F. Nourbakhsh, C. W. Ford, and D. W. Holden. 1997. Identification of Staphylococcus aureus virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. Molec. Microbiol. 26:399–407.

Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, C. M. Fraser, et al. 1999. Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of Thermotoga maritima. Nature 399:323–329.

O'Connor, C. D., M. Farris, R. Fowler, and S. Y. Qi. 1997. The proteome of Salmonella enterica serovar typhimurium: Current progress on its determination and some applications. Electrophoresis 18:1483–1490.

Overbeek, R., M. Fonstein, M. D'Souza, G. D. Pusch, and N. Maltsev. 1999. The use of gene clusters to infer functional coupling. Proc. Natl. Acad. Sci. USA 96:2896–2901.

Pappin, D. J. 1997. Peptide mass fingerprinting using MALDI-TOF mass spectrometry. Methods Molec Biol. 64:165–173.

Parkhill, J., M. Achtman, K. D. James, S. D. Bentley, C. Churcher, S. R. Klee, G. Morell, D. Basham, D. Brown, T. Chillingworth, R. M. Davies, P. Davis, K. Devlin, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Leather, S. Moule, K. Mungall, M. A. Quail, M.-A. Rajandream, K. M. Rutherford, M. Simmonds, J. Skelton, S. Whitehead, B. G. Spratt, and B. G. Barrell. 2000a. Complete DNA sequence of a serogroup A strain of Neisseria meningitidis Z2491. Nature 404:502–506.

Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell. 2000b. The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. Nature 403:665–668.

Pizza, M., S. V. Masignani, M. M. Giuliani, B. Arico, M. Comanducci, G. T. Jennings, L. Baldi, E. Bartolini, B. Capecchi, C. L. Galeotti, E. Luzzi, R. Manetti, E. Marchetti, M. Mora, S. Nuti, G. Ratti, L. Santini, S. Savino, M. Scarselli, E. Storni, P. Zuo, M. Broeker, E. Hundt, B. Knapp, and E. Blair. 2000. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science 287:1816–1820.

Polissi, A. P. A., G. Feger, M. Altieri, H. Mottl, L. Ferrari, and D. Simon. 1998. Large-scale identification of virulence genes from Streptococcus pneumoniae. Infect. Immunol. 66:5620–5629.

Ross, D. T., U. Scherf, M. B. Eisen, C. M. Perou, C. Rees, P. Spellman, V. Iyer, S. S. Jeffrey, M. Van De Rijn, M. Waltham, A. Pergamenschikov, J. C. Lee, D. Lashkari, D. Shalon, T. G. Myers, J. N. Weinstein, D. Botstein, and P. O. Brown. 2000. Systematic variation in gene expression patterns in human cancer cell lines. Nat. Genet. 24:227–235.

Scherf, U., D. T. Ross, M. Waltham, L. H. Smith, J. K. Lee, L. Tanabe, K. W. Kohn, W. C. Reinhold, T. G. Myers, D. T. Andrews, D. A. Scudiero, M. B. Eisen, E. A. Sausville, Y. Pommier, D. Botstein, P. O. Brown, and J. N. Weinstein. 2000. A gene expression database for the molecular pharmacology of cancer. Nat. Genet. 24:236–244.

Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden. 1996. Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. Proc. Natl. Acad. Sci. USA 93:2593–2597.

Shea, S., and D. W. Holden. 2000. Signature-tagged mutagenesis helps identify virulence genes. American Society for Microbiology News 66:15–20.

Shoemaker, D. D., D. A. Lashkari, D. Morris, M. Mittmann, and R. W. Davis. 1996. Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. Nat. Genet. 14:450–456.

Smith, D. R., L. A. Doucette-Stamm, C. Deloughery, H. Lee, J. Dubois, T. Aldredge, R. Bashirzadeh, D. Blakely, R. Cook, K. Gilbert, D. Harrison, L. Hoang, P. Keagle, W. Lumm, B. Pothier, D. Qiu, R. Spadafora, R. Vicaire, Y. Wang, J. Wierzbowski, R. Gibson, N. Jiwani, A. Caruso, D. Bush, J. N. Reeve, et al. 1997. Complete genome sequence of Methanobacterium thermoautotrophicum deltaH: Functional analysis and comparative genomics. J. Bacteriol. 179:7135–7155.

Sonnenberg, M. G., and J. T. Belisle. 1997. Definition of Mycobacterium tuberculosis culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect. Immunol. 65:4515–424.

Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin, and R. W. Davis. 1998. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science 282:754–759.

Tekaia, F., S. V. Gordon, T. Garnier, R. Brosch, B. G. Barrell, and S. T. Cole. 1999. Analysis of the proteome of Mycobacterium tuberculosis in silico. Tuber. Lung Dis. 79:329–342.

Tettelin, H. S. N., J. Heidelberg, A. C. Jeffries, K. E. Nelson, J. A. Eisen, K. A. Ketchum, D. W. Hood, J. F. Peden, R. J. Dodson, W. C. Nelson, M. L. Gwinn, R. DeBoy, J. D. Peterson, E. K. Hickey, D. H. Haft, S. L. Salzberg, O. White, R. D. Fleischmann, B. A. Dougherty, T. Mason, A. Ciecko, D. S. Parksey, E. Blair, and H. Cittone. 2000. Complete genome sequence of neisseria meningitidis serogroup B strain MC58. Science 287:1809–1815.

Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, J. C. Venter, et al. 1997. The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature 388:539–547.

Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson, J. R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B.

Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields, and J. M. Rothberg. 2000. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature 403:623–627.

Wasinger, V. C., J. D. Pollack, and I. Humphery-Smith. 2000. The proteome of Mycoplasma genitalium Chaps-soluble component. Eur. J. Biochem. 267:1571–1582.

White, O., J. A. Eisen, J. F. Heidelberg, E. K. Hickey, J. D. Peterson, R. J. Dodson, D. H. Haft, M. L. Gwinn, W. C. Nelson, D. L. Richardson, K. S. Moffat, H. Qin, L. Jiang, W. Pamphile, M. Crosby, M. Shen, J. J. Vamathevan, P. Lam, L. McDonald, T. Utterback, C. Zalewski, K. S. Makarova, L. Aravind, M. J. Daly, C. M. Fraser, et al. 1999. Genome sequence of the radioresistant bacterium Deinococcus radiodurans R1. Science 286:1571–1577.

Williams, J. M., G. C. Chen, L. Zhu, and R. F. Rest. 1998. Using the yeast two-hybrid system to identify human epithelial cell proteins that bind gonococcal Opa proteins: intracellular gonococci bind pyruvate kinase via their Opa proteins and require host pyruvate for growth. Molec. Microbiol. 27:171–186.

Winzeler, E. A., D. R. Richards, A. R. Conway, A. L. Goldstein, S. Kalman, M. J. McCullough, J. H. McCusker, D. A. Stevens, L. Wodicka, D. J. Lockhart, and R. W. Davis. 1998. Direct allelic variation scanning of the yeast genome. Science 281:1194–1197.

Winzeler, E. A., B. Lee, J. H. McCusker, and R. W. Davis. 1999a. Whole genome genetic-typing in yeast using high-density oligonucleotide arrays. Parasitology 118 (Suppl.):S73–80.

Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J. D. Boeke, H. Bussey, A. M. Chu, C. Connelly, K. Davis, F. Dietrich, S. W. Dow, M. El Bakkoury, F. Foury, S. H. Friend, E. Gentalen, G. Giaever, J. H. Hegemann, T. Jones, M. Laub, H. Liao, R. W. Davis, et al. 1999b. Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285:901–906.

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CHAPTER 1.10

Genomics and Metabolism in *Escherichia coli*

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Escherichia coli as a Model Organism

When did *Escherichia coli* attain the status of a model organism? Over many decades biochemical researchers used *E. coli* as a source of enzymes supplementing the more common sources such as rat liver, pigeon breast and spinach, but that practice in itself did not confer the status of model organism. A giant step was taken in 1955 when the Carnegie Institute of Washington Biophysics group headed by R.B. Roberts published a large monograph reporting its own work and the work of others on the intermediary metabolism of *E. coli* (Roberts et al., 1955).

Model organism status was gained when genetic transfer capabilities were discovered (Wollman et al., 1959; Lederberg and Tatum, 1946). In this same era, Maaloe and colleagues untangled the dynamics of *E. coli* cell physiology by documenting the effects of shifts in growth conditions on macromolecule synthesis (summarized in Maaloe and Kjeldgaard, 1966). Elucidation of the operon theory of regulation of gene expression (by means of a combination of bacterial genetics and enzyme assays; Pardee et al., 1959; Jacob and Monod, 1961) firmly established *E. coli* as a lead microorganism for molecular biology research.

Over 50 years of intense investigation of laboratory strains of *E. coli* have produced a vast amount of experimental information. The unique repository of summarized information on *E. coli* genetics, cell biology, physiology, metabolism, evolution and population biology is the two-volume, 155-chapter, and nearly 3000-page multi-author work coordinated by senior editor F. C. Neidhardt and published by ASM Press (Neidhart, 1996). The object of most of this study has been the nonpathogenic strain K-12. Medical microbiology throughout this time and to the present dealt with entero- and uropathogenic *E. coli* strains, but this essay is about the nonvirulent strain K-12.

Escherichia coli plays a prominent role in the new field of genomics, the study of entire genomes, their chemistry, and the molecular biology of all gene products. Inasmuch as gene cloning allows introduction of genes from any source into *E. coli* (Sambrook et al., 1989), this organism has become a tool for studying its own molecular biology as well as that of other taxa. One of perhaps 25 microorganisms for which an entire nucleotide sequence is known (Blattner et al., 1997), *E. coli* plays a prominent role in reaping biological information from the complete nucleotide sequences of genomes.

The Genomics of *Escherichia coli*

The complete sequence of the 4.7 Mb chromosome of *E. coli* strain MG1655 was announced in 1997 (Blattner et al., 1997). A substantial portion of another K-12 strain W3110 was completed independently (Itoh et al., 1999).

What is the point of having a complete genome sequence of an organism? When we understand how every gene product (protein or RNA) of a genome functions and how those proteins and nucleic acids interact, we will understand how a single-cell organism lives, derives energy and motility from foodstuffs, adapts its processes to changing circumstances, and regularly reproduces itself. The DNA sequence itself does not give this complete knowledge of course, but it does set us in this direction. With the genomic sequence in hand—even one from an organism as well studied as is *E. coli*—much remains to be discovered. At least some, if not all, future work on this organism will be based on the broad platform of knowledge from two sources: decades of experimentation and the sequence of the entire genome.

Complete genomic sequences also may be used to reconstruct evolutionary history (possible events, paths and mechanisms of evolution) from examining minutely the relationships between comparable sequences in many life forms. Sequences of ribosomal RNAs have been particularly successful information sources for

revealing the structure of the tree of life (Woese, 1987). Trees for proteins are presenting complexities difficult to untangle. Determining what proteins are truly comparable among a set of organisms (truly orthologous) is made easier by availability of complete genome sequences. In time with more information on contemporary organisms, reconstruction of the path of the evolution on earth will become more straightforward.

What has the complete sequence of the *E. coli genome* so far revealed about its biology and evolution? The great advantage of assembling information on a model organism is that so much experimental work has been done on its biochemistry, metabolism, cell biology, physiology, and genetics. As a result, detailed information on a major fraction of the total genomic sequence can be understood in terms of the known gene functions and gene products (RNA and proteins). As the databases on gene sequences from many organisms grow rapidly, chances that function by similarity to other known genes can be imputed continue to increase. Thus the fraction of totally unknown genes in *E. coli* is much lower than for other organisms. Table 1 summarizes the kinds of gene products made in *E. coli* K-12 strain MG1655, both known and imputed by sequence similarity (for updates see [{genprotec.mbl.edu}{GeneProtEC database]). About half of the gene products are known from experimental biology, about one quarter are assigned functions imputed from sequence similarity to known gene products, and the remainder are open reading frames of unknown function.

Of known and tentatively known genes, the largest category encodes enzymes, followed by categories that encode transporters and regulators. (Smaller functional categories include cellular structural components, factors, and RNAs.)

Table 1. Numbers of *Escherichia coli* genes of different types.

Category	Experimental	Putative	Total
Enzymes	955	361	1,316
Transport proteins	281	295	576
Regulators	208	121	329
Factors	103	44	147
Structures	93	37	130
RNAs	114		114
Membrane	50	49	99
Carriers	30	17	47
Leaders	12		12
External origin	284		284
Phenotype	117		117
ORFs			1,225
Total	2,247	924	4,396

Abbreviation: ORF, open reading frame.

The number of genes for enzymes is known to be even higher than the current tally because genes have not been identified as yet for 52 enzymes (known biochemically to be present in *E. coli*; Table 2). Information on transporters (http://www.biology.ucsd.edu/~iapulsen/transport/) and regulators (http://www.cifn.unam.mx/Computational_ Biology/regulondb/) (the next largest categories) has been assembled into accessible databases.

A framework for designating functions of gene products was devised (Riley, 1993; Riley and Labedan, 1996) and used to classify the physiological roles of gene products of sequenced genomes. The assignments for *E. coli* have been improved as understanding has increased. Current designations can be accessed at the [{GenProtEC database}{genprotec.mbl.edu}]. However, it has become increasingly apparent that the assignment of just one role to any one-gene product is often artificial. For instance, many enzymes and enzyme subunits as well as transport elements are counted as "enzymes" when in fact they are located in the inner membrane and thereby constitute an important part of the cell structure. Because they are not registered as membrane proteins, the count of total membrane proteins in the cell is artificially low. Other multi-role cases also exist, so the division of function in Table 1 is to some extent artificial.

How densely packed is the *E. coli* chromosome with coding information and regulatory sequences? Some genes abut precisely, such as genes within operons; a few even have an overlap of the stop codon of one protein with the f-Met initiating codon of the next. Two genes are particularly dense in information, giving rise to two polypeptides from each transcript (the DNA polymerase III gamma subunit from the *dnaX* gene and spermidine biosynthesis gene *speD*). How much is there of intergenic DNA, noncoding DNA that falls between coding regions? Rudd finds 11.4% of the genome in intergenic spaces and has analyzed them for presence of repeat sequences (Rudd, 1999).

Some genes clearly have been incorporated into the *E. coli* chromosome from other sources from time to time. Presence and absence of genes and operons in different *E. coli* strains may signal extrachromosomal origins. Genes that come from the outside and vary in number and genetic location from strain to strain are genes of prophages (most no longer viable as independent phages), insertion elements, or transposons that replicate themselves and move within the genome. These constitute 6.4% of the genome of *E. coli* K-12 strain MG1655 (see class of genes of extrachromosomal origin in the {GenProtEC database}({genprotec.mbl.edu}). An early

Table 2. Enzymes purified from *Escherichia coli*, but not as yet identified with genes.

EC[a] number	Enzyme
1.1.1.111	Imidazol-5-yl-lactate dehydrogenase
1.1.1.214	2-Dehydropantoyl-lactone reductase (B-specific)
1.1.1.5	Diacetyl reductase
1.1.1.75	(R)-Aminopropanol dehydrogenase
1.1.3.3	Malate oxidase
1.1.99.14	Glycolate dehydrogenase
1.2.1.16	Succinate semialdehyde dehydrogenase II, NAD-dependent
1.2.1.19	Aminobutyraldehyde dehydrogenase
1.2.7.1	Pyruvate synthase (pyruvate ferredoxin reductase)
1.3.1.10	Enoyl-ACP-reductase, NADPH-specific
1.6.2.5	NADPH-cytochrome-c2 reductase
1.6.99.2	NAD(P)H dehydrogenase (quinone)
1.8.4.2	Glutaredoxin reductase
2.3.1.4	Glucosaminephospho-*N*-acetyltransferase
2.4.2.5	Nucleoside ribosyltransferase
2.6.1.29	Diamine transaminase
2.6.1.31	Pyridoxamine-oxaloacetate transaminase
2.6.1.38	Histidine transaminase
2.6.1.54	Pyridoxamine-phosphate transaminase
2.7.1.121	Phosphoenolpyruvate-glycerone phosphotransferase
2.7.1.23	NAD kinase
2.7.1.24	Dephospho-CoA kinase
2.7.1.7	Mannokinase
2.7.7.18	Nicotinate-nucleotide adenylyltransferase
2.8.1.2	3-Mercaptopyruvate sulfurtransferase
3.1.1.17	Gluconolactonase
3.1.3.-	NADP phosphatase
3.1.2.2	Palmitoyl-CoA hydrolase
3.1.3.27	Phosphatidylglycerolphosphatase C
3.2.1.68	Isoamylase
3.2.2.14	NMN nucleosidase
3.2.2.5	NAD(+) nucleosidase
3.5.1.2	Glutaminase A
3.5.1.2	Glutaminase B
3.5.1.42	Nicotinamide-nucleotide amidase
3.6.1.-	Dihydroneopterin triphosphate pyrophosphohydrolase
3.6.1.-	Dihydroneopterin monophosphate dephosphorylase
3.6.1.21	ADP-sugar pyrophosphatase
3.6.1.22	NAD(+) pyrophosphatase
3.6.1.9	Nucleotide pyrophosphatase
4.1.1.36	Phosphopantothenoylcysteine decarboxylase
4.1.3.9	2-Hydroxyglutarate synthase
4.4.1.1	Cystathionine gamma-lyase/L-cysteine desulfhydrase
4.4.1.15	D-Cysteine desulfhydrase
4.5.1.2	3-Chloro-D-alanine dehydrochlorinase
4.6.1.2	Guanylate cyclase
5.1.99.1	Methylmalonyl-CoA epimerase
5.3.1.13	D-Arabinose-5-phosphate isomerase
5.4.2.3	Phosphoacetylglucosamine mutase
6.3.2.5	Phosphopantothenate cysteine ligase
6.3.4.3	Formate-tetrahydrofolate ligase
6.4.1.3	Propionyl-CoA carboxylase

Abbreviations: CoA, coenzyme A; NAD, nicotinamide adenine dinucleotide; NADP nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; NMN, nicotinamide mononucleotide.
[a]EC, Enzyme Comission.

approach to identifying such acquired genes was based on comparing genetic maps to find genetic information present in one bacterial genus not in a closely related genus (Riley and Anilionis, 1978). Comparing gene contents of laboratory strains and pathogens identifies "pathogenicity islands" that may have been incorporated by the pathogenic strains (Ohnishi et al., 1999; Perna et al., 1998). Methods of identifying horizontally transferred genes involve differences in base composition and codon usage of genes (Danchin et al., 2000; Lawrence and Ochman, 1998; Medigue et al., 1991; Mrazek and Karlin, 1999; Ochman, 2000). Estimates of the total content of relatively recently acquired genes in the *E. coli* K-12 MG1655 genome are around 17% (Lawrence and Ochman, 1998).

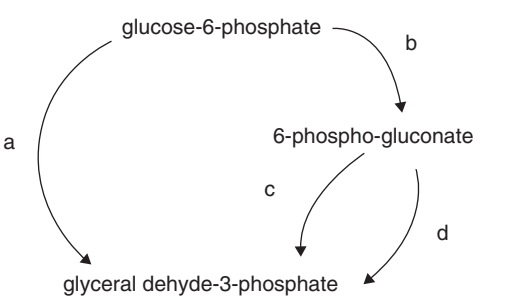
The Versatile Metabolism of *Escherichia coli*

Escherichia coli is a very versatile organism that can exist as an aerobe, anaerobe, or as a facultative anaerobe that can ferment sugars or amino acids (and can degrade proteins, polysaccharides and nucleic acids to provide the low molecular weight material). When oxygen is the ultimate electron acceptor for an oxidative chain, the organism is growing by aerobic respiration, but when nitrate or organic compounds such as fumarate are the final electron acceptors, the organism is growing by anaerobic respiration. Glucose can generate energy in conversion to triose phosphate by glycolysis, the pentose phosphate pathway or the Entner-Douderoff pathway (Fig. 1). Other sugars are converted to glucose or to intermediates in these pathways. When the available carbon source is further down the energy scale, such as acetate or succinate, the gluconeogenesis pathway is invoked to generate necessary precursors for biosynthetic pathways.

Thus many alternate paths from different starting points are available to support the life of *E. coli*, all generating energy and reducing power for biosynthesis, some more efficient than others. Regulatory machinery detects the availability of substrates for catabolism and the presence of suitable electron acceptors for oxidative pathways. Regulation induces or activates the appropriate enzymes for the appropriate pathways.

Escherichia coli can grow on rich media containing digests of proteins and many pre-made organic compounds, or it can make do on a minimal medium that provides one organic carbon source (such as glucose), an organic or inorganic source of nitrogen, and inorganic salts (as sources of K, Na, Cl, P, S, Mg, Fe, and trace elements). The versatility is reflected in the regulation machinery that induces synthesis of enzymes necessary for the available energy source and that turns on biosynthetic pathways when required substances are absent or shuts them down by feedback inhibition or repression when they are present in the environment.

It might be supposed that a chromosome containing over 4,400 genes (about 1,000 coding for enzymes) is necessary for *E. coli* to grow on defined minimal medium (Table 1). However, this is only part of the story. Versatility and a certain amount of redundancy account for some of the genetic capacity. Examples are present in the catabolic metabolism from glucose to glyceraldehyde-3-phosphate (Fig. 1). Also, the early steps of gluconeogenesis provide an example of alternate pathways to generate phosphoenol pyruvate from malate (Fig. 2). After pyruvate,



a. Glycolysis
b. Pentosephosphate pathway, oxidative branch
c. Entner-Doudoroff pathway
d. Pentosephosphate pathway, non-oxidative branch

Fig. 1. Degradation of glucose 6-phosphate via: a) glycolysis; b) pentose phosphate pathway, oxidative branch; c) Entner-Douoroff pathway; and d) pentose phosphate pathway, non-oxidative branch.

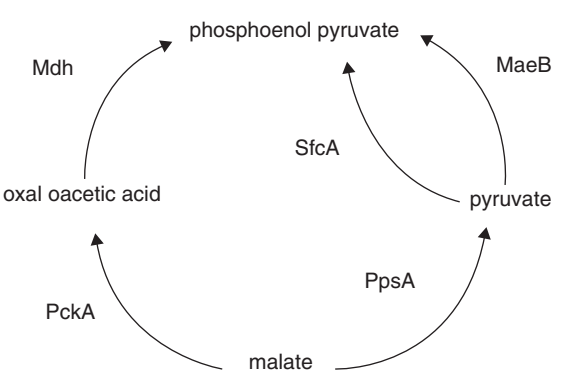


Fig. 2. Synthesis of phosphoenol pyruvate pathway from malate in the gluconeogenesis pathway. Mdh indicates Malate dehydrogenase; MaeB, Maleic enzyme; SfcA, NAD-linked Malate dehydrogenase; PpsA, Phosphoenolpyruvate synthase; PckA, Phosphoenolpyruvate carboxylase.

the path chosen depends on whether conditions are aerobic or anaerobic and what electron acceptors are present. During fermentative metabolism, *E. coli* may reduce phosphoenol pyruvate to a variety of compounds (Fig. 3), and alternatives are available at almost every turn. For instance, many "contingency" pathways evidently are present to deal with circumstances as they come into play. Fitting this description are the many pathways that bring carbohydrates (other than glucose) into glycolysis. Some of these feeder pathways are tailored for aerobic or anaerobic conditions. There are alternate glycerate dehydrogenases that cover this possibility.

In nitrogen metabolism of *E. coli*, inorganic ammonium ion, nitrite or nitrate is sufficient as a source of nitrogen. Should organic sources of nitrogen be available, many transaminases and amidotransferases can harvest amino or amido nitrogen from organic compounds.

Many pathways bifurcate where there are two enzymes available for a given step. In some cases, each one of the pair uses a different cofactor. This allows the organism greater flexibility in case one of the cofactors is not available in the cell. For examples of bifurcations, see Table 3. In the formyl tetrahydrofolate (THF) biosythesis pathway, THF is converted to 5,10-methylene-THF by four alternate paths (1a–d). Two of these paths contain 1 step (1a, b) whereas the other two (1c, d) carry out the conversion in two steps. Purine metabolism provides two examples of bifurcations: 1a, b (5-phosphoribosyl-glycinamide to 5′-phosphoribosyl-*N*-formylglycinamide) and 2a, b (xanthosine-5-phosphate to guanosine monophosphate [GMP]), all of which are one-step reactions. Table 4 lists instances of more than one enzyme known in *E. coli* to carry out the same or almost the same reaction. In some cases the physiological conditions under which each enzyme is active are known, but not all. Some of the multiple enzymes are used in different pathways with different end products and thus could be regulated differently, such as the three diaminohydroxypyrimidine (DAHP) synthetases (2-dehydro-3-deoxyphosphoheptonate aldolase) in aromatic amino acid biosynthesis. The three enzymes are regulated separately by tyrosine, phenylalanine or tryptophan (Pittard, 1996). Some of the enzymes listed in Table 4 are isozymes in that they are closely similar in amino acid sequence as well as reaction mechanism, whereas others carry out the same reaction but have quite different amino acid sequences (see Riley and Serres, 2000 for examples). Clearly *E. coli* possesses far more than a minimum set of enzymes needed to grow at 37°C aerobically on a glucose minimal medium. Many fewer than the 4,400 genes would be needed for that purpose.

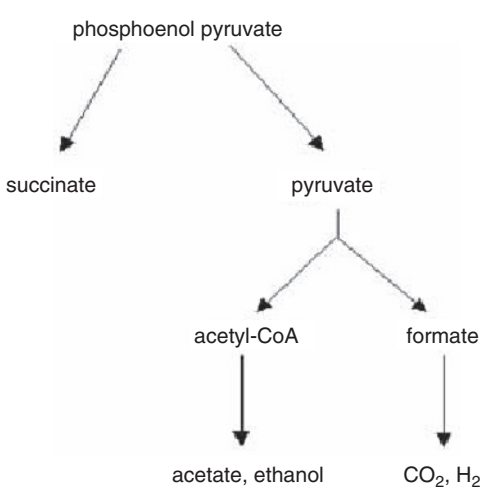


Fig. 3. Fermentative metabolism of phosphoenol pyruvate.

The multiplicity of genetic capacity in *E. coli* is not only present among enzymes but also extends to transporters and regulators. A system has been constructed for classifying transporters (http://www.biology.ucsd.edu/~ipaulsen/transport/) by type (Saier, 2000). *Escherichia coli* frequently has more than one type of transport system for any given compound to be transported. Examples are given in Table 5. As to regulators, action at different levels means that several regulators may act on a single operon to control its expression. The *lac* operon is under the control of the specific *lac* repressor and of the more general cyclic-AMP catabolism regulator, and it is subject to broad controls rendered for switches between aerobic and anaerobic growth.

Because *Escherichia coli* is rich with alternative mechanisms, multiplicity of options and some redundancy, its genome is far from minimal. There have been attempts to define a minimal bacterial genome (Hutchison et al., 1999; Kyrpides et al., 1999). It goes without saying that one must speak of a minimal genome under a stated set of conditions. Is it to allow growth as an intracellular parasite? Or is it to allow growth outside a host but in rich medium of a certain composition? Or is the condition to be growth in minimal medium of a certain composition? Is the intention to describe a genome that gives the organism a certain degree of adaptability or one that simply equips it for survival under only one condition of growth? Evidently there is not just one minimal genome, but rather a set of sparse genomes suitable to sustain life under different circumstances from rich to spare. It may be that the estimates of between 650 and 800 Kb for *Mycoplasma* spp. are at or near the minimum for those organisms.

Table 3. Examples of identical substrate-to-product conversions by multiple pathways.[a]

Pathway	Rx[b]	Gene Prod	EC[c]	Reactions
Asparagine biosynth.	1a	AsnA	6.3.1.1	L-Aspartate + ATP + NH_3 + H_2O = L-asparagine + Pi + AMP
	1b	AsnB	6.3.5.4	L-Aspartate + ATP + L-glutamine + H_2O = L-asparagine + L-glutamate + AMP
FormylTHF biosynth.	1a	Gcv[d]		THF + L-glycine + NAD = 5,10-methylene-THF + CO_2 + NH_3 + NADH + H^+
	1b	GlyA	2.1.2.1	THF + L-serine = 5,10-methylene-THF + H_2O + L-glycine
	1c	MetH	2.1.1.13	THF + L-methionine = 5-methyl-THF + homocysteine
		MetF	1.7.99.5	5-Methyl-THF + NAD = 5,10-methylene-THF + NADH
	1d	FolA	1.5.1.3	THF + NADP = 7,8-dihydrofolate + NADPH
		ThyA	2.1.1.45	7,8-Dihydrofolate + dTMP = 5,10-methylene-THF + dUMP
Glycogen catabolism	1a	AlP	2.4.1.1	Maltodextrins + phosphate = maltotetraose + glucose-1-phosphate
	1b	Isoamylase	3.2.1.68	Maltodextrins = maltotetraose
Ornithine degrad.[e]	1a	GabD	1.2.1.16	Succinate semialdehyde + NADP + H_2O = succinate + NADPH
	1b	Sad	1.2.1.24	Succinate semialdehyde + NAD + H_2O = succinate + NADH
Purine metabolism	1a	PurT	2.1.2.-	5-Phosphoribosyl-glycinamide + ATP + formate = 5′-phosphoribosyl-*N*-formylglycineamide + ADP + Pi
	1b	PurN	2.1.2.2	5-Phosphoribosyl-glycinamide + 10-formyl-THF = 5′-phosphoribosyl-*N*-formylglycineamide + THF
	2a	GuaA	6.3.4.1	Xanthosine-5-phosphate + ATP +NH_3 = GMP + AMP + pyrophosphate
	2b	GuaA	6.3.5.2	Xanthosine-5-phosphate + L-glutamine + H_2O + ATP = GMP + L-glutamate + AMP + pyrophosphate
Trehalose degrad.	1a	TreC	3.2.1.98	α,α-Trehalose 6-phosphate + H_2O = β-D-glucose + glucose-6-phosphate
	1b	TreE	3.1.3.12	α,α-Trehalose 6-phosphate + H_2O = trehalose + phosphate
		TreF	3.2.1.28	Trehalose + H_2O = 2 β-D-glucose

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; GMP, guanosine monophosphate; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide reduced; NADP, dihydronicotinamide adenine dinucleotide phosphate; NADPH, dihydronicotinamide adenine dinucleotide phosphate reduced; THF, tetrahydrofolic acid.

[a]Information from EcoCyc (http://ecocyc.DoubleTwist.com).

[b]Rx = reaction.

[c]EC = Enzyme Commission reaction classification.

[d]Gcv = GcvT, GcvH, GcvP and LpdA.

[e]Also 4-aminobutyrate degradation.

Table 4. Multiple enzymes that perform identical conversions in *Escherichia coli*.

Gene Product	EC number	Enzyme name
AdhE_2[a]	1.1.1.1	Alcohol dehydrogenase, Fe-requiring
AdhP	1.1.1.1	Alcohol dehydrogenase, propanol-preferring
AdhC_1	1.1.1.1	Alcohol dehydrogenase, class III
YqhE	1.1.1.-	2,5-Diketo-D-gluconate reductase A
YafB	1.1.1.-	2,5-Diketo-D-gluconate reductase B
ThrA_2	1.1.1.3	Homoserine dehydrogenase I
MetL_2	1.1.1.3	Homoserine dehydrogenase II
Dld	1.1.1.28	Lactate dehydrogenase, NADH-independent
LdhA	1.1.1.28	Lactate dehydrogenase, NAD-dependent
GarR	1.1.1.60	Tartronate semialdehyde reductase
GlxR	1.1.1.60	Tartronic semialdehyde reductase 2
GlpA,B,C	1.1.99.5	Glycerol-3-phosphate dehydrogenase, anaerobic
GlpD	1.1.99.5	Glycerol-3-phosphate dehydrogenase, aerobic
FdhF	1.2.1.2	Formate dehydrogenase H
FdnG,H,I	1.2.1.2	Formate dehydrogenase N
FdoG,H,I	1.2.1.2	Formate dehydrogenase O
AdhE_1	1.2.1.10	Acetaldehyde-CoA dehydrogenase
MhpF	1.2.1.10	Acetaldehyde-CoA dehydrogenase
GabD	1.2.1.16	Succinate semialdehyde dehydrogenase (NADP)
Sad	1.2.1.24	Succinate semialdehyde dehydrogenase (NAD)
AldA	1.2.1.22	Aldehyde dehydrogenase A (NAD)

Table 4. *Continued*

Gene Product	EC number	Enzyme name
AldB	1.2.1.22	Aldehyde dehydrogenase B (NAD)
AldH	1.2.1.3	Aldehyde dehydrogenase (NADP/NAD)
FrdA,B,C,D	1.3.99.1	Fumarate reductase
SdhA,B,C,D	1.3.5.1	Succinate dehydrogenase
PntA,B	1.6.1.1	Pyridine nucleotide transhydrogenase, membrane-bound
UdhA	1.6.1.1	Pyridine nucleotide transhydrogenase, soluble
TorA	1.6.6.9	Trimethylamine *N*-oxide reductase I, inducible
—	1.6.6.9	Trimethylamine *N*-oxide reductase II, constitutive
NarG,H,I,J	1.7.99.4	Nitrate reductase 1
NarZ,Y,V,W	1.7.99.4	Nitrate reductase 2
NapA,B,C,D	1.7.99.4	Nitrate reductase, periplasmic
CydA,B	1.10.3.-	Cytochrome BD-I oxidase
AppC,B	1.10.3.-	Cytochrome BD-II oxidase
CyoB,A,C	1.10.3.-	Cytochrome O ubiquinol oxidase
KatG	1.11.1.6	Catalase HPI
KatE	1.11.1.6	Catalase HPII
SodA	1.15.1.1	Superoxide dismutase (Mn)
SodB	1.15.1.1	Superoxide dismutase (Fe)
SodC	1.15.1.1	Superoxide dismutase (Cu-Zn)
NrdA,B	1.17.4.1	Ribonucleoside diphosphate reductase 1
NrdE,F	1.17.4.1	Ribonucleoside diphosphate reductase 2
HyaA,B	1.18.99.1	Hydrogenase 1
HybO,C	1.18.99.1	Hydrogenase 2
HycB,E	1.18.99.1	Hydrogenase 3
MetH	2.1.1.13	Homocysteine transmethylase, B12-dependent
MetE	2.1.1.14	Homocysteine transmethylase, B12-independent
Ada	2.1.1.63	O-6-Methylguanine-DNA methyltransferase, inducible
Ogt	2.1.1.63	O-6-Methylguanine-DNA methyltransferase, constitutive
PurN	2.1.2.2	Phosphoribosylglycinamide formyltransferase 1
PurT	2.1.2.-	Phosphoribosylglycinamide formyltransferase 2
ArgI	2.1.3.3	Ornithine carbamoyltransferase 1
ArgF	2.1.3.3	Ornithine carbamoyltransferase 2
TktA	2.2.1.1	Transketolase 1
TktB	2.2.1.1	Transketolase 2
TalA	2.2.1.2	Transaldolase A
TalB	2.2.1.2	Transaldolase B
RimI	2.3.1.128	Ribosomal-protein-alanine acetyltransferase (S18)
RimJ	2.3.1.128	Ribosomal-protein-alanine acetyltransferase (S5)
FabB	2.3.1.41	3-Oxoacyl-ACP synthase I
FabF	2.3.1.41	3-Oxoacyl-ACP synthase II
FabH	2.3.1.41	3-Oxoacyl-ACP synthase III
PflB	2.3.1.54	Pyruvate formate lyase I
TdcE	2.3.1.54	Pyruvate formate lyase IV
GlgP	2.4.1.1	Glycosyl transferase
MalP_2	2.4.1.1	Glycosyl transferase
DeoD	2.4.2.1	Purine nucleoside phosphorylase
XapA	2.4.2.1	Purine nucleoside phosphorylase
GabT	2.6.1.19	4-Aminobutyrate aminotransferase
GoaG	2.6.1.19	4-Aminobutyrate aminotransferase
PfkA	2.7.1.11	6-Phosphofructokinase I
PfkB	2.7.1.11	6-Phosphofructokinase II
IdnK	2.7.1.12	Gluconate kinase 1
GntK	2.7.1.12	Gluconate kinase 2
PdxK	2.7.1.35	Pyridoxal kinase 1
PdxY	2.7.1.35	Pyridoxal kinase 2
PykF	2.7.1.40	Pyruvate kinase I
PykA	2.7.1.40	Pyruvate kinase II
AroK	2.7.1.71	Shikimate kinase I
AroL	2.7.1.71	Shikimate kinase II
TdcD	2.7.2.-	Propionate kinase
AckA	2.7.2.-	Propionate kinase II
AckA	2.7.2.1	Acetate kinase A
AckB	2.7.2.1	Acetate kinase B

(Continued)

Table 4. *Continued*

Gene Product	EC number	Enzyme name
ThrA_1	2.7.2.4	Aspartokinase I
MetL_1	2.7.2.4	Aspartokinase II
LysC	2.7.2.4	Aspartokinase III
RelA	2.7.6.5	(p)ppGpp synthetase I
SpoT	2.7.6.5	(p)ppGpp synthetase II
AcpS	2.7.8.7	Holo-[acyl carrier protein] synthase
AcpT	2.7.8.7	Holo-[acyl carrier protein] synthase 2
TesA	3.1.1.5	Lysophospholipase I
PldB	3.1.1.5	Lysophospholipase II
TesA	3.1.2.-	Acyl CoA thioesterase I
TesB	3.1.2.-	Acyl CoA thioesterase II
RnhA	3.1.26.4	RNase HI
RnhB	3.1.26.4	RNAse HII
TreE	3.1.3.12	Trehalose-6-phosphate phosphatase, catabolic
OtsB	3.1.3.12	Trehalose-6-phosphate phosphatase, biosynthetic
PphA	3.1.3.16	Protein phosphatase 1
PphB	3.1.3.16	Protein phosphatase 2
PgpA	3.1.3.27	Phosphatidylglycerophosphatase A
PgpB	3.1.3.27	Phosphatidylglycerophosphatase B
PgpC	3.1.3.27	Phosphatidylglycerophosphatase C
GlpQ	3.1.4.46	Glycerophosphodiester phosphodiesterase, periplasmic
UgpQ	3.1.4.46	Glycerophosphodiester phosphodiesterase, cytosolic
AmyA	3.2.1.1	α-Amylase, cytoplasmic
MalS	3.2.1.1	α-Amylase, periplasmic
EbgA,C	3.2.1.23	β-D-galactosidase, cryptic
LacZ	3.2.1.23	β-D-galactosidase
TreA	3.2.1.28	Trehalase, periplasmic
TreF	3.2.1.28	Trehalase, cytoplasmic
BglA	3.2.1.86	6-Phospho-β-glucosidase A, cryptic
BglB	3.2.1.86	6-Phospho-β-glucosidase B, cryptic
AscB	3.2.1.86	6-Phospho-β-glucosidase (salicin, cellobiose), cryptic
CelF	3.2.1.86	6-Phospho-β-glucosidase (salicin, cellobiose, arbutin)
DacA	3.4.16.4	D-Alanyl-D-alanine carboxypeptidase (PBP-5)
DacB	3.4.16.4	D-Alanyl-D-alanine carboxypeptidase (PBP-4)
DacC	3.4.16.4	D-Alanyl-D-alanine carboxypeptidase (PBP-6)
DacD	3.4.16.4	D-Alanyl-D-alanine carboxypeptidase (PBP-6B)
AnsA	3.5.1.1	Asparaginase I
AnsB	3.5.1.1	Asparaginase II
AmiA	3.5.1.28	*N*-Acetylmuramoyl-l-alanine amidase I
AmiB	3.5.1.28	*N*-Acetylmuramoyl-l-alanine amidase II
FolE	3.5.4.16	GTP-cyclohydrolase I
RibA	3.5.4.25	GTP-cyclohydrolase II
GppA	3.6.1.11	Exopolyphosphatase, cytoplasmic
Ppx	3.6.1.11	Exopolyphosphatase, membrane associated
GadA	4.1.1.15	Glutamate decarboxylase, alpha
GadB	4.1.1.15	Glutamate decarboxylase, beta
SpeC	4.1.1.17	Ornithine decarboxylase, constitutive
SpeF	4.1.1.17	Ornithine decarboxylase, inducible
CadA	4.1.1.18	Lysine decarboxylase 1
LdcC	4.1.1.18	Lysine decarboxylase 2
AdiA	4.1.1.19	Arginine decarboxylase, biodegradative
SpeA	4.1.1.19	Arginine decarboxylase, biosynthetic
GatY	4.1.2.-	Tagatose-bisphosphate aldolase 1
AgaY	4.1.2.-	Tagatose-bisphosphate aldolase 2
FbaB	4.1.2.13	Fructose-bisphosphate aldolase class I
FbaA	4.1.2.13	Fructose-bisphosphate aldolase class II
AroF	4.1.2.15	DAHP synthetase, Tyr sensitive
AroG	4.1.2.15	DAHP synthetase, Phe sensitive
AroH	4.1.2.15	DAHP synthetase, Trp sensitive
AceB	4.1.3.2	Malate synthase A
GlcB	4.1.3.2	Malate synthase G
IlvB,N	4.1.3.18	Acetolactate synthase I
IlvG,M	4.1.3.18	Acetolactate synthase II

Table 4. *Continued*

Gene Product	EC number	Enzyme name
IlvI,H	4.1.3.18	Acetolactate synthase III
FumA	4.2.1.2	Fumarase A
FumB	4.2.1.2	Fumarase B
FumC	4.2.1.2	Fumarase C
AcnA	4.2.1.3	Aconitase 1
AcnB	4.2.1.3	Aconitase 2
SdaA	4.2.1.13	Serine dehydratase 1
SdaB	4.2.1.13	Serine dehydratase 2
TdcG	4.2.1.13	Serine dehydratase 3
IlvA	4.2.1.16	Threonine dehydratase, biosynthetic
TdcB	4.2.1.16	Threonine dehydratase, catabolic
EntC	5.4.99.6	Isochorismate synthase, inducible
MenF	5.4.99.6	Isochorismate synthase, menaquinone-specific
LysS	6.1.1.6	Lysine tRNA synthetase, constitutive
LysU	6.1.1.6	Lysine tRNA synthetase, inducible
AsnA	6.3.1.1	Asparagine synthetase A
AsnB	6.3.5.4	Asparagine synthetase B
DdlA	6.3.2.4	D-Alanine-D-alanine ligase A
DdlB	6.3.2.4	D-Alanine-D-alanine ligase B

Abbreviations: DTDP, deoxythymidine diphosphate; FKBP, FK-506-binding protein. Refer to footnotes in Table 3 for all other abbreviations.

[a]1, indicates the N-terminal, and 2, the C-terminal, part of the molecule has the activity.

Table 5. Examples of multiple transporters for the same compound.

Compound	Transporter	Gene(s)	Comments
Galactose	ABC superfamily	*mglA, B, C*	
	MFS family	*galP*	
Gluconate	GntP family	*gntU*	Low affinity transporter
	GntP family	*gntT*	High affinity transporter
	GntP family	*gntP*	Possibly additional "substrates"
	GntP family	*idnT*	Primarily L-idonate transporter
Nitrite	FNT family	*nirC*	Nitrite uptake
	MFS superfamily	*narK*	Nitrite extrusion
	MFS superfamily	*narU*	Nitrite extrusion
Nucleosides	MFS superfamily	*nupG*	
	NUP family	*nupC*	
Phenylalanine	APC family	*pheP*	
	APC family	*aroP*	Also transports tyrosine
Phosphate	ABC superfamily	*pstABCS*	High-affinity transporter
	PiT family	*pitA*	Low-affinity transporter
	PiT family	*pitB*	Low-affinity transporter
Proline	ABC superfamiliy	*proVWX*	Also transports glycine, betaine
	APC family	*proY*	Cryptic
	MFS superfamily	*proP*	Also transports betaine
	SSS family	*putP*	
Putrescine	ABC superfamily	*potFGHI*	
	ABC superfamily	*potDCBA*	Also transports spermidine
	APC family	*potE*	Also transports ornithine

Abbreviations: ABC, ATP-binding cassette; MFS, major facilitator superfamily; GntP, gluconate permease; FNT, formate nitrite transporter; NUP, nucleoside uptake permease; PiT, inorganic phosphate transporter; APC, amino acid polyamine choline; SSS, solute sodium symporter.

Genes and Enzymes

The gene-enzyme relationship can be complex. The insightful doctrine of "one gene-one enzyme," originally set forth by Beadle and Tatum (1941), paved the way for decades of fruitful progress in understanding what a gene does. Later Benzer articulated the relationship of a gene to *trans* complementation. Based on phage studies, he saw that the basic gene component,

Table 6. Stoichiometry of genes, polypeptides, enzymes and their reactions.

Gene	Enzyme	EC number[a]	Ratios of gene : polypeptides : enzyme:reaction
fumA	Homodimer	4.2.1.2	3 : 3 : 3 : 1
fumB	Homodimer	4.2.1.2	3 : 3 : 3 : 1
fumC	Homotetramer	4.2.1.2	3 : 3 : 3 : 1
fadB	Homodimer	5.3.3.8, 4.2.1.17, 1.1.1.35, 5.1.2.3	1 : 1 : 1 : 4
trpD	Homodimer	2.4.2.18	2 : 2 : 1 : 2
trpD, E	Heterotetramer 2 : 2	4.1.3.27	2 : 2 : 1 : 2
trpE	Homodimer	4.1.3.27	2 : 2 : 1 : 2
hisF,H	Heterodimer 1 : 1	2.4.2.-	2 : 2 : 1 : 1
hisF	Monomer	—	2 : 2 : 1 : 1
sdhA, sdhB, sdhC, sdhD	Heterotetramer 1 : 1 : 1 : 1	1.3.5.1	4 : 4 : 1 : 1
sped	Heterooctamer 4 : 4	4.1.1.50	1 : 2 : 1 : 1
dnaX	Gamma and tau subunits of complex 1 : 1	2.7.7.7	1 : 2 : 1 : 1
aceE, aceF, lpd	Multienzyme complex 24 : 24 : 12	1.2.4.1, 2.3.1.12,1.8.1.4	3 : 3 : 1 : 3

[a]EC indicates Enzyme Commission.

which he dubbed "the cistron" (Benzer, 1955), was able to function as an independent complementing unit and encode one polypeptide chain.

Today the extent and sequence of many genes and their products can be known with more precision, i.e., what they do in the cell and the phenotypes brought on by their absence. It can be seen that one functional protein complex may require many genes, and a single polypeptide with many functions may require only one gene. Clearly complex structures such as ribosomes require the coordinated action of several assembly proteins to put together in correct spatial and temporal relationship the many protein and RNA constituents of the structure. Some examples of complex gene-enzyme relationships are listed in Table 6. The stoichiometry of gene : polypeptide : enzyme : reaction can be 3 : 3 : 3 : 1 as for the three isozymes of fumarase, where there are three genes—one for each polypeptide—but only one reaction. For the fatty acid degradation polypeptide FadB there is one gene, one polypeptide, one enzyme, but four separate catalytic reactions. The example of TrpD (a tryptophan biosynthesis enzyme) is more complicated. The C-terminal part of TrpD is a straightforward 1 : 1 : 1 : 1 enzyme for EC reaction 2.4.2.18. In addition the N-terminal glutamine amidotransferase part of TrpD forms a heterotetrameric complex with TrpE to generate anthranilate synthase (EC 4.1.3.27). The TrpDE encoded enzyme uses glutamine or ammonia as a nitrogen donor, though TrpE can carry out the reaction by itself using ammonium as the nitrogen donor. This situation is similar to the reaction carried out by imidazole glycerol phosphate synthase (EC 2.4.2.-) where the histidine biosynthesis (HisFH) enzyme complex uses glutamine as the nitrogen donor and HisF alone uses ammonium as the nitrogen donor.

Succinate dehydrogenase is an example of a multiple subunit enzyme. Many dehydrogenases take this form. The relationships are 4 : 4 : 1 : 1, four genes and polypeptides and one four-subunit enzyme for one reaction. For spermidine biosynthesis gene *speD*, whose transcript is processed to give two different polypeptides that come together as a heterodimer, the ratio is 1 : 2 : 1 : 1, and in a similar case the *dnaX* gene is transcribed into two polypeptides that function as separate subunits in the DNA polymerase III holoenzyme. One of the most complex enzymes in *E. coli* is the large pyruvate dehydrogenase multienzyme complex. With three genes making three independent enzymes that complex together to carry out one overall reaction, the ratio can be expressed as 3 : 3 : 1 : 3. Yet another type of complexity not mentioned in Table 6 follows. The two homodimers PheA and TyrA of phenylalanine and tyrosine biosynthesis, respectively, both have chorismate mutase activity (EC 5.4.99.5). Each has a different second reaction: PheA has prephenate dehydratase activity (EC 4.2.1.51) and TyrA has prephenate dehydrogenase activity (EC 1.3.1.12). Each would need to be described as 1 : 1 : 1 : 2. These and other similar variations illustrate the complexity and variations involved in assembling the elements of enzymes for metabolic functions from the information encoded in the DNA of genes. It now is known that the simple view of one gene making one enzyme is not always the case. Interpreta-

tion of phenotypes of mutants and functions of genes takes this complexity into account.

Molecular Evolution

Multiple Enzymes

The versatility of *E. coli* is seen in the cases of both multiple enzymes used for one reaction and multiple reactions catalyzed by one enzyme that is a single protein used in more than one context. The *lpdA* gene encodes lipoamide dehydrogenase. This enzyme is a necessary component of the two similar dehydrogenase complexes, the pyruvate and oxalglutarate dehydrogenase complexes. It is also a necessary part of the glycine cleavage complex which releases CO_2 from glycine. This kind of multiple use of single proteins has been called "moonlighting" (Jeffery, 1999). Moonlighting also could play a role in molecular evolution. If a gene that has more than one function is duplicated, it is easy to picture that one of the two copies might evolve to better carry out one function while the other copy might concentrate on the other function. In time, three specialized *lpdA* genes and proteins, each especially fitted for one of the three roles, might be seen. This scenario is a variation of the evolutionary mechanism "enzyme recruitment" that pictures cooption of a protein with one function to accomodate to another related function (Ahmad and Jensen, 1988; Jensen, 1976).

Fused Genes

Fused genes also reflect changes over time. Some of the genes of *E. coli* act as if two or more independently functioning genes have fused to give a multimodular gene with two or more independent functions. Such fusions are easiest to find in enzymes, but also exist in regulators (such as those for both sensor and response, which are in the same gene, e.g., *arcB*). In transport, phosphotransferase enzymes IIA, IIB and IIC are found sometimes in one polypeptide (NagE), sometimes in pairs (FrvA = IIA and FrvB = IIBC), or divided into three separate genes and polypeptides (CelC = IIB, CelA = IIC and CelB = IIA).

Multimodular proteins seem to have undergone fusions relatively recently in evolution as is indicated by the different patterns of fusion found in different microorganisms (Crawford, 1975). Multimodularity can cause artifact in procedures that search for sequence similarities among proteins. The separate modules each with a unique evolutionary history must be recognized and separated, if true sequence relationships are to be identified and quantified (Riley and Labedan, 1996; 1997).

Molecular Mechanisms of Change

What other aspects of molecular evolution are brought forward by the study of genomics and comparative genomics? From the point of view of chemistry of DNA, molecular mechanisms of evolution have been proposed for many years. Point mutations and frameshifts predominate at close distances. Comparisons of either DNA or protein sequences of related genes in related organisms often reveal that small insertions and deletions have also occured, resulting in gaps in the alignments. One does not know whether the gaps represent replication errors or incorporation or losses of oligonucleotides from genes. New functions are generally believed to have been created over time first by duplication and then by divergence. Acquisition of DNA from the outside contributes to change when foreign DNA (integrated by recombination into the genome) is retained. Rearrangements of genetic material within the chromosome also occur, changing gene order between genera as between *E. coli* and *Salmonella enterica* (Riley and Labedan, 1997) and among strains of *E. coli*. These rearrangements do not seem to affect integrity of the chromosome or the phenotype of the organism.

Does knowledge of the complete sequence of a genome offer an opportunity to understand the molecular basis of evolution any better? Now with fully sequenced genomes, detailed analysis of chromosomal sequences and comparisons between organisms provide examples of the kinds of changes that can underlie evolutionary change. Comparisons of whole genomes give information on acquisition/loss of genes and occurrence of rearrangements. Construction of trees for comparable sequences among many organisms (orthologous relationships) does not depend on having complete genome sequences. On the other hand, with complete genome sequences true orthologous pairs can be identified with more confidence. In addition, with complete genome sequences, sets of related sequences within any one organism (paralogous relationships) can be studied and give information on families of proteins descended during ancient and recent evolution from common ancestors.

Ancestral Proteins

Ancestors of a family of proteins present in all branches of the tree of life are believed to have arisen early in evolution. Duplication within ancestral organisms provided copies of an ances-

tral gene that could become specialized by divergence. Because the functions of so many *E. coli* proteins are known, the composition and functions of groups of sequence-similar genes and proteins can clarify mechanisms of evolution (Labedan and Riley, 1999). The sequence-similar paralogous groups of earliest origins will have counterparts in all or most other organisms, such as the set of 80 ATP-binding-cassette (ABC) transport proteins. Sequence-similar groups of more recent origin can be identified by the fact that they exist only in some related species.

The paralogous groups (sets of sequence-related proteins) within *E. coli* range in size from pairs to a group of 82 (P. Liang and M. Riley, manuscript in preparation; [{GenProtEC} (genprotec.mbl.edu)]. The smaller groups are mostly enzymes whereas the largest groups are mostly transporters and regulators. Larger groups may contain proteins whose functional requirements limit much change and are therefore still visibly related by sequence, even though divergence was ancient. Smaller groups and single proteins with no known partner evidently diverged widely, so that it is difficult to trace their early evolutionary history. At the present only their closest relatives can still be detected. Most of the proteins of this type in *E. coli* are enzymes.

Mechanisms of Divergence to New Functions

How did metabolic pathways evolve? Did each sequential enzyme in a pathway evolve from the one before by duplication and divergence (Riley and Sanderson, 1990), or did an entire pathway duplicate, changing specificity toward substrates while retaining the same general mechanism of catalytic conversions down the pathway? Coopting one type of enzyme to produce another related type is referred to as "recruitment" (Jensen, 1976). The short-chain dehydrogenase reductase (SDR) family seems be an example.

Only a few examples exist of related enzymes that function in individual pathways, as visualized by Horowitz and Leupold (1951). Table 7 lists cases in *E. coli* (i.e., genes of methionine, histidine and purine biosynthesis pathways and the synthesis of peptidoglycan). There are more examples known of proteins of similar sequence catalyzing similar reactions serving many pathways. Iron-sulfur subunits of dehydrogenases form a big family of sequence-related proteins. The ATP-binding subunits of ABC transporters also form a large family. A family of aminotransferases comprises enzymes for the same type of reaction but each with different substrate specificities. These examples speak to the validity of the recruitment model (Jensen, 1976).

Table 7. Sequence-similar genes of *Escherichia coli* found in the same metabolic pathway.

Pathway	Sequence similar genes
Methionine biosynthesis	*metB, metC*
Histidine biosynthesis	*hisA, hisF*
Purine biosynthesis	*purK, purT*
Peptidoglycan biosynthesis	*murC, murD, murE, murF*

Although most of the proteins in any one sequence-similar group in *E. coli* are closely related in function, there are exceptions. These exceptions may be able to tell us something about the way divergence proceeded in evolution.

Most sets of sequence-similar dehydrogenases of *E. coli* using NAD(P) as cofactor are made up only of proteins that catalyze the dehydrogenase reaction but use different substrates. However, one sequence-similar group of dehydrogenases also contains certain NAD(P) requiring epimerases and dehydratases. These enzymes are known to belong to the SDR class of NAD-requiring enzymes. They are related by binding sites for the NAD coenzyme and they utilize similar mechanisms of reaction that pass through or terminate at an oxidation or reduction step. The SDR group may be an example of how functions can begin to diverge among the progeny of gene duplication events. A common type of dehydrogenase seems to have thrown off variants that have become epimerases, dehydratases, and, in organisms other than *E. coli*, also isomerases. This behavior represents the divergence part of the duplication and divergence process.

Unknown or Unused Genes and Functions

How will the functions of *E. coli* genes that have no sequence match in current databases be learned? Laboratory projects are under way to find the phenotypes of mutants deleted for every *E. coli* gene. Some of these mutants will have no observable phenotype either because of multiple capabilities in *E. coli* that act like redundancies or because the phenotype is subtle or only observable under circumstances different from the laboratory environment. Expression analysis will provide clues. Large grids of unique oligonucleotides will identify by hybridization the transcripts that predominate when the bacteria are grown under specific conditions.

Conclusions

It comes as something of a surprise that in spite of the decades of experimental work on *E. coli* K-12 by many scientists, and in spite of having the complete sequence of the genome in hand, a

great deal is still unknown about the genetic content of *E. coli*, the functions of many of its genes, and all the complex intertwined mechanisms for coordinating gene expression as environmental circumstances change. There is much more work to be done.

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Literature Cited

Ahmad, S., and R. A. Jensen. 1988. New prospects for deducing the evolutionary history of metabolic pathways in prokaryotes: Aromatic biosynthesis as a case-in-point. Orig. Life Evol. Biosph. 18:41–57.

Beadle, G. W., and E. L. Tatum. 1941. Genetic control of biochemical reactions in neurospora. Proc. Natl. Acad. Sci. USA 27:499–506.

Benzer, S. 1955. Fine structure of a genetic region in bacteriophage. Proc. Natl. Acad. Sci. 41:344–354.

Blattner, F. R., G. Plunkett 3rd, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of Escherichia coli K-12. Science 277:1453–1474.

Crawford, I. P. 1975. Gene rearrangements in the evolution of the tryptophan synthetic pathway. Bacteriol. Rev. 39:87–120.

Danchin, A., P. Guerdoux-Jamet, I. Moszer, and P. Nitschke. 2000. Mapping the bacterial cell architecture into the chromosome. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 355:179–190.

Horowitz, N. H., and U. Leupold. 1951. Some recent studies bearing on the one gene-one enzyme hypothesis. Cold Spring Harbor Symposia on Quantitative Biology: Genes and Mutations 16:65–74.

Hutchison, C. A., S. N. Peterson, S. R. Gill, R. T. Cline, O. White, C. M. Fraser, H. O. Smith, and J. C. Venter. 1999. Global transposon mutagenesis and a minimal mycoplasma genome. Science 286:2165–2169.

Itoh, T., T. Okayama, H. Hashimoto, J. Takeda, R. W. Davis, H. Mori, and T. Gojobori. 1999. A low rate of nucleotide changes in Escherichia coli K-12 estimated from a comparison of the genome sequences between two different substrains. FEBS Lett. 450:72–76.

Jacob, F., and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Molec. Biol. 3:318–356.

Jeffery, C. J. 1999. Moonlighting proteins. Trends. Biochem. Sci. 24:8–11.

Jensen, R. A. 1976. Enzyme recruitment in evolution of new function. Ann. Rev. Microbiol. 30:409–425.

Kyrpides, N., R. Overbeek, and C. Ouzounis. 1999. Universal protein families and the functional content of the last universal common ancestor. J. Molec. Evol. 49:413–423.

Labedan, B., and M. Riley. 1999. Genetic inventory: Escherichia coli as a window on ancestral proteins. *In:* R. L. Charlebois (Ed.) Organization of the Prokaryotic Genome. ASM Press. Washington DC, 365.

Lawrence, J. G., and H. Ochman. 1998. Molecular archaeology of the Escherichia coli genome. Proc. Natl. Acad. Sci. USA 95:9413–9417.

Lederberg, J., and E. L. Tatum. 1946. Novel genotypes in mixed cultures of biochemical mutants of bacteria. Cold Spring Harbor Symposia on Quantitative Biology 11:113–114.

Maaloe, O., and N. O. Kjeldgaard. 1966. Control of Macromolecular Synthesis. W. A. Benjamin. New York, NY.

Medigue, C., T. Rouxel, P. Vigier, A. Henaut, and A. Danchin. 1991. Evidence for horizontal gene transfer in Escherichia coli speciation. J. Molec. Biol. 222:851–856.

Mrazek, J., and S. Karlin. 1999. Detecting alien genes in bacterial genomes. Ann. NY Acad. Sci. 18:314–329.

Neidhart, F. C. 1996. Escherichia Coli and Salmonella. ASM Press. Washington DC, 2882.

Ochman, H., J. G. Lawrence, and E. A. Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405:299–304.

Ohnishi, M., C. Tanaka, S. Kuhara, K. Ishii, M. Hattori, K. Kurokawa, T. Yasunaga, K. Makino, H. Shinagawa, T. Murata, K. Nakayama, Y. Terawaki, and T. Hayashi. 1999. Chromosome of the enterohemorrhagic Escherichia coli O157:H7: Comparative analysis with K-12 MG1655 revealed the acquisition of a large amount of foreign DNAs. DNA Res. 6:361–368.

Pardee, A. B., F. Jacob, and J. Monod. 1959. The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by E. coli. J. Molec. Biol. 1:165–178.

Perna, N. T., G. F. Mayhew, G. Posfai, S. Elliott, M. S. Donnenberg, J. B. Kaper, and F. R. Blattner. 1998. Molecular evolution of a pathogenicity island from enterohemorrhagic Escherichia coli O157:H7. Infect. Immunol. 66:3810–3817.

Pittard, A. J. 1996. Biosynthesis of the aromatic amino acids. *In:* F. Neidhardt, R. Curtiss 3rd, J. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (Eds.) Escherichia Coli and Salmonella. ASM Press. Washington DC, 2822.

Riley, M., and A. Anilionis. 1978. Evolution of the bacterial genome. Ann. Rev. Microbiol. 32:519–560.

Riley, M., and K. E. Sanderson. 1990. Comparative genetics of Escherichia coli and Salmonella typhimurium. *In:* K. Drlica, and M. Riley (Eds.) The Bacterial Chromosome. ASM Press. Washington DC, 469.

Riley, M. 1993. Functions of the gene products of Escherichia coli. Microbiol. Rev. 57:862–952.

Riley, M., and B. Labedan. 1996. Escherichia coli: Physiological functions and common ancestries. *In:* F. Neidhardt, R. Curtiss 3rd, J. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (Eds.) Escherichia Coli and Salmonella: Cellular and Molecular Biology. ASM Press. Washington DC, 2118–2202.

Riley, M., and B. Labedan. 1997. Protein evolution viewed through Escherichia coli protein sequences: Introducing the notion of a structural segment of homology, the module. J. Molec. Biol. 268:857–868.

Riley, M., and M. H. Serres. 2000. Interim report on genomics of Escherichia coli. Ann. Rev. Microbiol. 54:341–441.

Roberts, R. B., D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. J. Britten. 1955. Studies of Biosynthesis in Escherichia Coli. Carnegie Institution of Washington. Washington DC, 607:521.

Rudd, K. E. 1999. Novel intergenic repeats of Escherichia coli K-12. Res. Microbiol. 150:653–664.

Saier Jr., M. H. 2000. A functional-phylogenetic classification system for transmembrane solute transporters. Microbiol. Molec. Biol. Rev. 64:354–411.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY. 1659.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Wollman, E. L., F. Jacob, and W. Hayes. 1959. Conjugation and genetic recombination in Escherichia coli K-12. Cold Spring Harbor Symposia on Quantitative Biology 21:141–162.

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CHAPTER 1.11

Origin of Life: RNA World versus Autocatalytic Anabolism

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Introduction

The deep past of the earth is unobservable. Therefore, the problem of the origin of life, the emergence of the first evolvable entity, which is the primordial ancestor of all extant organisms, can only be solved by a theory. Theories on the origin of life are scientific rather than myth, if they have empirical significance: empirical biological significance by providing evolutionary explanations for extant facts of biology and/or empirical chemical significance by predicting unknown but testable chemical reactions. Preferably, they also should have geological significance by being compatible with geological theories on the early history of the earth, which themselves, however, need to be scrutinized for their power to explain and predict facts of geology and chemistry. These requirements follow from Popper's methodology of science (Popper, 1959; Popper, 1963; Popper, 1972). They constitute the challenge of the field.

Only two theories on the origin of life are detailed enough for an evaluation by the above criteria:

1) The *RNA world theory* (Joyce, 1989; Gesteland et al., 1999) assumes that the first organism was a "living" RNA-like molecule replicating in a prebiotic broth of activated nucleotides.

2) The *autocatalytic anabolist theory* (Wächtershäuser, 1988; Wächtershäuser, 1990; Wächtershäuser, 1992; Wächtershäuser, 1997; Wächtershäuser, 2000) assumes that life began on minerals with an anabolic metabolism of synthetic, autocatalytic carbon fixation cycles.

These two theories will be discussed here in a comparative manner. For a detailed list of references and for a description of further studies, the readers should consult the available comprehensive reviews (Bengtson, 1994; Brack, 1998; deDuve, 1991; Fry, 2000; Lahav, 1999; Smith and Szathmary, 1995; Zubay, 2000). Given the huge body of literature, the specific references given here cannot be anything but incomplete; the emphasis is placed on references that are extraordinarily relevant for the present discussion, or very recent, or effectively forgotten.

Nutrients and Energy

All organisms require nutrients and energy for growth and reproduction. In the *RNA world theory*, activated nucleotides are required as nutrients. Because these nucleotides are quite complex, the theory is forced to assume a rather protracted "prebiotic chemistry" for generating a prebiotic pool of activated nucleotides. There is some experimental support, most notably the formation of adenine from HCN (Oró, 1960) and the formation of pyrimidines from cyanoacetylene (Ferris et al., 1968).

However, the various reactions reported require different and mutually incompatible reaction conditions (Shapiro, 1986). A further difficulty of this approach resides in high dilution and in the destructive force of hydrolysis, notably in a hot prebiotic broth. As a solution of this problem an adsorption to minerals, like clay, has been suggested. Another difficulty resides in the problem of chemical selectivity. Given the multitude of organic compounds in a presumptive prebiotic broth, any envisioned reaction would be accompanied by numerous competing reactions. It is presently not clear, which mechanism of chemical selection could keep these competing reactions at bay (Shapiro, 1986).

It should further be remembered that the main ingredients in a prebiotic broth, when judged by the composition of meteorites or by electric discharge experiments (Löb, 1908; Löb, 1913; Miller, 1953) are carboxylic acids, notably amino acids. For thermodynamic reasons, free (amino) acids in a dilute aqueous solution cannot combine to form larger compounds. As a remedy, it has been suggested that thioesters are formed by equilibration with mercaptanes under hot, acidic

conditions and subsequently quenched with cold, neutral water (deDuve, 1991). Hydrolysis, however, will occur as soon as aqueous nonequilibrium conditions are established.

The *autocatalytic anabolist theory* requires as nutrients the inorganic volatiles in magmatic exhalations, such as CO_2, CO; H_2S; N_2, NH_3; H_2, and H_2O. They are found in varying proportions in extant magmatic exhalations and must have been ubiquitous in the earliest days of the earth. Additionally, the theory requires mineral or cluster surfaces with catalytic centers of mainly iron, but also nickel or cobalt (notably with a combination of sulfido and CO ligands). At high temperature (e.g., 1,500°C), gaseous exhalations are equilibrated. They are quenched into a state of nonequilibrium when mixed rapidly with cool (e.g., 100°C) aqueous liquid. This provides the chemical potential for synthetic reactions. For example, at low temperatures (e.g., 100°C) H_2 and CO are reductants and FeS and H_2S form a potent reducing agent ($E^{\circ\prime} = -620$ mV):

$$FeS + H_2S \rightarrow FeS_2 + 2H^+ + 2e^-$$

Initiation and Reproduction

The two competing theories give characteristically different answers to the question of primordial reproduction, which chemically speaking is an autocatalysis.

According to the *RNA world theory*, a large variety of RNA-type molecules formed de novo in the prebiotic broth, and by chance one of these was capable of reproducing by replication from activated nucleotides, which started evolution. Strand separation required a temporary elevation of the temperature (Kuhn, 1972), which complicates the theory considerably. This theory was greatly promoted by the discovery of catalytic RNA (ribozymes). It was concluded that the first "living" RNA molecules were not only the carriers of genetic inheritance (genotype), but also the sole carriers of catalytic function (phenotype). All attempts to demonstrate a replication of an RNA-type molecule without enzymes have failed. This result, mainly due to L. E. Orgel and his school, is close to a conclusive falsification, and therefore, a most important scientific achievement in this field. Subsequent attempts to employ *in vitro* evolution to at least show the possibility of an RNA sequence with a polymerase function for its own replication from nucleoside triphosphates have resulted in the conclusion that the minimum RNA with such capability could only have arisen by Darwinian evolution and not in a prebiotic broth (Johnston, 2001).

The *autocatalytic anabolist theory* assumes a primordial reproduction cycle consisting of small, low-molecular-weight organic constituents, like the constituents of extant intermediary metabolism. These are seen as an autocatalytic carbon fixation process in a ligand sphere of catalytic transition metal complexes, clusters, or minerals. The initiation reaction (Fig. 1) involves a reaction sequence from CO and H_2S via methyl mercaptane (CH_3-SH) to activated acetic acid (CH_3-CO-SH and CH_3-CO-SCH_3) in the presence of Fe/Co/Ni-catalysts, e.g., coprecipitated FeS and NiS. This has been experimentally demonstrated at 100°C (Huber and Wächtershäuser, 1997). The reaction is very similar to the reductive acetyl-CoA pathway. This and other experiments have revealed the importance of freshly precipitated sulfide gels (presumably comprising a library of unknown cluster structures ranging from oligonuclearity through multinuclearity to crystal structures) as compared to highly ordered sulfide crystals.

For a primordial reproduction by an autocatalytic feedback mechanism, several possibilities, which may have come in sequentially or jointly by cooperation, have been considered:

1) Feedback by the accumulation of lipids on mineral/cluster surfaces. Lipid accumulation would have the effect of mineral/cluster modification and hydrophobization. This in turn would lower the water activity on the surface and inhibit hydrolytic reactions. At the same time, lipid synthesis would be promoted, which means feedback. The biosynthetic formation of lipids by the condensation of activated acetic acid requires a strong base for forming an anionic intermediate. Under primordial aqueous conditions, such a strong base cannot exist. As a solution, we may consider a hypothetical route (Fig. 1) via metal-bonded HS-CH_2-SH to activated

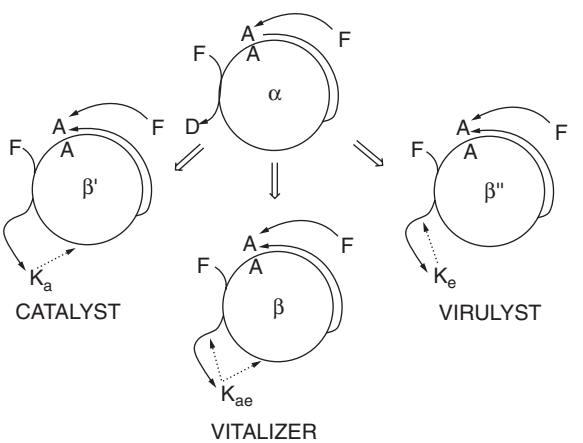

Fig. 1. Hypothetical reaction cascades and feedback effects of a primordial metabolism. (Bold arrows: experimentally demonstrated reactions; dotted arrows: hypothetical reactions; drawn-out arrows: extant enzymatic reactions.)

thioglycolic acid, which in the presence of FeS converts into an anionic species by nucleophilic attack and reductive elimination. Subsequent condensation to activated acetoacetic acid would open the way for formation of isoprenoid acids. It is an important aspect of this suggestion that an accumulation of these first surface-bonded lipids would constitute the common origin of structural and metabolic evolution.

2) Feedback by peptide ligand formation. It has been suggested that peptides bonded to surfaces or to transition metal centers preceded proteins and translation (Woese, 1972). Primordial peptide formation by carbon fixation (Fig. 1) is here seen as including the following steps (each one experimentally demonstrated in dilute aqueous systems around 100°C):

Reduction of CO via HS-CH_2-SH to CH_3-SH by H_2S and FeS/NiS (Huber and Wächtershäuser, 1997);

Double carbonylation of CH_3-SH to keto acids with Fe/Co/Ni catalysts (C. Huber and G. Wächtershäuser, manuscript in preparation);

Reductive amination of keto acids to amino acids with ammonia and Fe catalysts (Hafenbradl et al., 1995; C. Huber and G. Wächtershäuser, manuscript in preparation);

Activation of the amino acids (presumably as oxazolinones) with CO and H_2S on (Fe,Ni)S and conversion of the activated amino acids into peptides, which readily hydrolyze again to amino acids under the same conditions (Huber and Wächtershäuser, 1998).

This suggests the formation of a dynamic peptide library, which continuously scans the peptide sequence space as long as the energy source (CO + H_2S) lasts. Some of the peptides would bind strongly to the metal centers and would thereby be stabilized. Some of these in turn would increase the catalytic activity and/or selectivity for the above steps by ligand modification of the catalytic transition metal centers. This would constitute autocatalysis by self-selecting peptides (Wächtershäuser, 2000). Pressure is highly advantageous, as evidenced by the reported conversion of nonaqueous HCOOH via CO to pyruvate at 200 MPa and 250°C in the presence of FeS (Cody et al., 2000).

3) Reductive citric acid cycle as a primordial feedback cycle. A nonenzymatic archaic version of the reductive citric acid cycle would be autocatalytic and therefore could function on mineral surfaces as the first reproduction cycle, driven by a redox energy source and doubling by cleavage at the level of a thioester (or thioacid) of citric acid or malic acid.

4) Autocatalytic formose cycle as a primordial feedback cycle. It has been suggested that a primordial autocatalytic formose cycle based on CO and operating on mineral surfaces could function as primordial reproduction cycle (L. E. Orgel, 2000, personal communication).

Mechanism of Evolution

The mechanism of evolution is of singular importance for the problem of the origin of life. The *RNA world theory* assumes that evolution has always been based on replication of nucleic acids, on sequence variations, and on selection.

The *autocatalytic anabolist theory* assumes that nucleic acids and replication are a product of evolution. This means that there must have been a primordial mechanism of evolution, which was independent of nucleic acids. Such a primordial mechanism of evolution has been proposed (Wächtershäuser, 1988; Wächtershäuser, 1992). It is based on the by-products of autocatalytic metabolic reproduction (Fig. 2). Normally such by-products are products of decay. If they dominate, the rate of reproduction goes below 1 and the metabolic reproduction ceases to exist. But if such a by-product or a set of such by-products has a positive feedback effect either by being catalytic for at least one step of the pre-existing metabolism or by being converted into a constituent of the pre-existing metabolism, an accelerated or expanded metabolism arises. This positive feedback will persist as long as the production rate of the by-product is greater than its rate of decay. If the rate of decay is very slow, as with lipids, even a slow production will lead to a positive feedback effect by accumulation. For (catalytic) by-products with a high rate of decay, we entertain the following additional consideration: As long as such a (catalytic) by-product or set of by-products is produced solely by the pre-existing chemical conditions (*de novo* synthesis), its concentration changes in synchrony with the changes of the chemical environment. A dramatically different situation exists, however, if a by-product or a set of by-products shows the following "dual feedback" effect: an "altruistic feedback" effect for promoting at least one step of the pre-existing metabolism, and an "egotistic feedback" effect for promoting at least one step of the pathway of synthesis of the by-product. Now, the (catalytic) by-product with this dual feedback ("vitalizer") may persist by virtue of its egotistic feedback even though the chemical conditions for its *de novo* synthesis have vanished. Therefore, with every new autocatalytic dual feedback, the metabolism switches into a new and relatively stable expanded state. This constitutes a memory effect. A catalyst that has a purely egotistic feedback effect (a "virulyst") is strictly parasitic and may be considered the most primitive forerunner of a virus.

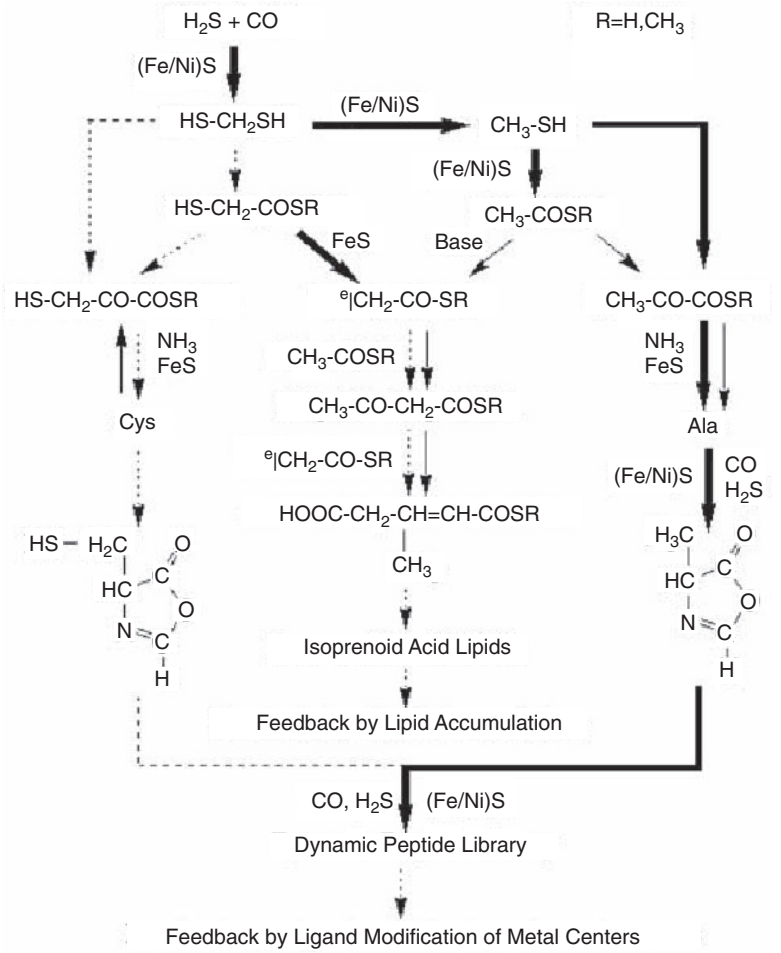

Fig. 2. Generalized mechanism of evolution by catalytic feedback. (Arrows signify expansions of the metabolism from α-stage to β-stage; Abbreviations: F, food; A, autocatalyst; D, product of decay; K_a, catalyst with altruistic feedback; K_e, catalyst with egotistic feedback; and K_{ae}, catalyst with dual feedback.)

Extant cellular organisms are replete with vitalizers: Coenzymes catalyzing a step in their own biosynthesis, ribosomes catalyzing the synthesis of all proteins including ribosomal proteins, translocases catalyzing the introduction of membrane proteins including translocase proteins into the cell membrane, as well as nucleic acids coding for all proteins including polymerases. As candidates for the earliest mutations by vitalizers, we may consider the emergence of new ligands (e.g., new amino acids and sets of peptides) modifying the catalytic metal centers or the emergence of new lipids modifying lipophilicity or lipid membranes.

Cellularization

All extant organisms are cellular owing to lipid membranes. However, primitive lipid membranes without transporter proteins are essentially impermeable for hydrophilic or ionic organic molecules. This creates a major paradox for the *RNA world theory*, which is dependent on the existence of a prebiotic broth. Any increase in barrier effectiveness of the primitive lipid membrane means on the one side an advantage in terms of keeping the constituents inside, but on the other side a disadvantage in terms of keeping the organic broth components outside (isolation/starvation paradox).

According to the *autocatalytic anabolist theory*, cellularization does not give rise to an isolation/starvation paradox. The neutral inorganic nutrients (H_2S, CO, CO_2, N_2 and NH_3) pass freely through a lipid membrane. The catalytic metal ions are provided by the mineral itself, and phosphate ions may be provided as an adsorbate of the mineral surface.

The overall cellularization process is seen as a protracted evolutionary affair. The earliest lipid function is seen in a modification of the inorganic structures (developing from cluster structures through colloidal structures to mineral structures), notably by (surface) lipophilization. This leads to a decrease of water activity and therefore it promotes lipid formation (egotistic feedback effect) as well as pre-existing metabolic

reactions (altruistic feedback effect). Most importantly, however, the accumulation of lipids elicits new kinds of reactions. This effect may be generalized and termed "feedforward effect." It greatly accelerates evolution. Eventually, with the accumulation of lipids, closed membrane vesicles are formed, first supported by the inorganic structures and later self-supporting. Subsequently, translocases and membrane proteins emerge.

An automatic consequence of this development is the possibility of an ion (proton) gradient over the vesicle membrane of a closed membrane envelope. This is the prerequisite for the emergence of Fe/S-dependent electron transport chains and chemiosmosis. Chemiosmosis may at first have been restricted to a combination of exergonic and endergonic electron transport chains. Membrane-bound ATPases would then have been added later (Wächtershäuser, 1997).

The early organisms with a primitive membrane envelope (termed "precells") must have undergone frequent fusions and fissions and thus did not have an effective function of individuation (Kandler, 1998; Woese, 1998). True cells capable of stable organismic individuation emerged as soon as vesicle fusions became inhibited, perhaps by mutually immiscible enantiomeric lipid types, which forced the physicochemical separation of the domains of life (Wächtershäuser, 1988; Wächtershäuser, 1992) and/or by cell walls.

Chirality

Homochirality unites biochemistry and sets it apart from the abiotic world. Therefore, it is a touchstone for any theory on the origin of life.

The *RNA world theory* requires homochiral building blocks for forming well-folded ribozymes. A prebiotic broth, however, can only be racemic (Bada and Miller, 1987). This seems to constitute an insurmountable dilemma. For under racemic conditions, replication is subject to enantiomeric cross-inhibition (Joyce et al., 1984; Schmidt et al., 1997).

According to the *autocatalytic anabolist theory*, the first ligands are small organic compounds, like lipids, amino acids or peptides. Homochirality is not required for a feedback effect by these ligands. It would even be detrimental by impoverishing structural variability. Moreover, optically active amino acids, even if they had come about, would have quickly racemated. It has been experimentally shown that such racemization of amino acids occurs rapidly under the conditions of activation with CO/H_2S, probably due to oxazolinone intermediates. This means that homochirality would have been impossible under primordial conditions. Homochirality would have occurred much later, perhaps only after the takeover of activation by ATP, and perhaps in conjunction with the emergence of homochiral polymers, like nucleic acids (Bolli et al., 1997).

Temperature

The *RNA world theory* of an origin of life in a prebiotic broth requires a cold ocean to avoid decomposition of the thermally sensitive constituents. Therefore, it is incompatible with geological theories of a hot early ocean. The *surface metabolist theory* is compatible with high temperatures because binding to metal centers of minerals or clusters stabilizes the constituents. The theory even requires a hot environment, for otherwise the reaction rates may not be high enough. Hot aqueous local environments exist today and surely must have existed at the dawn of life.

The discovery of hyperthermophilic Bacteria and Archaea, occupying deep and short branches in Woese's tree of life (Woese, 1987) has made room for the previously unthinkable possibility of a hyperthermophilic last common ancestor, and by extension, of a hyperthermophilic origin of life, from which mesophiles would have been derived polyphyletically. This notion contrasts with the conventional notion of a mesophilic or even psychrophilic origin of life and a mesophilic last common ancestor from which hyperthermophiles would have derived polyphyletically by adaptation to high temperatures.

The adherents of a mesophilic origin of life make the implicit assumption of an essential symmetry between thermally upward and thermally downward evolution. This view is not tenable. An adaptation from a mesophilic lifestyle to a hyperthermophilic lifestyle is effectively impossible for the following reason. Unlike any other parameter, temperature is all-pervasive. It influences practically all structures and processes of an organism profoundly. Therefore, an "upward adaptation" of a whole organism to higher temperatures requires a concerted, simultaneous adaptation of all processes and all structures. This is very demanding. For example, an upward adaptation of each polymer structure would require several specific covariations for folding stabilization at high temperatures. The probability of all these changes occurring simultaneously is extremely low. Therefore, the conclusion seems inescapable: an upward adaptation is probabilistically forbidden. It means that evolution proceeded irreversibly from hyperthermophily to mesophily. This must have occurred by

a gradual loss (one at a time) of a few sets of strong covalent bonds, e.g., S-metal bonds, in favor of a gradual appearance of a multitude of weak noncovalent bonds, i.e., by degeneration rather than by adaptation. Such degeneration may take a relatively long time, but its overall probability is not effectively zero. This explains a striking fact concerning many ancient proteins (e.g., aminoacyl synthetases for Val, Ile, Leu, Met, His, Phe and Pro; ribosomal proteins L33, S17, L24, S14, L36, S4, L31, L28 and S18). In hyperthermophiles, these proteins have patterns of sequence signatures with 4n Cys units (n = 1 to 6), which are typical for strong covalent metal (e.g., Zn) bonding. But in mesophiles, these signatures are partially to wholly absent. Their gradual loss is readily possible in a thermally downward evolution, whereas their simultaneous reintroduction at the right places in a thermally upward evolution is highly improbable.

The theory of a hyperthermophilic origin has been contradicted recently by a paper (Galtier et al., 1999), which has attempted a reconstruction of the G+C contents of rRNAs and tRNAs of the universal ancestor. The reconstruction was based on complete extant sequences with conserved as well as variable positions. The projected G+C contents were rather low, which was taken to be indicative for a mesophilic lifestyle of the universal ancestor. Subsequently, however, it has been shown (Di Giulio, 2000) that a reconstruction method that ignores highly variable positions produces very high G+C contents not only for the universal ancestor, but also for the common ancestor of all bacteria, the common ancestor of all archaea, and to a lesser extent the common ancestor of all eukaryotes. This strongly supports a hyperthermophilic origin of life.

It has been demonstrated that random RNAs do not fold well at temperatures of hyperthermophilic organisms. From this it was correctly concluded that the *RNA world theory* and the notion of a hyperthermophilic origin are incompatible. Based on the presumption of truth of the *RNA world theory*, a mesophilic origin was concluded (Moulton, 2000). This latter conclusion, however, is at odds with the simple fact that in hyperthermophilic organisms' RNAs have no trouble folding, owing to a high G+C content. Therefore, on the basis of the *autocatalytic anabolist theory*, we merely have to assume that the earliest RNAs were all bonded to surfaces and that only those with a high G+C content lifted off as well-folded self-supporting structures. Later, after the emergence of enzymes for stabilizing RNAs by secondary modifications, the restriction to a sequence space with high G+C content was relaxed. Similarly, we assume that the earliest peptides and proteins were all bonded as ligands to surfaces of clusters and minerals and that only those with sequence signatures for strong bonding to metal centers turned into self-supporting structures. Later, after the emergence of folding catalysts (chaparones), the sequence space for hyperthermophilic proteins became expanded.

Time Frame

The *RNA world theory* of an origin of life in a prebiotic broth requires a sufficiently long period of time for a slow accumulation of the presumptive constituents of the broth and for playing out the so-called "prebiotic (broth) chemistry" until the onset of replication. Early on, it was assumed that more than a billion years were available for prebiotic chemistry. Two geological findings narrowed the window of time dramatically. First, fossils of bacteria (much like cyanobacteria) were discovered in 3.5-billion-year-old sedimentary rocks in Australia (Schopf, 1993). Moreover, it was discovered that 3.85-billion-year-old, highly metamorphosed sedimentary rocks in Greenland have a C-isotope signature that is characteristic for life (Schidlowski, 1988; Mojzsis, 1996). Second, heavy bombardment with ocean-boiling impactors is calculated to have lasted from the origin of the earth (4.6 billion years ago) until about 3.8 billion years ago. This nearly removes the window of time required for the occurrence of a mesophilic origin of life in a prebiotic broth.

The *autocatalytic anabolist theory* avoids this dilemma. It does not require the preaccumulation of a prebiotic broth or even the existence of a large body (ocean) of water. It only requires enough aqueous fluid for solvating mineral surfaces, colloidal structures or catalytic clusters.

The *RNA world theory* sees the origin of life in a prebiotic broth as a one-time event, never to recur after the first inception of life. By contrast, the *autocatalytic anabolist theory* of a chemoautotrophic origin leads to the conclusion of perpetual or recurrent reactions of origin as long as suitable inorganic conditions prevail. It makes room for the realistic possibility of an origin of life on numerous celestial bodies with magmatic outgassing, including Mars. Moreover, it assumes a rapid succession of synthetic reaction steps giving rise to activated intermediates undergoing further synthetic reactions, which concatenate to pathways and cycles. These reactions must go on at a rate higher than the rate of hydrolysis. Thus this theory assumes a rapid origin of life by metabolic cycles instead of a protracted prebiotic chemistry in a slowly accumulating prebiotic broth.

These considerations are most important for the experimental research programs that transpire from the two theories. Experiments within

the *RNA world theory* can only have the role of demonstrating the principal possibility of certain partial chemical aspects of the theory. By contrast, the experimental program for testing the *autocatalytic anabolist theory* sets its ultimate target beyond such partial plausibility and aims at eventually bringing about an origin and early evolution of life in vitro.

Phylogenetic Connection

Extant organisms are the result of some four billion years of evolution. They are highly complex and far removed from primordial life. There are attempts to employ comparative biochemistry and genomics for reconstructing the last common ancestor to get closer to the origin of life. However, numerous lateral transfers, notably of genes concerning the metabolism, complicate this program of reconstruction considerably (Woese, 1998; Woese, 2000). Therefore, formal sequence comparisons alone cannot resolve major questions of the metabolism of the last common ancestor, e.g., whether it was autotrophic or heterotrophic.

Bacterial and archaeal genomes comprise long and highly conserved clusters of genes concerning the machineries of transcription and translation. These machineries are highly complex, comprising a large number of cooperating components. A lateral transfer of a long cluster of many genes encoding components of such machineries may be expected to lead to hybridized machineries with severely impaired functional competence. Therefore, these clusters are prime candidates when we search for cases of forbidden lateral transfer. By an overlapping alignment of these clusters of different organisms, it was possible to reconstruct a hypothetical segment of the genome of the last common ancestor (Wächtershäuser, 1998). It comprises genes for transcription (polymerases and antitermination factors), translation (ribosomal proteins and elongation factors), and protein translocation. This means that the universal ancestor had already an organized DNA genome, RNA, a closed precell lipid membrane, a genetic code, and sets of bases and amino acids much like in extant cells. This suggests the following broad stages of early evolution, which are not sharply separated, but overlapping.

Stage 1: Anabolic autotrophic surface metabolists with metal catalysts;
Stage 2: Formation of precells and the earliest genetic machinery;
Stage 3: Metabolic takeover by genetically encoded enzymes, perfection of the genetic machinery, emergence of true cells and isolation.

Genetic Machinery

The genetic machinery has the central function in all extant organisms. It is extremely complex. Therefore, it must have arisen in a long evolutionary process with numerous steps. The two theories discussed here give characteristically different accounts of this process.

The *RNA world theory* assumes that the pioneer process of life was replication of RNA or a similar nucleic acid in a prebiotic broth of activated nucleotides, and that RNA was catalytic for this process as a template and perhaps also as a ribozyme with the function of a polymerase. Other ribozyme functions followed, and it is assumed that the set of biocatalysts of the earliest forms of life consisted exclusively of ribozymes. Hence the name "RNA world." One of the later ribozymes was a proto-ribosomal RNA, which had the function of translating preexisting RNA sequences into protein sequences. This was the starting point for an enzymatic takeover, which eliminated almost all ribozymes. Subsequently DNA replication as an extension of RNA polymerization took over the role of information conservation.

The *autocatalytic anabolist theory* assumes that the pioneer process of life was a self-expanding synthetic autocatalytic metabolism. Proto-nucleic acids emerged as a product of this metabolism, the bases having first a catalytic (acid-base) function and the backbone having a surface anchoring function. The earliest nucleic acids may have been homopolymers, and therefore, would have been devoid of sequence information. Nevertheless base pairing could have already been in service of catalyzing the formation of short peptides by positioning amino acids. After the appearance of more and more amino acids, sequence information for genuine translation emerged. Now peptide sequences became more and more determined by the base sequences of the nucleic acids and thus faithful replication began to become important. Now longer and longer protein sequences appeared, and enzymes replaced the short peptide ligands. With the appearance of more and more complex RNA folding, additional ribozymes for catalyzing RNA chemistry emerged by dual feedback. Whether DNA came after RNA or coevolved with RNA is presently not crucial in this theory.

Explanatory Power

Popper has suggested assessing the empirical or scientific merit of a theory by assessing the explanatory power of the theory, i.e., its power to explain many facts by using few assumptions. Theories on the origin of life have to explain

extant facts by linking them to hypothetical primordial functions going back some four billion years. Therefore, the possibility of an explanatory success of a theory on the origin of life is largely dependent on how many radical biochemical takeovers are assumed to have occurred and obliterated the remains of the earliest evolution.

The *RNA world theory* makes several assumptions of different strength:

The strong assumption of an emergence of naked "living" RNA molecules that evolved by replication and selection in a prebiotic broth would be explanatory, if a linkage could be drawn from the presence or absence of constituents of the broth to the presence or absence of constituents of extant RNA.

The theory makes the strong assumption that, early on, ribozymes (RNA) were the sole biocatalysts. It remains to be explained how this strong assumption could be explanatory for extant organisms in which most of the biocatalysts are enzymes.

The much weaker postulate that ribozymes and enzymes had a role in early life is explanatory for the extant coexistence of the catalytic roles of enzymes and ribozymes, like rRNAs and tRNAs.

The postulate that RNA came before DNA has explanatory power for viral RNA genomes, RNA primers of DNA replication, RNA in telomerases, and the biosynthetic conversion of ribonucleotides into deoxyribonucleotides.

For the development of the *autocatalytic anabolist theory* a methodology of biochemical retrodiction has been employed, whereby a common precursor function is postulated for two or more disparate successor functions. This generates explanatory power. At the same time it produces a backward convergence toward the origin as the ultimate common precursor function. Here are some illustrative examples:

Primordial surface bonding to clusters/minerals is a functional precursor of cell enclosure, enzyme surfaces, catalytic transition metal centers and protein folding.

The requirement of strong surface bonding of anionic bioorganics to cationic metal centers is explanatory for the domination of the central metabolism by anionic carboxylate and thiolate groups and notably for the phosphate groups in sugar metabolism and nucleic acids.

A primordial CO-based metabolism is explanatory for the role of CO in the reductive acetyl-CoA pathway and for the CO ligands in hydrogenases.

A primordial sulfide-based metabolism is explanatory for the central role of SH and sulfido groups in extant enzymes and coenzymes.

A primordial iron-sulfur-based metabolism explains the central role of iron-sulfur clusters, notably in anaerobes; it explains iron-oxo clusters and hemes as later substitutes.

A primordial nickel-based metabolism explains the critical role of nickel in hydrogenases, CO-dehydrogenase, acetyl-CoA synthase and methane synthase as leftovers.

A primordial activation of amino acids as oxazolinones by CO/H_2S as a direct source of energy for peptide formation and prototranslation, and as an indirect source of energy for pyrophosphate or ATP formation, explains the emergence of extant amino acid activation by ATP in synthetases as a reaction reversal.

A general mechanism of evolution by (dual) autocatalytic feedback explains the possibility of an evolution by low-molecular-weight metabolic by-products. It further explains why central components of extant cells (e.g., ribosomes, translocases and coenzymes) exhibit dual feedback. And finally, it explains the takeover by a mechanism of evolution based on high-molecular-weight nucleic acids simply as an extension of the primordial mechanism. Metaphorically speaking, it explains early evolution as a changeover from the analog information of feedback loops to the digital information of nucleic acids and the ribosome as a digital-to-analog converter.

Conclusion

The scientific problem of the origin of life can be characterized as the problem of finding the chemical mechanism that led all the way from the inception of the first autocatalytic reproduction cycle to the last common ancestor. All present theories fall far short of this task. While we still do not understand this mechanism, we now have a grasp of the magnitude of the problem.

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Literature Cited

Bada, J. L., and S. L. Miller. 1987. Racemization and the origin of optically active organic compounds in living organisms. BioSystems 20:21–26.

Bengtson, S. (Ed.). 1994. Early Life on Earth. Columbia University Press. New York, NY.

Bolli, M., R. Micura, and A. Eschenmoser. 1997. Pyranosyl-RNA: Chiroselective self-assembly of base sequences by ligative oligomerization of tetranucleotide-2′,3′-cyclophosphates (with a commentary concerning the origin of biomolecular homochirality). Chem. Biol. 4:309–320.

Brack, A. (Ed.). 1998. The Molecular Origins of Life. Cambridge University Press. Cambridge, UK.

Cody, G. D., N. Z. Boctor, T. R. Filley, R. M. Hazen, J. H. Scott, A. Sharma, and H. S. Yoder Jr. 2000. Primordial carbonylated iron-sulfur compounds and the synthesis of pyruvate. Science 289:1337–1340.

DeDuve, C. 1991. Blueprint for a Cell: The Nature and Origin of Life. Neil Patterson. Burlington, NC.

DiGiulio, M. 2000. The universal ancestor lived in a thermophilic or hyperthermophilic environment. J. Theor. Biol. 203:203–213.

Ferris, J. P., A. Sanchez, and L. E. Orgel. 1968. Studies in prebiotic synthesis. III: Synthesis of pyrimidines from cyanoacetylene and cyanate. J. Molec. Biol. 33:693–704.

Fry, I. 2000. The Emergence of Life on Earth. Rutgers University Press. New Brunswick, NC.

Galtier, N., N. Tourasse, and M. Gouy. 1999. A nonhyperthermophilic common ancestor to exctant life forms. Science 283:220–221.

Gesteland, R. F., T. R. Cech, and J. F. Atkins (Eds.). 1999. The RNA World: The Nature of Modern RNA Suggests a Prebiotic RNA. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Hafenbradl, D., M. Keller, G. Wächtershäuser, and K. O. Stetter. 1995. Primordial amino acids by reductive amination of α-oxo acids in conjunction with the oxidative formation of pyrite. Tetrahedron Lett. 36:5179–5182.

Huber, C., and G. Wächtershäuser. 1997. Activated acetic acid by carbon fixation on (Fe,Ni)S under primordial conditions. Science 276:245–247.

Huber, C., and G. Wächtershäuser. 1998. Peptides by activation of amino acids with CO and (Ni,Fe)S surfaces: implications for the origin of life. Science 281:670–672.

Johnston, W. K., Unrau, P. J., Lawrence, M. S., Glasner, M. E., & Bartel, D. P. 2001. RNA-catalyzed RNA-polymerization: Accurate and general RNA-templated primer extension. Science 292:1319–1325.

Joyce, G. F., G. M. Visser, C. A. A. van Boeckel, J. H. van Boom, L. E. Orgel, and J. van Westrenen. 1984. Chiral selection in poly(C)-directed synthesisof oligo(G). Nature 310:602–604.

Joyce, G. F. 1989. RNA evolution and the origin of life. Nature 338:217–224.

Kandler, O. 1998. The early diversification of life and the origin of the three domains: A proposal. *In:* J. Wiegel and M. W. W. Adams (Eds.) Thermophiles: The Keys to Molecular Evolution in the Origin of Life. Taylor & Francis. London, 19–31.

Kuhn, H. 1972. Selbstorganisation molekularer Systeme und die Evolution des genetischen Apparats. Angew. Chem. 84:838–862.

Lahav, N. 1999. Biogenesis. Oxford University Press. Oxford, UK.

Löb, W. 1908. Die Einwirkung der stillen elektrischen Entladung auf feuchtes Methan. Ber. Dtsch. Chem. Ges. 41:87–90.

Löb, W. 1913. Über das Verhalten des Formamids unter der Wirkung der stillen Entladung: Ein Beitrag zur Frage der Stickstoff-Assimilation. Ber. Dtsch. Chem. Ges. 44:684–697.

Miller, S. L. 1953. A production of amino acids under possible primitive earth conditions. Science 117:528–529.

Mojzsis, S. J., G. Arrhenius, K. D. McKeegan, T. M. Harrison, A. P. Nutman, and C. R. L. Friend. 1996. Evidence for life on earth before 3800 million years ago. Nature 384:55–59.

Moulton, V., P. P. Gardner, R. F. Pointon, L. K. Creamer, G. B. Jameson, and D. Penny. 2000. RNA folding argues against a hot-start origin of life. J. Molec. Evol. 51:416–421.

Oró, J. 1960. Synthesis of adenine from ammonium cyanide. Biochem. Biophys. Res. Commun. 2:407–412.

Popper, K. R. 1959. The Logic of Scientific Discovery. Hutchinson. London,

Popper, K. R. 1963. Conjectures and Refutations: The Growth of Scientific Knowledge. Routledge & Kegan Paul. London, 1–431.

Popper, K. R. 1972. Objective Knowledge: An Evolutionary Approach. Clarendon Press. Oxford, UK.

Schidlowski, M. 1988. A 3800-million-year isotopic record of life from carbon in sedimentary rocks. Nature 333:313–318.

Schmidt, J. G., P. E. Nielsen, and L. E. Orgel. 1997. Enantiomeric cross-inhibition in the synthesis of oligonucleotides on a nonchiral template. J. Am. Chem. Soc. 119:1494–1495.

Schopf, J. W. 1993. Microfossils of the early archean apex chert: New evidence of the antiquity of life. Science 260:640–646.

Shapiro, R. 1986. Origins: A Sceptic's Guide to the Creation of Life on Earth. Summit Books New York, NY.

Smith, J. M., and E. Szathmary. 1995. The Major Transitions in Evolution. W. H. Freeman. Oxford, UK.

Wächtershäuser, G. 1988. Before enzymes and templates: Theory of surface metabolism. Microbiol. Rev. 52:452–484.

Wächtershäuser, G. 1990. Evolution of the first metabolic cycles. Proc. Natl. Acad. Sci. USA 87:200–204.

Wächtershäuser, G. 1992. Groundworks for an evolutionary biochemistry: The iron-sulfur world. Prog. Biophys. Molec. Biol. 58:85–201.

Wächtershäuser, G. 1997. The origin of life and its methodological challenge. J. Theor. Biol. 187:483–494.

Wächtershäuser, G. 1998. Towards a reconstruction of ancestral genomes by gene cluster alignment. Syst. Appl. Microbiol. 21:473–477.

Wächtershäuser, G. 2000. Life as we don't know it. Science 289:1307–1308.

Woese, C. R. 1972. The emergence of genetic organization. *In:* C. Ponnamperuma C. (Ed.) Exobiology. North-Holland Publishing. Amsterdam, The Netherlands. 301–341.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Woese, C. R. 1998. The universal ancestor. Proc. Natl. Acad. Sci. USA 95:6854–6859.

Woese, C. R. 2000. Interpreting the universal phylogenetic tree. Proc. Natl. Acad. Sci. USA 97:8392–8396.

Zubay, G. 2000. Origins of Life on Earth and in the Cosmos. Academic Press. San Diego, CA.

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CHAPTER 1.12

Biotechnology and Applied Microbiology

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Introduction

There is no agreed upon definition of biotechnology. Definitions vary from the most noble—the use of biology for the benefit of man—to the most prosaic—the use of biology to make money. A balanced definition might be the use of living organisms (or materials from living organisms) to perform defined processes for industrial application. Applied microbiology is a large part of biotechnology because microorganisms have a broadly diverse synthetic and degradative potential, which is easily manipulated genetically, and they can readily grow under controlled conditions in large fermenters. The so-called "new biotechnology" generally refers to the use of recombinant DNA technology to construct useful new strains. However, a considerable amount of "new biotechnology" does not use recombinant DNA technology but rather structural biology as a basis for rational drug design, monoclonal antibodies as biosensors, and enzymes from extremophiles to facilitate industrial production. In addition, conventional industrial microbiological processes apply many of the new biotechnologies.

This edition of the Prokaryotes contains an entire section dealing with the following specific biotechnology and applied microbiology topics: bioremediation, bioleaching, microbial biofilms, biodeterioration, bacterial enzymes, bacteria in food and beverage production, organic acid and solvent production, scale-up of bacterial fermentations, amino acid and vitamin production, recombinant DNA protein production in bacteria, bacterial pharmaceutical products and bioemulsifiers. In addition, each chapter dealing with a particular genus (or a group of genera) has a section on applications. In this introductory chapter, I will attempt to put applied microbiology and biotechnology into an historical perspective and give an overview of some of the general principles related to the biotechnology of prokaryotes. It should be emphasized at the outset that both applied and basic microbiology have been and always will be intertwined. Applied microbiology without basic microbiology is lame and limited. A major driving force of basic microbiology is the solution of applied problems.

The Fermentation Controversy

For thousands of years, microbiological processes (such as those for producing wine, beer, vinegar, bread, pickles, sauerkraut and various milk products such as yogurts, cheeses and butter) were developed by trial-and-error, without any understanding of the underlying principles. During the last half of the nineteenth century, the controversy regarding the causative agent of fermentation stimulated experimentation that serves as the scientific basis of today's applied microbiology and biochemistry.

For centuries fermentation had a significance that was almost equivalent to what we would now call a chemical reaction, an error that probably arose from the vigorous bubbling seen during the process. The conviction that fermentation was strictly a chemical event gained further support during the early part of the nineteenth century. French chemists led by Lavoisier and Gay-Lussac determined that alcoholic fermentation could be described as the chemical conversion of one molecule of glucose ($C_6H_{12}O_6$) into two molecules of ethanol (C_2H_5OH) and two molecules of CO_2. It was, of course, known that yeast must be added to the glucose solution to ensure a reproducible and rapid fermentation. The function of the yeast, according to the chemists, was merely to act as a chemical catalyst. Then, in 1837, Theodor Schwann and Charles Cagniard-Latour independently published studies that indicated yeast was a living microorganism. Prior to their publication, yeast was considered merely as a proteinaceous chemical substance. One reason it was difficult to ascertain whether yeasts were alive was that, like most other fungi, they are not motile. The cellular nature of yeast was discovered only when improved microscopes became available, and it is most likely that improved microscopes enabled the two workers to come up with the

same observation at approximately the same time. Schwann and Cagniard-Latour also observed that alcoholic fermentation always began with the first appearance of yeast, progressed only with their multiplication, and ceased as soon as their growth stopped, so both scientists concluded that alcohol is a by-product of yeast growth.

The biological theory of fermentation advanced by Cagniard-Latour and Schwann was immediately attacked by the leading chemists of the time. The eminent Swedish physical chemist, Jons Jakob Berzelius, reviewed the papers of Cagniard-Latour and Schwann in his *Jahresbericht* for 1839 and dismissed microscopic evidence as of no value in what was obviously a purely chemical problem. According to Berzelius, nothing was living in yeast. "It was only a chemical substance which precipitated during the fermentation of beer and which had the usual shape of a non-crystalline precipitate." In addition, Liebig published a paper containing several important arguments against the biological theory of fermentation. Liebig's two major points can be summarized as follows:

1. Certain types of fermentation such as the lactic acid (souring of milk) and acetic acid (formation of vinegar) can occur in the complete absence of yeast.
2. It is not necessary to conclude that, even if yeast were a living organism, fermentation is a biological process. Yeast is a remarkably unstable substance, which, as a consequence of its own death and decomposition, catalyzes the splitting of sugar. Thus fermentation is essentially a chemical change catalyzed by breakdown products of the yeast.

Liebig's views were widely accepted, partly because of his powerful influence in the scientific world and partly because of a desire among chemists to avoid conceding an important chemical change to the domain of biology.

In 1857, Pasteur published his first paper in the field of fermentation. The publication dealt with lactic acid fermentation, not alcoholic fermentation. Utilizing the finest microscopes of the time, Pasteur discovered that souring of milk was correlated with the growth of a microorganism, but one considerably smaller than the beer yeast. During the next few years Pasteur extended these studies to other fermentative processes, such as the formation of butyric acid and lactic acid. In each case he was able to demonstrate the involvement of a specific and characteristic microorganism; alcoholic fermentation was always accompanied by yeasts, lactic acid fermentation by nonmotile bacteria, and the butyric acid fermentation by motile, rod-shaped bacteria. Thus Pasteur not only disposed of one of the opposition's strongest arguments, but also provided strong circumstantial evidence for the biological theory of fermentation. Pasteur then attacked the crucial problem, alcoholic fermentation. Liebig had argued that the proteinaceous material, released from yeast during its decomposition, catalyzed the splitting of sugar. Pasteur countered this argument by developing a protein-free medium for the growth of yeast. He found that yeast could grow in a medium composed of glucose, ammonium salts and some incinerated yeast. If this medium was kept sterile, neither growth nor fermentation took place. As soon as the medium was inoculated with even a trace of yeast, growth commenced, fermentation ensued, and the quantity of alcohol produced paralleled the multiplication of the yeast. In this protein-free medium, Pasteur was able to show that fermentation took place without the decomposition of yeast. In fact, the yeast synthesized protein at the expense of the sugar and ammonium salts. Thus, Pasteur concluded in 1860 that "Fermentation is a biological process, it is the subvisible organisms which cause the changes in the fermentation process. What's more, there are different kinds of microbes for each kind of fermentation. I am of the opinion that alcoholic fermentation never occurs without simultaneous organization, development and multiplication of cells, or continued life of the cells already formed. The results expressed in this memoir seem to me to be completely opposed to the opinion of Liebig and Berzelius" (Dubos, 1950).

Pasteur argued effectively and, more importantly, all the data were on his side. Thus, the so-called vitalistic theory of fermentation predominated until 1897, when an accidental discovery by Eduard Buchner finally resolved the controversy and threw open the door to modern catalytic biochemistry based on enzymes.

Buchner was attempting to obtain from yeast an extract that might have medicinal value. After several unsuccessful trials, he discovered that mixing yeast cells with fine sand and grinding the mixture in a mortar and pestle disrupted the cells. After filtering the mash to remove the sand and any unbroken cells, a clear yeast juice was obtained. The juice, however, soon became contaminated with bacterial growth. Since the extract was for human consumption, Buchner could not utilize ordinary antiseptics to prevent the spoilage. Therefore, he attempted to preserve the yeast extract by adding large quantities of sugar. The yeast extract began to bubble soon after the sugar was added. Careful analysis revealed that the sugar was decomposing to carbon dioxide and ethyl alcohol. Fermentation had proceeded *in the absence of living cells*. Buchner's achievement inaugurated a new era in the study of alcoholic

fermentation and other metabolic processes. Reactions that normally took place only in vivo could now be studied in vitro. The agents which are present in cell extracts and which catalyze these reactions were called enzymes, from the Greek en zyme, meaning "in yeast." In 1907 Eduard Buchner received the Nobel Prize in Chemistry "for his biochemical researches and his discovery of cell-less fermentation."

Classical Applied Microbiology (1900–1940)

After it was demonstrated that living yeast was the causative agent for the alcohol fermentation, rapid progress was made in isolating and identifying the microorganisms responsible for other fermentations. As a result, considerable practical knowledge was gained, commercial media developed, producer strains improved, and the fermentation industry grew and diversified (Table 1). As an example, commercial bio-production of citric acid was begun in New York in 1923, and by 1940, over 10-million kgm were produced per annum. Much of this early work on industrial fermentations has been reviewed by Prescott and Dunn (1940).

With the advent of World War I, it became necessary to develop a fermentation method for making acetone, used for the manufacture of explosives. The United States government purchased two large distilleries in Indiana and established the Weizmann process, which made acetone from maize by *Clostridium acetobutylicum* fermentation. After the war, the Weizmann process was used mainly to produce butyl alcohol needed in the manufacture of automobile lacquers. During the development of the Weizmann and other industrial fermentation processes, several important and general aspects of applied microbiology became established.

Isolation of Cultures

Isolation of a pure culture of the desired microorganism is often the first step in an applied microbiology project. Selective media and growth conditions developed for the isolation and cultivation of specific groups of Bacteria and Archaea are described throughout this book. In addition, microorganisms that carry out a specific selectable function can be isolated by enrichment culture procedures (Krieg, 1981; Veldamp, 1970; Jones and Krieg, 1984). For example, if the applied goal is to obtain a xylanase that is active at pH 9.0 and 70°C, then an enrichment culture that contains xylan as the sole carbon and energy source would be adjusted to pH 9.0 and incubated at 70°C. Also, the preferred inoculum would be a soil sample taken from a high temperature and pH site that contains decaying plant material. After several transfers in the same medium, the mixed culture is streaked onto an agar medium containing the same nutrients. The resulting colonies of microorganisms must contain an extracellular xylanase that is active at 70°C and pH 9.0. Enrichment cultures of this type have been successfully employed to obtain a wide variety of useful strains. The power of the enrichment culture technique depends on the enormous metabolic diversity of microorganisms and on the ingenuity of the investigator to establish proper restrictive growth conditions.

Screening Procedures

The enrichment culture technique is valuable only when growth conditions select for the desired strain. The selection is usually based on the degradation of a particular substrate or the resistance to a particular environmental parameter, e.g., high and low temperature, salt and pH

Table 1. Production of industrial chemicals by fermentation.[a]

Fermentation	Microorganism	Commercial Use
Ethyl alcohol	*Saccharomyces cerevisiae*	Industrial alcohol
Acetone-butanol	*Clostridium acetobutylicum*	Synthetic rubber, explosives, lacquers and solvents
Acetone-ethanol	*Bacillus acetolyticus*	Solvents
Acetic acid	*Acetobacter*	Vinegar
Lactic acid	*Lactobacilli, Streptococci*	Textile and leather industries
Propionic acid	Propionic acid bacteria	Solvent
Citric acid	*Aspergillus niger*	Medicines, food and beverages
Gluconic acid	*Penicillium chrysogenum*	Pharmaceutical industry, cleaning
Gallic acid	*Penicillium glacum*	Dye industry
Fumaric acid	*Rhizopus*	Food and beverages
Mannitol	White *Asperg*	Resin production, food and beverages
Dihydroxy acetone	*Acetobacter*	Artificial tanning and chemical industry

[a]See chapter on Organic Acid and Solvent Production.

values, antibiotics and toxic metals. However, it is generally not possible to select directly for microorganisms that produce or overproduce useful chemicals, such as fermentation products, antibiotics and other drugs. In such cases, screening procedures have to be employed. The success of the Weizmann process for producing acetone and butanol depended on screening a large number of microorganisms for a strain (*C. acetobutylicum*, Weizmann) that produced high levels of these solvents. In recent years, many new screening procedures have been developed and automated for the discovery of new products (White et al., 1986). Screening procedures are based on either chemical or functional assays, using intact cells or subcellular preparations. An example of a functional assay that uses intact cells is antibacterial screening by the agar plate diffusion assay (Gerhardt, 1981). The lambda prophage induction assay for screening anticancer drugs is an example of an assay that uses subcellular preparations (Price et al., 1964).

Strain Improvement

Microorganisms freshly isolated from nature have highly regulated metabolic systems designed to prevent the overproduction of biochemicals. Genetically altering the strain so that it overproduces a desired product is an important step in industrial microbiology. Three such general procedures have been used successfully: mutation, genetic recombination, and gene cloning. Each technique has its advantages and disadvantages. Often two or three of these techniques can be used in tandem to obtain a stable improved strain. Success in bringing a fermentation product to market and consequently competing in that market depends on continuous strain improvement programs. Screening and selection methods for strain improvement have been reviewed by Elander (1966), Aharonowitz and Cohen (1981), Queener and Lively (1986), and Silverman et al. (1998).

Historically, mutant screening was the first systematic method to improve industrial strains. The lineage of strain improvement for penicillin production is shown in Table 2. After an exhaustive screening of natural *Penicillium* strains, one isolate (NRRL-1951) was obtained that produced ten times more penicillin than the initial isolate of Fleming. Starting in 1944, strain NRRL-1951 was used as the parent strain for a program of mutation and selection by several groups of investigators. The results were remarkable: strain X-1612, an x-ray-induced mutant of NRRL-1951, produced 0.3 g of penicillin per liter; strain Q-176, an ultraviolet light-induced mutant of X-1612, yielded 0.55 g per liter; and descendants of strain Q-176 (e.g., strain 49-133) in turn produced 1.2 g; and currently used commercial strains yield more than 7 g per liter. The significance of this strain improvement program is that today it is possible to produce enough penicillin at a low cost to treat anyone who needs the antibiotic, whereas before only a few serious cases could be treated at a high cost.

Genetic recombination of advantageous mutations from several mutant strains is a useful procedure for strain improvement. It allows one to combine advantageous mutations from different sources. Also, genetic recombination makes possible the removal of deleterious secondary mutations. For example, when a culture is mutagenized and then a bacterium is selected which overproduces the desired product, the bacterium may also contain mutations that interfere with growth. By backcrossing the mutant strain into the wild type, it is possible to screen for strains that contain the useful mutation without the deleterious one. This procedure of "cleaning up" the strains is particularly important when multiple mutation steps are employed.

Gene manipulation is the third and most recent technique of strain improvement. This method requires a good understanding of the molecular genetics and biochemical pathway that is involved in the biosynthesis of the desired product. Gene manipulation can be used to overcome rate-limiting reactions by increasing the production of specific enzymes. This can be obtained by cloning the gene and increasing its copy number, by altering promoter strength and

Table 2. Strain improvement in penicillin production.

Year	Penicillin strain	Origin	Yield (g/l)
1929	Penicillium (Fleming)	Chance contamination	0.01
1941	NRRL-832	Isolated in Belgium	0.04
1943	NRRL-1951	Isolated from a melon	0.15
1944	X-1612	X-ray mutant of NRRL 1951	0.30
1945	Q-176	UV mutant of X-1612	0.55
1949	49–133	Spontaneous mutants of Q-176[a]	1.2
1990	Commercial strains	Nitrogen mustard mutants of 49–133[a]	>7.0

[a]Several steps of mutation and selection were used to obtain this overproducing strain.

ribosome binding sites, and eliminating undesirable properties such as product inhibition. In addition, gene manipulation can be used to generate new products by combining genes from different microorganisms. This latter technique—combinatorial genetics—has been used successfully to produce new macrolide antibiotics (Hutchinson and Fujii, 1995).

Biochemical Engineering

To commercialize the production of fermentation products by microorganisms, it was necessary to scale-up productions from flasks to large fermentation tanks. This requires a combination of microbiological and engineering skills. The first problem that had to be overcome was the need for sterilization and prevention of contamination by undesirable microorganisms. The fundamental principles of sterilization, developed by Pasteur and others, had to be modified considerably when going from flasks and Petri dishes to very large stainless steel tanks. Not only did engineering techniques have to be applied to sterilize the medium, but techniques had to be developed for the sterile introduction of the inoculum, maintenance of pH and temperature, and the introduction of large quantities of oxygen (air) under sterile conditions.

Understanding and controlling a fermentation process depends on the data obtained from biosensors and instrumentation. The most important parameters to measure and control are pH, temperature, and concentrations of dissolved oxygen and substrate. If these parameters are not maintained within a narrow range, the synthesis of the desired product will decline and in the worst case, the culture will die. Each of these parameters must be measured on-line. In modern fermenters, the information obtained by the sensors is fed into computers that then automatically adjust the conditions of the fermentation broth to a predetermined value. Dissolved oxygen can be regulated by increasing air flow into the broth or agitation speed of the rotors, pH can be adjusted by pumping in acid or base, and temperature can be controlled by heating or cooling with circulating cold water. Additional parameters of biological significance that can be measured include culture turbidity, exit O_2 and CO_2, and product concentration. Instrumentation for measuring and regulating fermentation parameters and for computer control of microbial processes has been reviewed by Wang (1981) and Guerreiro et al. (1997).

Scale-up of fermentation processes generally proceeds from flasks to small fermenters to large fermenters (Gaden, 1981). Certain physiological parameters can be determined in flask experiments, such as optimum temperature for growth and product formation, growth rates, growth yields, changes in pH values during growth, and nutrient requirements. Since the cost of producing a fermentation product is often influenced largely by the costs of the nutrient feed stocks, it is important to examine inexpensive commercial sources of nutrients, such as corn syrup and casein hydrolysates, even in the flask experiments. Small fermenters (1 to 50 liters) are used to determine the optimum biochemical engineering parameters that are required in large-scale industrial fermentations, e.g., oxygen demand. Many economic evaluations can not be performed accurately using laboratory equipment and, therefore, pilot plants must sometimes be built. Scale-up of fermentations has been reviewed by Trilli (1981) and Reisman (1993).

The series of steps used to concentrate and purify the desired product is referred to as the downstream process. Each downstream process is tailored to fit the specific properties of the desired product. The first step is usually the separation of the cells from the broth by continuous centrifugation or hollow fiber filtration. If the product is in the cells, then the cells are disrupted and the product purified by specific biochemical procedures. If the product is in the cell-free broth, then the next step is concentration of the product by removal of the water. Depending on the molecular size and charge of the product, the water can be removed by ultrafiltration, precipitation, or distillation of the product. Again, the final purification of the fermentation product depends on its specific biochemical properties. Typical procedures include liquid-liquid extractions, adsorption chromatography, ion-exchange chromatography and gel filtration.

The Antibiotic Era

(See chapter on Bacterial Pharmaceutical Products.)

The Discovery of Penicillin and Aftermath

The discovery and development of antibiotics is one of the great scientific achievements of the twentieth century. Like many events of major historical significance, the contributing factors were varied and complex. Microbiologists, chemists, fermentation engineers, medical doctors, businessmen, industrialists, lawyers and government officials played major roles. The motivating forces ranged from pure scientific curiosity and the desire to alleviate suffering to economic gain and the pressures of the Second World War.

The story, which has been told many times, begins in 1928 with a fortuitous observation by Alexander Fleming, a bacteriologist working in St. Mary's Hospital in London. Fleming was growing a disease-causing staphylococcus in Petri dishes containing nutrient agar. An airborne fungal spore fell inadvertently onto the agar and began to multiply. As the contaminating mold grew, a halo, or clear area, developed around the mold colony. Such accidents must surely have happened to hundreds of bacteriologists before Fleming, but the ruined agar plates were simply discarded in disgust. Fleming, however, realized the significance of the clear area around the mold colony. The fungus must have secreted something into the medium that inhibited the growth of the staphylococci. He therefore isolated the fungus and repeated the experiment, this time intentionally adding the fungus to the bacterial culture. Again the fungus secreted a product that killed bacteria in the neighborhood of the mold colony. Fleming went one step further. He grew large batches of the mold and then separated the mycelial mass from the culture medium. The mold-free juice he prepared was still lethal to the bacteria. Since the mold was a species of *Penicillium*, Fleming called the antibacterial material in the juice penicillin. Shortly thereafter he attempted to concentrate and purify penicillin, failed, and abandoned the project. There followed a period of ten years during which there were no significant developments in penicillin research. Several suggestions have been put forth to explain this ten-year lag between the discovery of penicillin and its development as a potent chemotherapeutic agent. Although Fleming wrote in 1929 that penicillin "may be an efficient antiseptic for applications to, or injection into, areas infested with penicillin-sensitive microbes," he failed to convince the scientific community. Fleming gave up because he was not a good enough chemist to purify the unstable molecule and lacked the money necessary to hire chemists to help him. With the millions of dollars being spent today on medical research, it is difficult to comprehend how an established scientist like Fleming could not then obtain the $5000 per year that he needed. Finally, it should be mentioned that scientists are not always the best promoters of their own ideas. Fleming represents the classic example of a scientist whose original findings were subsequently rediscovered, developed, and exploited by others.

As a consequence of the rise of fascism in the 1930s, many outstanding scientists were forced to flee from Hitler's Germany. One of these refugees was the biochemist Ernst Chain. Shortly after arriving in England, Chain joined with Howard Florey, a pathologist at Oxford, in a systematic search for antibacterial substances. A careful library study led Chain to select penicillin as the target of research. A relatively short time after receiving the penicillin-producing mold from Fleming, the team of Chain and Florey succeeded in developing techniques for the purification of penicillin. The first animal experiment with the partially purified penicillin was extremely encouraging. Subsequent studies demonstrated that penicillin was not toxic to mammals, including humans. Preliminary tests with humans suffering from incurable bacterial infections showed that penicillin was a miraculous drug. By 1941, the pressing problem was how to produce enough penicillin for general clinical use. With the outbreak of World War II, the need for drugs effective against battle wounds became even more urgent. Florey and Chain went to the Ministry of Health for help. A decision was made at the highest level of government to assist the Oxford group in making more penicillin. Florey was sent to Peoria, Illinois, the headquarters of the newly established Fermentation Division of the U.S. Department of Agriculture. The laboratory in Peoria was ideally suited for the project. The proper equipment was available and, more importantly, the research staff was experienced in mold fermentations. After a few days of intense discussions, Florey returned to England, leaving his trusted assisstant, Norman Heatley, to teach the Americans what was then known about growing *Penicillium* and measuring the quantity of penicillin produced. Progress was rapid. As described above, strain improvement by mutation and selection yielded strains that produced higher and higher levels of antibiotic (Table 2). By 1943, the major bioengineering problems had been resolved by a cooperative effort of the Peoria laboratory, universities and industry, and the first aerated stainless steel tank for penicillin production was put into operation.

One of the ironies of the penicillin saga is that England, where the drug was first discovered and shown to be effective, had to pay United States' companies royalties after the Second World War for the technical know-how to produce penicillin. Fleming, of course, never attempted to patent penicillin. It is unfortunate but true that even if he had, the patents would have expired before the drug was produced commercially. The Oxford group, on the other hand, could have obtained considerable financial reward for developing the penicillin extraction procedure, but instead they gave their knowledge freely to the world. The pioneering research on penicillin, however, did not go unrecognized: The 1944 Nobel Prize in medicine was shared by Fleming, Chain and Florey. For the immigrant biochemist Chain, there was the added pleasure of knowing that he was a major contributor to the develop-

ment of a drug that saved the lives of thousands of young men who fought to liberate his former homeland—sweet justice.

Limitations of Penicillin and the Development of Other Antibiotics

There are at least three serious limitations to the chemotherapeutic use of penicillin. First, it cannot be taken orally. The molecule is rapidly decomposed in the acid of the human stomach. Second, it is not effective against several bacteria, including a number of Gram-negative pathogens. Third, a significant number of people are allergic to it. At least three approaches have been used by applied microbiologists and chemists to overcome these limitations: 1) synthesis of chemical derivatives of penicillin (semisynthetic penicillins) that are more resistant to acid and penicillinase and inhibitory to a wider range of bacteria than the natural penicillin G, 2) use of natural inhibitors of penicillinase, such as clavulanic acid, that can work synergistically with β-lactam antibiotics to extend their range, and 3) development of many other antibiotics for clinical use.

The great success of penicillin encouraged microbiologists to search for other antibiotics. A key figure in this search was the soil microbiologist Selman Waksman of Rutgers University in New Jersey. Between 1939 and 1945, Waksman and his associates examined thousands of microbes for their ability to produce antibacterial substances. A major conclusion that emerged from the Rutgers study was that spore-forming bacteria in the soil, especially the *Streptomyces*, were a rich source of antibiotics. From the thousands of antibacterial substances tested, Waksman isolated, characterized and patented several important antibiotics, including streptomycin, neomycin and actinomycin. Streptomycin, first reported in 1944, quickly emerged as a valuable treatment for tuberculosis and other infections caused by penicillin-resistant Gram-negative bacteria. With Waksman pointing his finger to the soil, the "gold rush" was on for new and better antibiotics. Thousands of active compounds have been found, identified chemically, and screened for potential clinical use. Although most of the active compounds were shown to be too toxic for general use, about 50 of them have proven therapeutic value and are currently produced commercially for medical and veterinary use. A partial list of important antibiotics is shown in Table 3.

What are the common characteristics of antibiotics? 1) From the chemical point of view, they are an extremely diverse group of organic molecules. The only chemical feature antibiotics seem to share is their relatively small size. Molecules have fewer than 40 carbon atoms. 2) Ecologically, antibiotics are produced almost exclusively by spore-forming soil microorganisms. Furthermore, antibiotics are synthesized at precisely the time the cells are sporulating. Because of these correlations, some microbiologists have hypothesized that the natural role of antibiotics is to regulate the formation of spores in microbes. However, many mutants do not produce antibiotics yet sporulate normally. It is, therefore, reasonable to conclude that antibiotics have no single function, playing different natural roles in different microorganisms. 3) Medically, the most important trait of an antibiotic is high therapeutic index, an indicator of selective killing and low toxicity. Each antibiotic interferes with an essential and specific microbial function.

The Role of Antibiotic Research in the Development of Microbial Biochemistry, Molecular Genetics and Biotechnology

Much of what we now know about the bacterial cell wall and biopolymer synthesis has come from studies using specific antibiotics to inhibit synthesis. Furthermore, antibiotics were key tools in the development of microbial genetics. For example, most auxotrophic mutants were obtained using the penicillin selection method (Davis, 1948). Antibiotic resistance continues to be the most important selection marker for genetic recombination. The discovery of plasmids was initially connected to research on multi-drug resistance or R factors. Even today most of DNA recombinant technology relies heavily on the use of antibiotics and antibiotic-resistance genes to construct useful strains. Antibiotic research is an excellent example of how applied and basic research overlap, interact and mutually benefit each other.

The New Biotechnology: Applications of Genetic Engineering

(See chapter on DNA Recombinant Protein Production in Bacteria.)

Cloning genes in bacteria is already a valuable technology in both basic biological research and practical applications (Glazer and Nikaido, 1995). In basic research, genetic engineering in bacteria has been used to examine the underlying mechanisms of DNA replication, gene recombination, and gene expression of viral, bacterial and eukaryotic genes. The number of commercial applications of gene engineering continues to grow (Table 4). Recombinant DNA technology can be used to more efficiently carry

Table 3. Antibiotics used in chemotherapy.

Antibiotic	Source	Used medically in treating	Mode of action
Penicillin	Fungus (Penicillium)	Gram-positive infections	Blocks bacterial cell-wall synthesis
Ampicillin (Penbritin)	Semi-synthetic pencicillin	Gram-positive and gram-negative infections	Blocks bacterial cell-wall synthesis
Cephalosporins	Fungus and *Streptomyces*[a]	Gram-positive and gram-negative infections	Blocks bacterial cell-wall synthesis
Streptomycin	*Streptomyces*	Tuberculosis; gram-negative intestinal tract infections	Inhibits protein synthesis in bacteria
Oxytetracycline (Terramycin)	*Streptomyces*	Most bacterial infections	Inhibits protein synthesis in bacteria
Chlortetracycline	*Streptomyces*	Most bacterial infections	Inhibits protein synthesis in bacteria
Chloramphenicol (Chloromycetin)[b]	*Streptomyces*	Typhoid fever; Rocky Mountain spotted fever	Inhibits protein synthesis in bacteria
Polymyxin (Aerosporin)	*Bacillus*	Gram-negative wound infections	Destroys bacterial cytoplasmic membrane
Bacitracin	*Bacillus*	Topical infections of eye and skin; burn infections	Blocks bacterial cell-wall synthesis
Novobiocin	*Streptomyces*	As penicillin; also penicillin-resistant staphylococci	Blocks bacterial nucleic acid synthesis
Erythromycin	*Streptomyces*	As penicillin; also penicillin-resistant staphylococci	Inhibits protein synthesis in bacteria
Griseofulvin	Fungus and *Streptomyces*[a]	Fungal infections (taken orally)	Destroys fungal cytoplasmic membrane
Polyenes (Nystatin)	*Streptomyces*	Systemic fungal infections	Destroys fungal cytoplasmic membrane

[a]Initially discovered as a fungus, now known also to be produced by *Streptomyces* species.
[b]Initially discovered in a *Streptomyces*, now made chemically.

Table 4. Some of the commercial applications of recombinant DNA technology.

Applications	Examples
Production of mammalian proteins in bacteria	Insulin, blood proteins, interferons, growth hormones and tumor necrosis factor
Vaccines	Hepatitis B, rabies, measles, polyvalent vaccines and DNA vaccines
Fermentation products	Increased yields of antibiotics, restriction enzymes, proteases, xylanases, amino acids and vitamins
Diagnostics	Diagnosis of pathogenic microbes and genetic diseases
New antibiotics	Hybrid polyketides
Transgenic plants and animals	Herbicide, insect and microbial disease resistant plants; cows that produce pharmaceutical products in their milk
Gene therapy	Introduction of cloned adenosine deaminase (ADA) into immune deficient patients lacking ADA
Environmental biotechnology	Cloning and overexpression of genes involved in the degradation of pollutants

out an existing process or develop entirely new products. One of the first examples was the production of human insulin in *Escherichia coli*. After the insulin gene was chemically synthesized and inserted downstream from a suitable *E. coli* promoter, the cell was able to synthesize a fusion protein, which was then split into the separate insulin peptides. These peptides, once connected by disulfide bonds, were the final product that is identical to human pancreatic insulin and now commercially available. Microbially produced human insulin is not immunogenic and is purer and less expensive to produce than porcine or bovine insulin, which was previously used. Several other human protein pharmaceuticals are now produced in microorganisms including anticancer agents, such as α- and β- interferon; human growth hormone for the treatment of dwarfism; nerve growth factor; lysozyme for the treatment of inflammations; and several blood proteins, such as hemoglobins used as blood substitutes (Kumar, 1995), erythroprotein to treat anemia, urokinase to assist in blood clotting, and tissue plasminogen activator to dissolve clots.

Safe and highly effective vaccines can be produced by recombinant DNA technology. Instead of using killed or attenuated pathogenic bacteria or viruses, subunit vaccines are produced by cloning part of the protein that is on the outer surface of a bacterium or virus. The subunit protein vaccine is then produced in a microorganism, usually *E. coli*. The subunit vaccines produce a rapid and high level of protective immunity with no possibility of transmitting the infection. Furthermore, vaccines can be produced against microbes that are difficult to grow in vitro. The first recombinant subunit vaccine approved for use in humans was against hepatitis B virus. Recombinant vaccines are also available for animal diseases, such as Newcastle's and fowl pox diseases in poultry. Several trials are currently being carried out with polyvalent (subunits from more than one protein) vaccines against acquired immunodeficiency syndrome (AIDS). A new approach is to use specific, cloned DNA itself as the vaccine. The DNA is taken up by the animal cell and then transcribed and translated into an active immunogenic protein. If successful, these DNA vaccines would be safe and inexpensive.

The fermentation industry has benefited greatly from recombinant DNA technology. The technology is used in strain improvement programs to obtain not only more efficient processes but also new products. Most of the DNA restriction enzymes used in biotechnology are produced in *E. coli* from cloned DNA that has been put into high expression vectors. Similarly, many of the commercial enzymes (such as proteinases used in the detergent and food industries, xylanases and amylases used in the textile and paper industries, and glucose isomerase used to make high fructose corn syrup) are produced in bacteria that are easy to grow in fermenters, e.g., *E. coli* and *Bacillus* species (Godfrey and West, 1996). An interesting approach to the production of new antibiotics is to place selected genes from two different polyketide antibiotic-producing bacteria into a recipient bacterium that can then synthesize a new polyketide antibiotic (McDaniel et al., 1993). Another important application of recombinant DNA technology is the construction of transgenic plants and animals. Such organisms have great potential for enhancing agricultural productivity and improving the nutritional and storage properties of meats and vegetables. Also in development are crop plants, used for the production of recombinant vaccines, including edible vaccines against cholera and enteric diarrhea. In many of the plant engineering constructs, *Agrobacterium tumefaciens* is used to introduce desired genes into the plant. Monsanto Company (St. Louis, MO) is presently marketing a transgenic soybean plant that has an *Agrobacterium-derived* gene

that confers resistance to Roundup, a glyphosate herbicide. Genetic engineering also has been used to protect plants from viral diseases. Calgene Corporation (Davis, CA) produces a transgenic tomato that spoils more slowly than normal tomatoes because it contains an inhibitor of pectinase transcription. Transgenic animals are used extensively in research as well as for the commercial production of proteins of pharmaceutical value, such as α-1-antitrysin in sheep and tissue plasminogen activator in goats. Biotechnology is also involved with human genes and, in the long run, recombinant DNA technology will probably have its most dramatic effect on the human genome. As Robert Sinsheimer stated thirty years ago:

"For eon after eon, creature has given rise to creature upon this earth—blindly, each generation usually like the former, occasionally—by accident—a little different. Of all the creatures that have lived upon this earth we are the first to understand this process. . . . The ultimate significance of this understanding of the very basis of heredity is incalculable. It will change all the eons to come" (Rosenberg, 1991).

Already, PCR technology is widely used in forensic medicine to determine parentage, to analyze evolutionary relationships between modern man, early man, and pre-man, and so forth. In one of the largest biological research programs ever conducted, the human genome project, the entire nucleotide sequence of the human genome will soon be determined. By comparing the DNA sequence of healthy and diseased individuals, the precise position and aberration in the "bad" gene may be found. Gene therapy, through genetic engineering, offers the hope of replacing disease-causing genes with healthy ones, thereby providing preventive medicine in the most profound sense.

Applied Environmental Microbiology: Bioremediation

(See chapters on Bioremediation and Bioemulsifiers.)

Albert Einstein defined the environment as "anything which is not me." This definition is interesting for two reasons: it has enormous scope, and it points the finger away from each of us. Individuals, industries and societies have assumed incorrectly that if the environment is not "them," they do not have to be responsible for it. The result is world-wide pollution, which degrades the quality of our lives and threatens our very existence.

When considering pollution, one must focus on the turnover of matter, which is fundamentally a series of microbial processes. It is primarily by microorganisms that waste products, including most pollutants, are broken down. Microbes have enormous biodegradation potential. They occupy all possible ecological niches, from deep in the earth to high in the atmosphere, and collectively are able to grow over a wide range of temperatures, pH values, salt concentrations and oxygen tensions. There is not a single natural organic compound that cannot be degraded by one or more microbial species.

If microbes have such enormous degradative power, why do we have pollution? For two fundamental reasons: 1) man-made conditions are unfavorable to rapid degradation and 2) certain man-made synthetic compounds are resistant. The former is dictated by the "law of the minimum," i.e., growth is limited by one essential component. For example, if a large amount of petroleum is dumped into the sea, biodegradation may not be at an appreciable rate because utilizable forms of nitrogen and phosphorus are not available—even if many hydrocarbon-degrading bacteria are at the site. Nitrogen and phosphorus are, of course, essential components for all living cells. Another condition, which is unfavorable for biodegradation, is high concentration of organic material. Two good examples of this are whiskey and jam in which ethyl alcohol and sugar are respectively at levels that resist breakdown. One of the reasons that organic matter is degraded so slowly in garbage dumps is that the consolidation and concentration of materials make biodegradation difficult. In the above examples, natural bacteria can completely degrade (mineralize) these materials, whether they are hydrocarbons in crude oil or cellulose in newspaper, if the conditions are favorable. However, several man-made unnatural compounds, such as certain halogenated aromatics and plastics, are not biodegradable in any circumstances. They simply have not been on the planet long enough for bacteria to have evolved degradative mechanisms.

Bioremediation is the use of living organisms, usually bacteria, to prevent or clean-up pollution. Bioremediation is a large and growing area of applied microbiology (Bitton, 1994). Hundreds of companies around the world are involved in treating water and soil that is polluted with industrial wastes, such as petroleum products, herbicides, pesticides, explosives like TNT, toxic metals, dioxenes and polychlorinated biphenyls (PCBs). There are two general approaches to bioremediation. The first and most successful method is to optimize conditions so bacteria native to the polluted site can more rapidly degrade the pollutants. The second method, which has had limited success to date, is to introduce more efficient degraders to the polluted

site, or in the case of nonbiodegradable synthetic pollutants, to construct genetically engineered microorganisms (GEMs) that can degrade the pollutant. The following examples will illustrate these two approaches.

Petroleum Pollution and Bioremediation

During the twentieth century the demand for petroleum as a source of energy and as a primary raw material for the chemical industry has increased world production to about 3,500 million metric tons per year. Approximately 1% or 35 million tons finds its way to soils, lakes and seas. The toxicity of petroleum to microorganisms, plants, animals and man is well established. The polycyclic aromatic hydrocarbon (PAH) fraction of petroleum is particularly toxic and is on the United States Environmental Protection Agency priority pollutant list (Cernilia, 1992).

It has been known for over 80 years that certain bacteria are able to degrade petroleum hydrocarbons and use them as a sole source of carbon and energy. Effective bioremediation of petroleum pollution relies heavily on a fundamental understanding of the requirements for petroleum biodegradation (Table 5). Generally, the presence of hydrocarbon-degrading microorganisms is not the limiting factor in petroleum bioremediation. Hydrocarbon-oxidizing bacteria have been isolated from a wide variety of natural aquatic and terrestrial environments (Rosenberg, 1991), including hydrothermal vent sites (Baylinski et al., 1989). Furthermore, the addition of oil causes a rapid shift to increased numbers of hydrocarbon degraders. For example, Song and Bartha (1990) demonstrated that the number of hydrocarbon-degrading microorganisms increased from 410^4 to 310^{10} per g of soil 4 weeks after addition of jet fuel and inorganic nutrients. The practical conclusion from this and other studies is that seeding an oil-polluted area with microorganisms is generally not necessary, and GEMs are not required for petroleum bioremediation.

In addition to having the biochemical potential for degrading one or more classes of hydrocarbons, an efficient hydrocarbon degrader must be able to adhere to and desorb from water-insoluble hydrocarbons. The role of cell surface hydrophobicity, adhesins, and bioemulsifiers in the growth process has been reviewed (Rosenberg and Ron, 1996).

The most important factor determining the success of a petroleum pollution bioremediation project is the ability to provide the optimum conditions for the hydrocarbon-degrading bacteria. The major requirements are adequate sources of oxygen, nitrogen and phosphorus. Oxygen can be added to soils by frequent plowing and to underground reservoirs by pumping aerated water through them. Soils can be amended with utilizable nitrogen and phosphorus salts. However, no satisfactory method exists that provides the nitrogen and phosphorus necessary for bioremediation of oil spills in open systems, such as the sea. The ideal nitrogen and phosphorus source should adhere to the oil, rather than be diluted with water. An example of bioremediation of beach sand by addition of nitrogen and phosphorus is shown in Table 6. Overall, the bioremediation treatment resulted in the degradation of 88% of the oil after 4 months. During the same period, there was virtually no change in the oil concentration of the untreated control plot.

The Use of GEMs in Bioremediation

The first GEM to be patented in the United States was a Pseudomonas that was designed for degrading different hydrocarbons and constructed from genes derived from different bacteria (Chakrabarty and Gunsalus, 1971). The great potential of GEMs for bioremediation was publicized by the news media, which referred to them as "superbugs." Unfortunately, the potential of GEMs in bioremediation has not been

Table 5. Requirements for biodegradation of petroleum.

A. Microorganisms with:
   1. Hydrocarbon-oxidizing enzymes
   2. Ability to adhere to hydrocarbons
   3. Mechanism for desorption from hydrocarbons
   4. Emulsifier-producing potential
B. Water
C. Oxygen
D. Relatively large amounts of utlizable nitrogen and phosphorus
E. Iron, magnesium, and other essential elements

Table 6. Bioremediation of hydrocarbon-contaminated sand at an Israeli beach.

	Hydrocarbon degradation (%)	
Day	Control (untreated)	Experimental (N_3P added)[b]
10	10	20
20	20	41
30	22	50
40	18	60
50	20	65
70	10	70
90	0	75
120	0	88

[a]Data from Rosenberg et al. 1996.

[b]Approximately 30,000 m^2 of heavy oil contaminated beach were treated with 15 g fertilizer (containing utilizable N and P) per kg sand. The area was plowed twice weekly.

realized as yet for several reasons. 1) Safety regulations have restricted the release of GEMs into the environment; in fact, most of the research carried out on GEMs and bioremediation has been diverted to studying the fate of GEMs in the environment. 2) Genetically engineered microorganisms do not appear to have any significant advantage over a mixture of existing bacteria for the in situ degradation of natural pollutants, such as crude oil. 3) Also, to construct GEMs that effectively degrade recalcitrant synthetic pollutants has proved difficult. In spite of these problems, progress is being made in constructing GEMs for degrading haloaromatic compounds (Reineke, 1998; Timmis et al., 1994) and other pollutants (Chen et al., 1999). When enhanced biodegradative pathways are combined with the genetic potential to catabolize these pollutants in the natural environment, then mineralization rates will likely improve. One possible approach is to clone genes for biosurfactant production into GEMs that have good catabolic potential.

Health-Related Applied Microbiology

(See chapter on Bacterial Pharmaceutical Products.)

Drug Discovery

One of the most exciting areas of applied microbiology involves the discovery of new drugs for human and animal health. Secondary metabolites produced by microorganisms provide a vast array of potentially useful drugs. While the search for new drugs from *Streptomyces* and other spore-forming bacteria continues to be productive, new sources of bacteria are now being exploited, such as marine bacteria (Fenical, 1993, 1997). In addition to expanding the pool of microorganisms in the search for novel pharmaceuticals, researchers are now employing new technologies for drug screening (Steele and Stowers, 1991; Lloyd, 1998; Schmid et al., 1999). These include robotic techniques for plating test bacteria and delivering dilutions of bacterial extracts, use of monoclonal antibodies and fluorescent techniques and combinatorial biosynthesis (Hutchinson, 1998), and screens based on specific enzymatic reactions. These new techniques allow for a more rapid and comprehensive screening for new drugs.

Diagnostic Microbiology

Detecting, classifying and enumerating microorganisms in drinking water, foods and beverages, and human tissues and fluids is a large area of applied microbiology that is constantly undergoing improvements. During most of the twentieth century, microbiology laboratories developed many dependable approaches for detecting microorganisms, especially pathogens, using microscopy coupled to staining techniques, culturing in selective media, and immunoserology (Koneman et al., 1992). This field of diagnostic microbiology has proved invaluable to physicians treating patients, food producers controlling the quality of their products, and health officials monitoring water supplies and consumer products. However, many of these diagnostic tests are slow, expensive and insensitive. For example, culturing bacteria from urine or blood and then testing them for antibiotic sensitivity can take several days. During this time the patient may be at risk, or in the case of a water supply or consumer product, many people could be infected during the diagnosis period. Therefore, it would be advantageous to have more rapid and sensitive techniques.

During the last ten years, many new biotechnology companies have emerged that have as their major focus the development of specific diagnostic tests that do not depend on culturing microorganisms. Rather, these new biotechnology methods rely on amplification of a species-specific DNA target, of the probe, or of the signal that is generated (Hammes and Higgins, 1995; Whelen and Persing, 1996). In addition to great sensitivity and rapidity, these methods can detect pathogens that are difficult to culture, such as *Tropheryma whippelii* (Relman et al., 1992), *Chlamydia trachomatis* (Chapin-Robertson, 1993) and *Neisseria gonorrhorea* (Hale et al., 1993). Nucleic acid probes have already replaced classical morphology and biochemical tests in clinical laboratories for the definitive identification of mycobacteria (Keller, 1993). Considerable effort is now being employed to develop biotechnology-based detection methods for drug resistance in clinically important organisms (Eron et al., 1992). Advances in approaches to DNA-based diagnostics have recently been reviewed (Whitcombe et al., 1998).

Food Microbiology

(See chapter on Bacteria in Food and Beverage Production.)

Food microbiology, one of the traditional areas of applied microbiology, is changing dramatically. Microorganisms play a direct role in producing and processing the healthy foods we eat and drink. Meat and milk are available to us only because microbes, in symbiotic relationships with certain animals, are able to digest cel-

lulose. Different microorganisms convert milk to a variety of yogurts, cheeses and butter. Still other microbes preserve plant materials in the form of tasty products such as pickles and sauerkraut. Alcoholic fermentation by yeast is needed for bread as well as all alcoholic beverages. Hagedom and Kaphammer (1994) have reviewed the role of microbial biocatalysis in the generation of flavor and fragrance chemicals.

An interesting recent development in food microbiology is the use of "probiotic bacteria" to improve animal and human food (Bengmark, 1998; Holzapfel, 1998; Guarino, 1997). Probiotic bacteria are generally isolated from the mouth, stomach and upper and lower bowel of healthy humans and animals. These normal resident bacteria are beneficial, providing vitamin K, helping to process nutrients, preventing colonization of transient bacteria (nonspecific defense mechanism), developing the immune system, and stimulating phagocytic activity. The future of probiotics will depend upon the development of safe strains that are well adapted to occupy specific niches in the body and promote health. When added to food or used as starters in food fermentations, these strains can provide the beneficial effects of probiotics to man and animals.

Biosafety and Legal Protection in Biotechnology

The safety of research microbiologists and legal protection for their discoveries are two subjects which have become more important in recent years in applied microbiology and biotechnology. Unfortunately, most microbiologists do not get an adequate education in these topics. Many laboratory techniques create aerosols, which can lead to the inhalation of undetected infectious agents and the occupational illnesses among laboratory workers who handle infectious materials. Based upon available data, preventative measures have been developed which provide safeguards for scientific personnel and the environment. These safeguards are collectively called containment practices. Leiberman et al. (1986) have written a concise review on biosafety in biotechnology, which should be studied by all practicing microbiologists.

To derive financial benefit from an original and potentially useful discovery in biotechnology, it should be patented. This is particularly important for research scientists at universities because once a patent application has been filed, it is possible to disclose the invention (scientific publications, grant applications, seminars, etc.) and still have legal protection. In the United States it is possible to file a patent within one year of the publication date. The culture of science demands free exchange of ideas and data. Patenting makes this possible, while still protecting the rights of the scientist. Another reason for patenting a discovery is that it allows the owner of the patent to more safely market the invention. An applied microbiologist at a university or research institution cannot generally convert his discovery into a commercial product. It is therefore necessary for the institution to find a partner or sell the patent and know-how. The procedures for obtaining a patent have been outlined by Saliwanchik (1986).

Outlook

The border between basic and applied microbiology, which has always been tenuous, is disappearing. The period in which a discovery in basic microbiology becomes an applied project is often very short. Some of the most basic discoveries in microbiology are now made by scientists working on applied problems in universities, research institutions and biotechnology companies. The rapid progress taking place in applied microbiology and biotechnology, made possible by almost a century of fundamental work on microbial biochemistry, physiology and genetics, makes it difficult to predict which areas will develop most rapidly in the next few decades. Clearly, the use of bacteria in DNA-based technologies will continue to grow and find new applications. Already, a large percentage of the corn grown in the United States contains cloned *Bacillus thuringiensis* protein as an endogenous insecticide. In addition, I predict the following three general subjects will soon emerge as highly significant in applied microbiology and biotechnology.

Environmental Microbiology

The rapid increase in human population and technological developments is degrading our environment at an alarming rate, causing a lowering of the quality of life and serious health problems. Since microorganisms are largely responsible for the turnover of matter on this planet, applied environmental microbiology will become more important. Not only will bioremediation develop further scientifically, technologically, and commercially, but also selected microbes will be used under controlled conditions to recycle waste and to prevent pollution by replacing pollution-producing chemical processes. The so-called "green revolution" will be carried out by microorganisms supplied by a new generation of applied microbiologists.

An outcome of research in environmental microbiology will be the emergence of new prin-

ciples of ecology. For the same reasons that microbes were ideal organisms to study the principles of biochemistry and genetics, they will be used to develop fundamental principles of ecology. The use of higher animals and plants for deriving general ecological principles is limited by our inability to carry out controlled experiments. Animal and plant ecologists can make accurate observations, ask interesting questions and present hypotheses, but the microbial ecologist can obtain quantitative data in controlled experiments, using molecular probes and other tools of modern microbiology. The combined effort of animal, plant and microbial ecologists should lead to a better understanding of how organisms interact with their environment.

Microbial Associations with Animals, Plants and Humans

It is clear that natural bacteria play a key role in maintaining the health of animals, plants and humans by providing nutrients and conditions that deter pathogens (Falk et al., 1998). As we learn more about these interactions, use of selected and improved bacterial strains will grow: in agriculture, for improving yields and preventing spoilage; in animal husbandry, for improving yields and nutritional value of meat, milk and eggs; in humans, for maintaining health and deterring diseases. The use of probiotics in animal food and milk products for human consumption is just the beginning of this development.

Microbial Enzymes

Microbial enzymes are currently being used commercially, primarily for degradative reactions, e.g., proteinases, cellulases, amylases and lipases. This area will be further developed with the introduction of more efficient enzymes and enzymes from extremophiles with increased heat tolerance and extended pH range. However, the commercial potential of microbial enzymes used to synthesize drugs and other fine and bulk chemicals is even greater. With the accumulating data on biosynthetic pathways and the ability to overproduce specific enzymes, it should be possible to mix and match enzymes both in vivo and in vitro to efficiently produce existing valuable products as well as new materials. In this regard, bacterially produced polyhydroxybutyrate and its copolymers have attracted considerable attention as environmentally degradable plastics for a wide range of agricultural, industrial and medical applications (Anderson and Dawes, 1990).

This short list is certainly not comprehensive; many other subjects of applied microbiology will expand and new areas will be created. In short, the future of applied microbiology and biotechnology is bright.

Literature Cited

Aharonowitz, Y., and G. Cohen. 1981. The microbiological production of pharmaceuticals. Sc. American 245:106–119.

Anderson, A. J., and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–477.

Baylinski, D. A., C. E. Wirsen, and H. W. Jannasch. 1989. Microbial utilization of naturally occurring hydrocarbons at the Guaymas Basin hydrothermal vent site. Appl. Environ. Microbiol. 55:2832–2836.

Bengmark, S. 1998. Ecological control of the gastrointestinal tract. Gut 42:2–7.

Bitton, G. 1994. Wastewater Microbiology. Wiley-Liss. New York.

Cernilia, C. E. 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368.

Chakrabarty, A. M., and I. C. Gunsalus. 1971. Degradative pathways specified by extrachromosomal gene clusters in Pseudomonas. Genetics 68:510.

Chapin-Robertson, K. 1993. Use of molecular diagnostics in sexually transmitted diseases: critical assessment. Diagn. Microbiol. Infect. Dis. 16:173–184.

Chen, W., F. Bruhlmann, R. D. Richins, and A. Mulchandani. 1999. Engineering of improved microbes and enzymes for bioremediation. Curr. Op. in Biotechn. 10:137–141.

Davis, B. D. 1948. Isolation of biochemically deficient mutants of bacteria by penicillin. J. Am. Chem. Soc. 70:4267.

Elander, R. P. 1966. Two decades of strain development in antibiotic-producing microorganisms. Dev. Ind. Microbiol. 7:61–73.

Eron, J. J., P. Gorczyc, J. C. Kaplan, and R. T. D'Aquila. 1992. Susceptibility testing by polymerase chain reaction DNA quantitation: a method to measure drug resistance of human immunodeficiency virus type 1 isolated. Proc. Natl. Acad. Sci. 89:3241–3245.

Falk, P. G., L. V. Hooper, T. Midvedt, and J. I. Gordon. 1998. Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology. Microbiol. and Mol. Biol. Rev. 62:1157–1170.

Fenical, W. 1993. Chemical studies of marine bacteria: Developing a new resource. Chem. Rev. 93:1673–1683.

Fenical, W. 1997. New pharmaceuticals from marine organisms. Trends in Biotechnol. 15:339–341.

Gaden, E. L. 1981. Production methods in industrial microbiology. Sc. American 245:134–145.

Gerhardt, P. (ed). 1981. Manual of Methods for General Bacteriology. ASM Press. Washington, D.C.

Glazer, A. N., and H. Nikaido. 1995. Microbial Biotechnology: Fundamentals of Applied Microbiology. W. H. Freeman. New York.

Godfrey, T., and S. West. 1996. Industrial enzymology. Godfrey, T. and S. West. The Application of Enzymes in Industry 2nd Edition. Stockton. New York.

Guarino, A. 1997. Oral bacterial therapy reduces the duration of symptoms and of viral excretion in children with milk diarrhea. J. Pediatric Gastroenterol. Nutr. 25:516–519.

Guerreiro, M. A., S. R. Andrietta, and F. Maugeri. 1997. Expert system for the design of an industrial fermentation plant for the production of alcohol. J. Chem. Technol. Biot. 68:163–170.

Hagedom, S., and B. Kaphammer. 1994. Microbial biocatalysis in the generation of flavor and fragrance chemicals. Ann. Rev. Microbiol. 48:773–800.

Hale, Y. M., M. E. Melton, J. S. Lewis, and D. E. Willis. 1993. Evaluation of PACE 2 Neiseria gonorrhoeae assay by three public health laboratories. J. Clin. Microbiol. 31:451–453.

Hammes, B. D., and S. J. Higgins. 1995. Gene Probes: A Practical Approach. Oxford University Press. New York, 1–288.

Holzapfel, H. H. 1998. Overview of gut flora and probiotics. International Food Microbiol. 41:85–101.

Hutchinson, C. R., and I. Fujii. 1995. Polyketide synthase gene manipulation: a structure-function approach in engineering novel antibiotics. Ann. Rev. Microbiol. 49:201–238.

Hutchinson, C. R. 1998. Combinational biosynthesis for new drug discovery. Curr. Opin. Microbiol. 1:319–329.

Jones, D., and N. R. Krieg. 1984. Seriology and chemotaxonomy. N. R. Krieg, and J. C. Holt. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, 15–18.

Keller, G. H. 1993. DNA Probes: Background, Applications and Procedures, 2nd Edition. Stockton Press. New York.

Koneman, E. W., S. D. Allen, V. R. Dowell, Jr., W. M. Janda, H. M. Sommers, and W. C. Winn, Jr. 1992. Color Atlas and Textbook of Diagnostic Microbiology, 4th Edition. J. B. Lippincott. Philadelphia, PA.

Kreig, N. R. 1981. Enrichment and isolation. P. Gerhardt. Manual of Methods for General Bacteriology. ASM. Washington, DC, 112–142.

Kumar, R. 1995. Recombinant hemoglobins as blood substitutes: a biotechnology perspective. Proc. Soc. Exp. Biol. 208:150–158.

Leiberman, D. G., R. Fink, and F. Schaffer. 1986. Biosafety in biotechnology. A. L. Demain, A. L. and N. A. Solomon. Industrial Microbiology and Biotechnology. ASM Press. Washington, DC, 402–409.

Lloyd, A. 1998. High-throughput screening. Drug Discovery Today 3:566–569.

McDaniel, R., S. Ebert-Khosla, D. A. Hopwood, and C. Khosla. 1993. Engineered biosynthesis of novel polyketides. Science 262:1546–1550.

Prescott, S. C., and C. G. Dunn. 1940. Industrial Microbiology. McGraw-Hill Inc. New York, NY.

Price, K. E., R. E. Buck, and J. Lein. 1964. System for detecting inducers of lysogenic Escherichia coli W1709 (gamma) and its applicability as a screen for antineoplastic antibiotics. Appl. Microbio. 12:428–435.

Queener, S. W., and D. H. Lively. 1986. Screening and selection for strain improvement. A. L. Demain and N. A. Solomon. Industrial Microbiology and Biotechnology. ASM Press. Washington, D.C., 155–169.

Reineke, W. 1998. Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. Ann. Rev. Microbiol. 52:287–332.

Reisman, H. B. 1993. Problems in scale-up of biotechnology production processes. Crit. Rev. Biotechn. 13:195–253.

Relman, D. A., T. M. Schmidt, R. P. MacDermott, and S. Falkow. 1992. Identification of the uncultured bacillus of Whipple's disease. N. Eng. J. Med. 327:293–301.

Rosenberg, E. 1991. Hydrocarbon-oxidizing bacteria. A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer. The Prokaryotes, 2nd Edition. Springer-Verlag. New York, NY. 446–459.

Rosenberg, E., and E. Z. Ron. 1996. Bioremediation of petroleum contamination. R. L. and D. L. Crawford. Bioremediation: Principles and Applications. Cambridge University Press. New York, NY. 100–124.

Rosenberg, E., R. Legmann, A. Kushmaro, E. Adler, H. Abir, and E. Z. Ron. 1996. Oil bioremediation using insoluble nitrogen source. J. Biotechnol. 51:273–278.

Saliwanchik, R. 1986. Legal protection for biotechnology. A. L. Demain and N. A. Solomon. Industrial Microbiology and Biotechnology. ASM Press. Washington, D.C., 389–401.

Schmid, I., I. Sattler, S. Grabley, et al. 1999. Natural products in high throughput screening: Automatic high-quality sample preparation. J. Biomol. Screen. 4:15–25.

Silverman, L., R. Campbell, and J. R. Broach. 1998. New assay technologies for high-throughput screening. Curr. Opin. Chem. Biol. 2:397–403.

Song, H. G., and R. Bartha. 1990. Effects of jet fuel spills on the microbial community in soil. Appl. Environ. Microbiol. 56:646–651.

Steele, D. B., and M. D. Stowers. 1991. Techniques for selection of industrially important microorganisms. Ann. Rev. Microbiol. 45:89–106.

Swaminathan, B., and P. Feng. 1994. Rapid detection of foodborne pathogenic bacteria. Ann. Rev. Microbiol. 48:401–426.

Timmis, K. W., R. J. Steffan, and R. Unterman. 1994. Designing microorganisms for the treatment of toxic wastes. Ann. Rev. Microbiol. 48:525–557.

Trilli, A. 1981. Scale-up of fermentation. A. L. Demain and N. A. Solomon. Industrial Microbiology and Biotechnology. ASM Press. Washington, D.C., 277–307.

Veldamp, H. 1970. Enrichment cultures of prokaryotic organisms. J. R. Noris and D. W. Ribbons. Methods in Microbiology–Vol. 6. Academic Press Inc. New York, NY. 305.

Wang, H. Y. 1981. Bioinstrumentation and computer control of fermentation processes. A. L. Demain and N. A. Solomon. Industrial Microbiology and Biotechnology. ASM Press. Washington, D.C., 308–320.

Whelen, A. C., and D. H. Persing. 1996. The role of nucleic acid amplification and detection in the clinical microbiology laboratory. Ann. Rev. Microbiol. 50:349–373.

Whitcombe, D., C. R. Newton, and S. Little. 1998. DNA-based diagnostics. Curr. Op. in Biotechnol. 9:602–608.

White, R. J., W. M. Maiese, and M. Greenstein. 1986. A. L. Demain and N. A. Solomon. Industrial Microbiology and Biotechnology. ASM Press. Washington, D.C., 24–31.

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CHAPTER 1.13

The Structure and Function of Microbial Communities

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Introduction

For the greater part of its history, microbiology has been a science of the single cell. The cell has dominated thought and experiment. So much so that discussion of other forms of organization, if addressed at all, was most often the subject of hallway speculation rather than a serious question of investigation. It is heartening that within the last decade or so the science of microbiology has begun to address the dimensions of organization that transcend the single cell. For example, the study of biofilms as organized systems is now more acceptable, and several high profile publications have emerged that examine the physiological basis of structure and function at a multicellular level of monospecific biofilm communities (Fuqua et al., 1994; Fuqua et al., 1996; Hastings and Greenberg, 1999; Whiteley et al., 2001). Although there remains an essential reductionism to these studies, they have demonstrated the importance of intrapopulation, and possibly interpopulation, communication systems in controlling the structure and activity of multi-species microbial systems.

A decade ago one of the authors contributed a short article to the American Society for Microbiology (ASM) News (Stahl, 1993), noting a fundamental variance between microbiology and general biological sciences. Microbiology was not built upon a foundation of natural history, for lack of tools to observe and categorize microbes in the field. Morphology was of little utility—the simple shapes of microorganisms conceal their remarkable diversity. Culture-based studies provided only a sketchy census of natural diversity, and conventional biochemical tests did not serve a phylogenetically based system of classification. It was only through comparative sequencing, first of proteins and later of nucleic acids, that inferences of phylogenetic relationship among microorganisms could be made (Zuckerkandl and Pauling, 1965; Jukes and Cantor, 1969). Today we see a field transformed by comparative analyses of genes and more recently of complete genomes. The metric provided by a molecular clock introduced an evolutionary perspective and phylogenetic dimension most forcefully represented by a universal tree of life (Woese, 1987; Woese et al., 1990). This single phylogeny revealed the vast diversity of microbial life, reducing the plants and animals to a peripheral branch (Fig. 1). This has fueled more general recognition that the primary biology of our planet is microbial and provided an essential framework to conduct a census of diversity (Stahl et al., 1984; Olsen et al., 1986; Ward et al., 1990; DeLong et al., 1994; Amann et al., 1995). Within that framework, both cultured and uncultured organisms could be related through a common metric based on the sequence divergence of common biopolymers (e.g., DNA encoded RNA and protein components of the cellular translational and transcriptional machinery). As environmental surveys were initiated, and today continue, microbiologists are confronted with a natural diversity that far exceeds that represented in pure culture, revealing major lines of descent (kingdom and phylum level) that were previously unrecognized. The astounding implication is that we are only now beginning to explore significantly the biological diversity of our planet.

This introduction must include a disclaimer. Although our subject is microbial community structure, the accepted unit of community structure, the species, remains poorly conceptualized. Complete genome sequences and expansive molecular diversity surveys have not provided much illumination on the "species problem." For example, comparative analyses of different strains of *Escherichia coli* have revealed a remarkable plasticity in genome sequence among organisms coherent by traditional phenotypic criteria (Wren, 2000; Hayashi et al., 2001; Perna, 2001; Pennisi, 2001; Dobrindt et al., 2003). Microbial geneticists were startled by the large fraction of genes of unknown function revealed in the genome sequence of *E. coli*. Each newly sequenced genome contains a similarly high fraction of unknowns (20–30%). Nonetheless, clinical labs would assign *E. coli* strains differing by as much as 30% to the same species without difficulty. In contrast, strains of other well

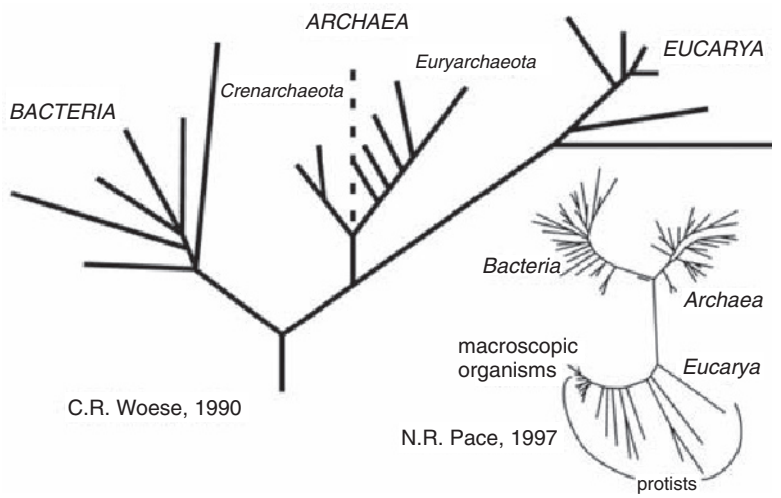

Fig. 1. Universal phylogenetic tree in rooted form shows the three domains. Branching order and branch lengths are based upon rRNA sequence comparisons (Woese et al., 1990). Inset tree illustrates elaborations of existing branches (Pace, 1997) but maintenance of the fundamental framework determined in the earlier phylogeny.

described genera and species are much more uniform in genome sequence and organization. For example, the genome sequence of *Mycobacterium bovis* is greater than 99.95% identical to *M. tuberculosis*, having no genes that uniquely distinguish it from *M. tuberculosis* (Garnier et al., 2003). This apparent incongruity illustrates one of the limitations of characterizing microorganisms in populations of clonally derived cells—the pure culture—and of emphasizing selected features (such as host range or pathogenicity) in formal descriptions. Here, we emphasize that the laboratory culture is not an appropriate context for fully appreciating any organism—divorced from a native habitat of which we have little understanding. The high proportion of genes of unknown function in every completed genome is another reflection of the pure culture paradigm. The environment is the context in which genomes evolved, function, and continue to evolve. It is the only context in which they can be fully understood.

The habitat in which microbial communities reside includes the interplay between biotic and abiotic factors. Microbiologists, again largely because of the pure culture paradigm, have tended to emphasize the abiotic features of habitat (electron donor, electron acceptor, salinity, temperature, pressure, etc.). These are the standards by which organisms have been defined. However, these factors are in most instances only a thin slice of the parameter space defining their ecology and evolution. As was long ago expressed by Darwin, "... the most important of all causes of organic change is one which is almost independent of altered physical conditions, namely, the mutual relation of organisms to organisms. If some of these many species become modified and improved, others will have to be improved in a corresponding degree or they will be exterminated." Thus, more complete understanding of any organism must encompass the features of higher order organization that have shaped the organism's evolution and contemporary "niche."

A recent retrospective by Moselio Schaechter suggests that we have so far experienced two golden ages in microbiology (Schaechter, 2003). The first age followed recognition of microorganisms in general terms—as examples of the unity of biochemical processes, as agents of disease and spoilage, and the primary engines of biogeochemical transformations. The technological development that heralded the beginning of this first golden age was the pure culture method. Advances in molecular biology and associated methods of genetic analysis introduced the second golden age. We are now poised at the beginning of a third golden age which will develop a more complete understanding of systems of organization (ecology), their origins, and mechanisms of change (evolution). This age will be fostered not only by the rapid advances in technology (e.g., high throughput sequencing, proteome and transcriptome analyses, nanotechnology, and bioinformatics) we now see influencing research in all areas of inquiry where life intrudes, but also by the coalescing of disciplines needed to address the complexity of systems-level organization. An empirical observation has been that predictions of higher levels of organization in biological systems are not readily derived from understanding gained at lower levels (Mayr, 1982). Thus, at least for biological systems, it is essential that the tools of analysis accommodate the complexity encountered at higher organizational levels. Advances in technology in concert with disciplinary coalescence will foster investigations that move far beyond the study of clonal lines in laboratory culture to studies of natural systems comprised of diverse interacting populations. This will continue to be

true and the final section of this chapter is devoted to limited discussion of the changing landscape of technology.

Structure and Function of Microbiological Systems

Microbiological systems can be characterized in three general ways: 1) the historical (considering system origins and evolution), 2) the proximate (characterizing the immediate structure and function of a system), and 3) the temporal (addressing the interplay of biotic and abiotic elements that shape a system over diel and annual temporal periods). No one way of characterization provides complete understanding—all three must be considered. In turn, each characterization must incorporate the different dimensions of structure and function. These dimensions encompass both the biotic (phylogenetic, evolutionary, and metabolic) and the abiotic (chemical and physical). We have organized this chapter by considering both the different ways microbiological systems are characterized and the multiple dimensions of structure and function that serve characterization.

The Historical System

The Phylogenetic Dimension

The evolution of our planet is intertwined with the evolutionary history of its microorganisms. Neither the planet nor any one organism is fully intelligible apart from that ancestry. Today, a reasonable representation of the phylogenetic relationships among all life is available (Woese et al., 1990; Woese, 2000). The "universal tree," inferred by comparative sequencing of the small ribosomal subunit rRNA, remains the canonical structure (Fig. 1). Although it is recognized that horizontal gene transfer may have eroded some of the fine detail of structure (Doolittle, 1999), there is an emerging consensus that microorganisms display an organismal genealogy and that many genes are phylogenetically informative (Ochman et al., 2000; Daubin et al., 2003).

Recognition that a microbial genealogy could be inferred from comparative analysis of appropriate gene homologs (orthologs) had significant impact on our perspective and understanding of the structure and function of microbiological systems. The most immediate impact derived from recognition that our census of biological diversity was incomplete; the several thousand named species of microorganisms scattered among thinly branched parts of a tree was not an adequate representation of diversification spanning the greater part of the evolutionary history of this planet. Subsequent application of molecular methods to directly describe environmental microbial diversity has confirmed that the greater part of biological diversity is microbial and that the greater part of microbial diversity has yet to be described (Amann, 2000a; DeLong and Pace, 2001; Torsvik et al., 2002a; Torsvik et al., 2002b).

The phylogenetic dimension embodies the connection between the diversification of life represented in the canonical tree and the emergence of functional groups (e.g., methanogens, acetogens, sulfate reducers, and nitrifiers) in both an evolving and contemporary biosphere. If microorganisms have diversified to fill both general and specific niches, the record of this diversification will be captured by their genealogy. However, there is considerable confusion concerning the ability to infer physiology from phylogenetic affiliation. There are many examples of close phylogenetic relationship between organisms that have remarkably different physiological attributes, for example, close relationships between phototrophs and autotrophs, and between autotrophs and heterotrophs (for examples, see Lane et al., 1992). Conversely, there are also examples of phylogenetically defined groups that are remarkably coherent in physiological characteristics. The *Desulfovibrio* (Devereux et al., 1989; Devereux et al., 1990), methanogenic groups (Raskin et al., 1994), and nitrifiers (Head et al., 1993; Teske et al., 1994) are notable examples of phylogenetic and physiological coherence. Part of this apparent discordance almost certainly derives from failure to recognize ecologically significant features. This is a consequence of using the pure culture phenotype to infer environmental activity outside of the context of the complex environment and interactions between organisms of a microbial community. As we develop methods for direct observations of populations within an environmental setting, we anticipate that many additional phylogenetically cohesive characters will be revealed.

A general relationship between habitat and taxonomic groups has long been recognized. However, molecular tools are refining the characterization of that relationship. An early observation of direct correspondence was made in a microbial mat community in which the depth distribution of different sulfate-reducing populations corresponded with members affiliated with discrete phylogenetic clades (Risatti et al., 1994). A similar correspondence between phylogenetic affiliation and aquatic habitat has been noted. The ubiquitous (SAR11) group, first identified by Giovannoni et al. (1990), appears to be exclusively marine (Morris et al., 2002). However, the

greater assemblage of organisms to which the marine SAR11 belongs includes more distantly related freshwater representatives (Bahr et al., 1996; Field et al., 1997; Morris et al., 2002). *Prochlorococcus* appears to be an exclusively marine assemblage of unicellular cyanobacteria. An early suggestion that genetic variation among *Prochlorococcus* isolates corresponded to different light adapted populations was subsequently confirmed (Moore et al., 1998). The population structure and thus phylogeny of planktonic bacterial communities are associated with general features of habitat (marine versus freshwater, and depth-associated changes in physical and chemical variables).

Although a correlation between closely related populations and habitat is not unexpected, we also ask the more general question—how are the most ancient of evolutionary divergences (domain and division level; Hugenholtz et al., 1998) related to the structure and activities of contemporary systems? If the emergence and diversification of a new lineage were primarily explained by a key evolutionary innovation, then retention of that innovation among contemporary representatives would serve to characterize the lineage and the defining evolutionary innovation. A few notable examples support this, suggesting a centrality of the phylogenetic dimension (Fig. 2). The first example is one of remarkable biochemical innovation, the invention of oxygenic photosynthesis. There is good support that this innovation was a consequence in part of horizontal gene transfer between early anoxygenic phototrophs, each having distinct photosystems. The melding of those photosystems into a two-photosystem apparatus defined the emergence of the cyanobacteria (Buttner et al., 1992). This ancestral metabolic innovation defines all contemporary representatives of the group. In contrast, the spirochetes provide an example of structural innovation. The spiral body form combined with axial flagella offered advantage for moving in viscous environments, and today they are abundant residents in many such habitats, including microbial biofilms and mats. A final example of both structural and physiological innovation is the endospore of named species of *Bacillus* and *Clostridium*. The bacterial endospore was apparently invented only once and likely in large part defines both the origin and successful radiation of major Gram-positive lineages.

The Evolutionary Dimension

The reflections of past innovations in microbial phylogeny raise immediate questions about mechanism and context of innovation. Complete genome sequencing is beginning to reveal the contribution of horizontal gene transfer as a mechanism of biological innovation. However, it

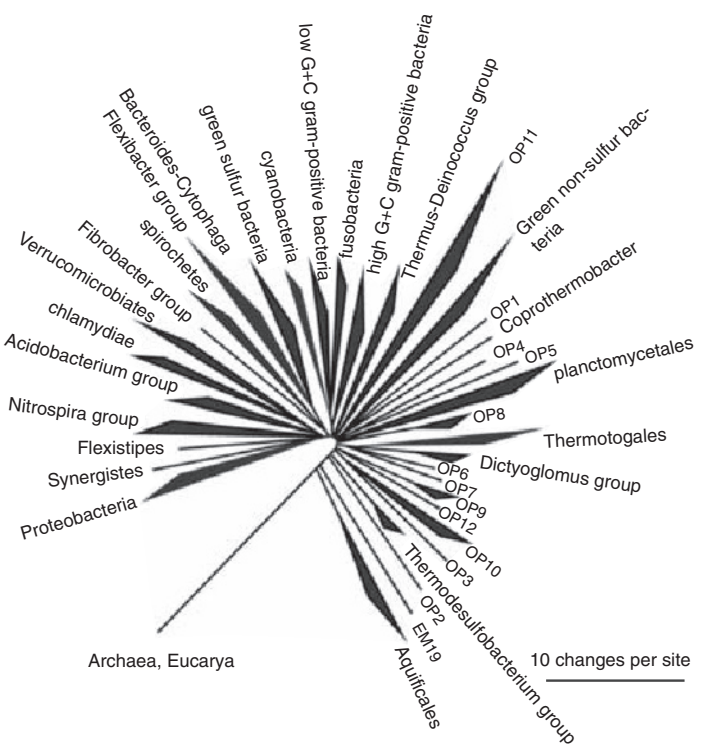

Fig. 2. Evolutionary distance tree of the bacterial domain shows currently recognized divisions and putative (candidate) divisions. The tree was constructed using the ARB software package (with the Lane mask and Olsen rate-corrected neighbor-joining options) and a sequence database modified for the March, 1997, ARB database release.

is too early to constrain its contribution to major innovations in the microbial world since the genomic data sets are still quite sparse in coverage of the major prokaryotic lineages. The context, or environment, in which innovation is fostered can't be separated from mechanism. If horizontal gene transfer is an important mechanism, then the fodder of innovation (genetic diversity) will be determined by opportunities for interaction among contributing populations. Microbial mat communities provide a specific example of this point. Microbial mats are highly active, highly compact, and highly diverse microbial communities providing ample opportunity for intimate interactions among genetically diverse populations. If some early mat communities were based on anoxygenic photosynthesis, this community would be a plausible context for the development of oxygenic photosynthesis via horizontal gene exchange among intimately associated phototrophs. If early mats provided viscous habitats rich in organic substrates produced by phototrophs, this would also have been an ideal context for the emergence of spirochetes. These primarily heterotrophic organisms are adapted to move rapidly in highly viscous environments like microbial mats and biofilms.

Novelty can also arise through isolation limiting genetic exchange and contributing to genetic drift and local adaptation. The degree to which this phenomenon occurs in bacteria is contentious. The prevailing opinion has been that bacterial species are cosmopolitan and exhibit a worldwide distribution. Indeed, surveys using the 16S rRNA suggest a global distribution of many species that can be poles apart (Fuhrman et al., 1992; Fuhrman, 1993; Giovannoni and Cary, 1995; Staley and Gosink, 1999). The adage "Everything is everywhere and nature selects" (Beijerinck, 1913) suggests that geographic barriers do not restrict bacterial dispersal. In this view, bacterial distribution is solely determined by the global dispersal of pre-adapted populations. This view is primarily based on the 16S rRNA gene divergence, which may not be representative of changes in other genes that define specific adaptive traits. The alternative, a biogeography in part determined by evolutionary adaptation to the local environment, is now receiving some support. Several studies have shown that geographically separated populations sharing identical or very similar16S rRNA sequences differ at other genetic loci (Moore et al., 1998; Casamayor et al., 2002; Rocap et al., 2002b). A recent study by Whitaker et al (2003) found that strains of the extremophile *Sulfolobus* clustered geographically rather than by environmental variables that characterized different hot springs. Their multilocus analysis revealed that the genetic distances between populations increased proportionally with geographic distance, suggesting that dispersal of populations and exchange of genetic material between geographically distant groups was limited. Using the 16S rRNA gene as a marker, Papke et al. (2003) also observed genetic differences among thermophilic cyanobacteria from different geographic regions. Their distribution patterns were also ascribed to biogeographic isolation. These and other cumulative data suggest that global distribution patterns reflect both endemic and cosmopolitan groups. This is also supported by recent studies of pure cultures maintained over many generations in the laboratory, demonstrating the capacity for continued adaptive change within a single clonal line of descent (Elena and Lenski, 2003).

Although specific examples are limited, available data point clearly to the importance of contingency in metabolic innovation and adaptive radiation of microorganisms. For example, widespread dispersal may follow major metabolic innovations that derive from interspecies horizontal gene transfer within a complex community, whereas adaptive radiation reflected by biogeographic patterns may arise from more restrictive mechanisms of genetic change. A less speculative discussion of the evolutionary dimension of microbial community structure and function must await more comprehensive descriptions of natural communities.

The Proximate System

Chemical and Physical Dimensions

A central consideration in discussing the structure of microbial communities concerns the interplay between physical and biological controls of organization. Microorganisms are small and experience low Reynolds numbers—viscosity and diffusion dominate their world rather than the mixing and turbulence more familiar to us. Only the fastest and largest bacterium known, *Thiovulum majus*, is able to significantly increase substrate availability by generating advective flow through the entire colony via coordinated communal organization (slime veil formation) and motility (Fenchel and Glud, 1998; Schulz and Jørgensen, 2001; Thar and Fenchel, 2001). More generally, diffusive delivery of nutrient solutes is more important than advective transport (Purcell, 1977; Blackburn and Fenchel, 1999), and diffusion determines structure at both microscopic and macroscopic scales. At microscopic dimensions, a diffusive "sphere" surrounds every metabolically active prokaryotic cell such that substrate concentrations only approach that of the bulk solution several cell diameters away from the microbe's surface.

Microorganisms have developed a variety of strategies to enhance nutrient recovery including the production of siderophores, exoenzymes, smallness and motility (Button, 1994; Blackburn et al., 1998; Blackburn and Fenchel, 1999). Directed movement along a concentration gradient gives the prokaryotic cell a mechanism to move towards regions of higher nutrient concentration, and by doing so increases the flux of nutrient through the cell's diffusive sphere. A recent publication (Fenchel, 2002) suggests that even in a turbulent water column, "nutrient micropatches" derived from cell lysis and excretion by protozoa have life spans sufficiently long to increase nutrient availability to bacteria, not to mention the occurrence of particles or marine snow. The implications for global processes are significant; motile bacteria converging transiently on microscale nutrient patches act to accelerate nutrient uptake and secondary production on a global scale. The ability of microorganisms to utilize available substrates in a competitive manner, and the distribution of these substrates, governs the structure of the microbial communities.

In environments experiencing limited mixing, diffusion and light attenuation contribute to stable architecture at the macroscopic dimensions of millimeters to meters. These systems are layered both chemically and biologically. We briefly discuss several types of layered communities common to aquatic habitats, although we recognize there are terrestrial layered communities as well.

SEDIMENTS. Within a sediment, in the absence of significant advection or bioturbation, mass transport occurs primarily through molecular diffusion. Sediments vary in coarseness and porosity, but in general the sediment matrix limits or prevents water to advect through it. Gradients are formed whenever the production or consumption of a product or nutrient (reactant) exceeds the diffusion of that product or reactant. Substrate concentration reaches a minimum (a boundary condition) at a depth at which the rate of diffusion from the bulk phase matches the consumption rate needed to sustain the minimum free energy required for maintenance. Under these conditions, substrate-concentration gradients reach steady state, yielding characteristic profiles (Nealson and Stahl, 1997; Brusseau et al., 1998; Fig. 3). A key variable is the amount of organic substrate received via sedimentation of organic matter derived from primary produc-

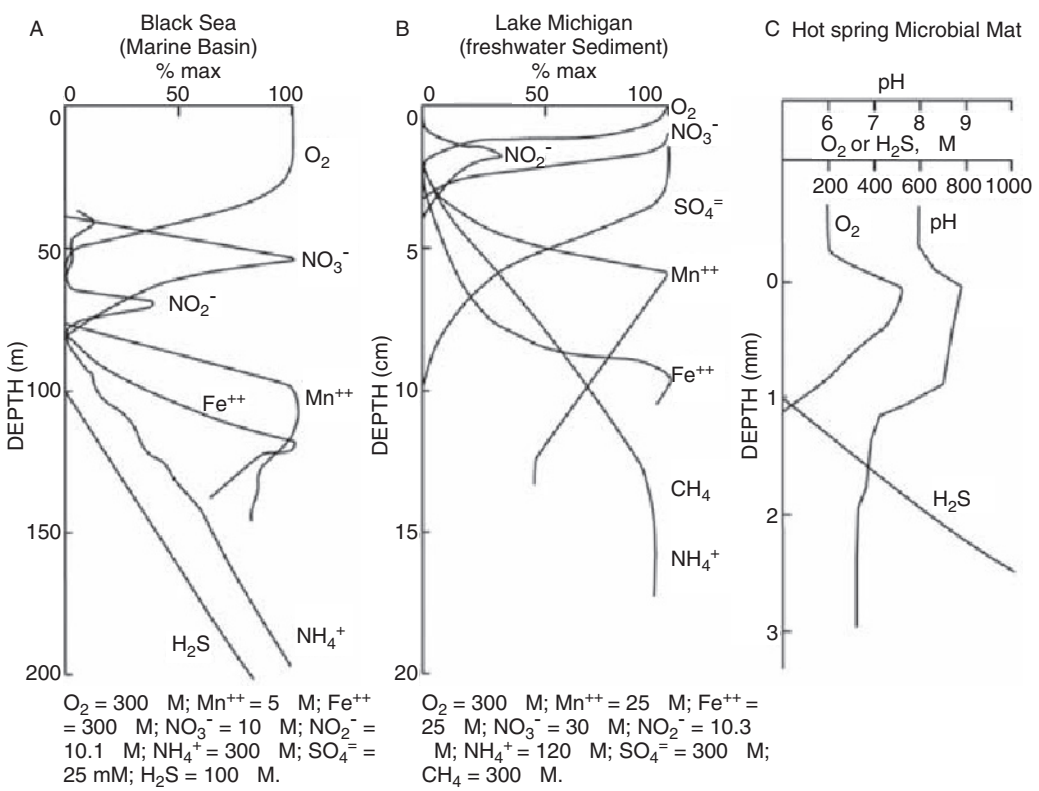

Fig. 3. Chemically stratified freshwater sediment from Lake Michigan and chemically stratified water column of the Black Sea. Although the distributions of the chemicals are similar, the scales over which the chemicals are distributed vary from centimeters in lake sediment to meters in the Black Sea water column.

tion in the water column or from terrestrial input or sediment surface photosynthesis in shallow systems that allow light penetration to the bottom. The observed depth-related changes in chemical composition correspond to a progression of thermodynamically predictable redox changes. Given a variety of possible respiratory modes, those that yield the greatest free energy prevail at each depth under steady state conditions. This begins near the surface with oxygen depletion and typically ends with the reduction of sulfate (in marine environments) to produce sulfide or the reduction of CO_2 (in freshwater environments) to produce methane.

THE WATER COLUMN. The water column of marine and freshwater systems may also exhibit similar depth related chemical structure, most comparably when oxygen depletion extends into the water column. This is observed in small eutrophic lakes and in the permanently stratified Black Sea (Nealson and Stahl, 1997; Taillefert et al., 2002). In addition to diffusion-controlled structure characteristic of sediments, the water column is divided by changes in density, temperature and light. The attenuation of light in the world's oceans provides the most easily resolvable of gradients along which different bacterial populations distribute. For example, as previously noted, different "ecotypes" of *Prochlorococcus* are adapted to different light intensities (Moore et al., 1998; West and Scanlon, 1999; Rocap et al., 2002). Physiological analyses have shown that one ecotype is adapted to the higher light intensities of the upper water column, having a low chlorophyll (Chl) b/a_2 ratio, and the other (higher Chl b/a_2) to life deeper in the euphotic zone. Genome sequence comparisons have also pointed to specific adaptive differences (Rocap et al., 2003). For example, the ecotype adapted to the lower euphotic zone has lost the photolyase gene which is involved in the light-driven enzymatic catalysis of DNA damage repair, presumably because there is little selective advantage to maintain this function under low light conditions.

Variables other than light also contribute to depth-related structure of oceanic provinces. Unlike the relationship to thermodynamic preference observed in chemically stratified lakes and sediments, the controlling variables in the marine water column have yet to be fully resolved. For example, the archaeal phylotypes common in the open ocean vary in depth-related abundance patterns (Massana et al., 2000). Studies of the Antarctic Peninsula showed that planktonic euryarchaeotes were most abundant in surface waters (Masanna, 1998). Increasing archaeal abundance with depth (ca. 25% of total rRNA) was associated with a shift in dominance from euryarchaeal to crenarchaeal rRNA (Massana, 1998). A more recent study at the Hawaii Ocean Time-series (HOTS) station examining the depth-related abundance of these archaeal groups (pelagic euryarchaeota versus pelagic crenarchaea) revealed a similar depth-related pattern of abundance (Karner et al., 2001). Pelagic creanarchaea comprised a large fraction of total picoplankton below the euphotic zone (>150 m), approaching 39% of total DNA-containing picoplankton detected. These groups are surely physiologically distinct, as suggested by a time series study in the Santa Barbara Channel that showed the abundance patterns of these two archaeal groups correlated with general environmental variables (Massana et al., 1997). Other abundant marine picoplankton such as the clade defined by *Pelagibacter ubique* (SAR11) and SAR211 and SAR203 marine clusters also show characteristic depth-related abundance patterns (Giovannoni et al., 1996; Field, 1997; Wright et al., 1997; Morris et al., 2002). Recent success in culturing representatives of the SAR 11 cluster suggests that temperature, not light (Rappe et al., 2002; Zengler et al., 2002), may be an important factor in the growth and distribution of members of this assemblage. The soon to be available genome sequences for these abundant marine picoplankton will undoubtedly provide insights into their physiology and likely provide some explanatory basis for the patterns of structure observed in the open oceans.

MICROBIAL MATS. Microbial mats are among the most visibly conspicuous of layered communities (Fig. 4). Built by photosynthetic or chemolithotrophic bacteria, they share features of structure similar to sediment and water column communities, in that population distribution is governed by light availability and diffusive flux of substrates and metabolites. However,

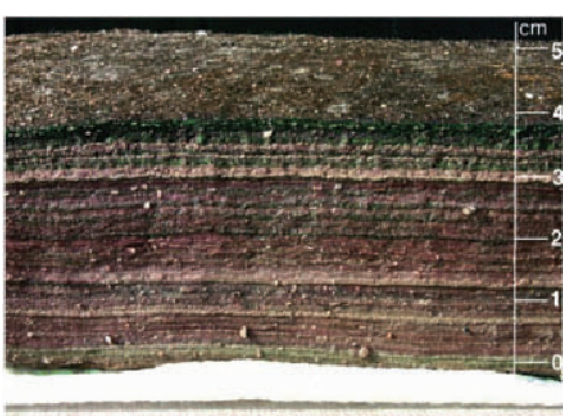

Fig. 4. An example of a layered community from Guerrero Negro Baja California Sur. The colors denote pigments from different microbial groups. Photograph courtesy of Jesse Dillon.

mats provide for much closer physical and metabolic interactions among contributing populations. The most abundant and versatile photosynthetic mat builders today are the oxygen-producing cyanobacteria (Cohen et al., 1989). The most common types of chemolithotrophic mats are comprised of filamentous sulfur-oxidizing bacteria, generally occurring on sediment surfaces at the interface between gradients of reduced sulfur species and the oxidants oxygen or nitrate (Jørgensen and Revsbech, 1983; Jannasch et al., 1989; Sassen et al., 1993).

The cyanobacterial mats are complete microbial ecosystems, comprised of primary producers (cyanobacteria) and populations of consumers that together mediate all key biogeochemical cycles (Fig. 5). Remarkably, this ecosystem can be represented by a fragment of microbial mat only several centimeters on a side. Although this general type of microbial community is thought to have existed for over 3.5 billion years (Cohen and Rosenberg, 1989), the evolution of metazoan grazers, competition with macrophytes, and changing oceanic carbonate chemistry triggered the decline of the extensive mat communities (represented by stromatolithic fossils) at the end of the Proterozoic (Grotzinger and Knoll, 1999). Today's mats develop conspicuously only in aquatic environments where environmental stress limits or excludes grazing, most commonly in hypersaline or thermal habitats. These are among the best studied of microbial communities and have provided a superb context for studies relating structure and function (Cohen and Rosenberg, 1989).

Hypersaline cyanobacterial mats are characterized by intense oxygen production during the day in the photic surface layer and by highly active sulfate reduction throughout the mat. These gradients virtually disappear at night when the entire mat turns anoxic and sulfidic (Revsbech and Jørgensen, 1983; Canfield and DesMarais, 1993). Both molecular and cultivation studies of the oxic surface layer of cyanobacterial mats have revealed high numbers of sulfate-reducing bacteria (SRB; Risatti et al., 1994; Teske et al., 1998; Minz et al., 1999a; Minz et al., 1999b). Although SRBs are classical anaerobes, oxygen supersaturation apparently does not interfere with the activity of near surface populations in these mats (Jørgensen and Cohen, 1977; Canfield and DesMarais, 1991; Canfield and DesMarais, 1993; Fründ and Cohen, 1992; Jørgensen, 1994; Teske et al., 1998). As yet, there is no physiological or ecological basis to explain these unexpected patterns of distribution.

The chemolithotrophic *Thioploca* mats on the Chilean and Peruvian continental shelf are the most extensive microbial mats on earth (Gallardo, 1977; Fossing et al., 1995). Abundant *Thioploca* populations residing in the upper centimeter of these mats participate in an intense sulfur cycle. The high rate of sulfate reduction (up to 1500 nmol $\cdot$ cm^{-3} $\cdot$ d^{-1}) is balanced by the oxidation of sulfide by *Thioploca* such that sulfate is not appreciably depleted (Thamdrup and Canfield, 1996; Ferdelman et al., 1997). This highly efficient sulfur cycle has been attributed to a close physical association between sulfate-

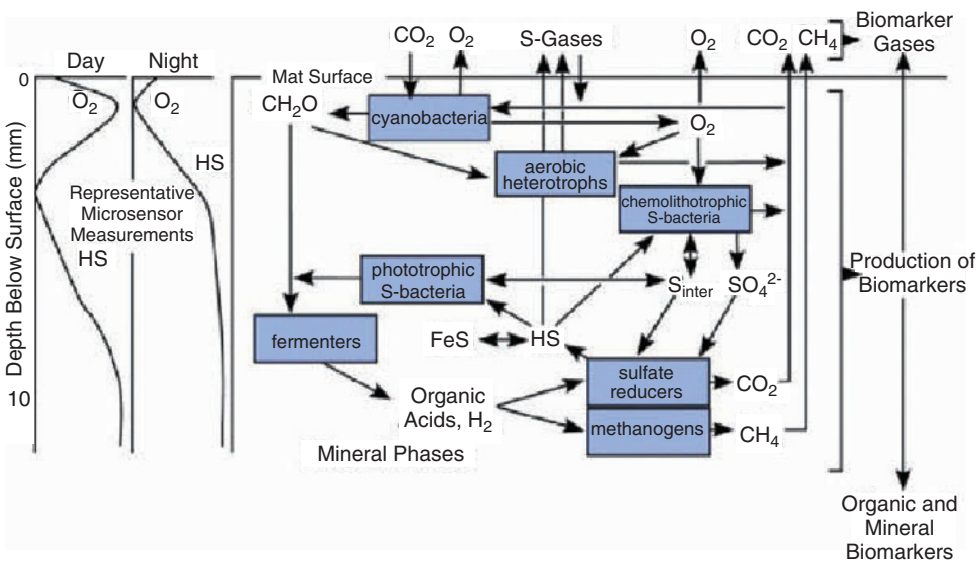

Fig. 5. The distribution and conversions of oxygen, sulfur, hydrogen and carbon dioxide within a layered cyanobacterial mat community.

reducing and sulfur-oxidizing bacteria. Filamentous sulfate reducers of the genus *Desulfonema* were observed growing within the *Thioploca* sheaths, suggesting a complete cycle of sulfate reduction and re-oxidation within individual *Thioploca* bundles, representing an example of syntrophy (Fukui et al., 1999).

Some mat populations rely on motility to follow shifting chemical gradients. Microbes such as the filamentous microaerophilic sulfide-oxidizing bacterium *Beggiatoa* monitor local chemical and physical dimensions of habitat, using that sensory input to relocate to environments better suited to their physiology. *Beggiatoa* in the cyanobacterial mats of Guerrero Negro follow the diel up-and-down movement of the oxygen-sulfide interface closely (Garcia-Pichel et al., 1994). Other organisms have developed a strategy to bridge spatially separated resources. This is exemplified by the recent discovery that *Thioploca* species use large internal vacuoles to store high concentrations of nitrate for anaerobic respiration. Much like a scuba diver fills diving tanks with oxygen to dive in an oxygen limited water environment, *Thioploca* migrate to the sediment surface, partially emerge from their sediment-embedded sheaths to partially enter the water column, and charge internal vacuoles with high concentrations of nitrate (Hüttel et al., 1996). They then return ("dive") to the anoxic depths of the sediment (gliding at a speed of 3–5 $mm \cdot h^{-1}$) to use their stored nitrate for sulfide oxidation (Maier et al., 1990; Fossing et al., 1995).

BIOFILMS. The term "biofilm" is the generally accepted term for microorganisms attached to a solid surface in a relatively thin film. Biofilms pervade virtually all environments and surfaces, often dominating the microbial activity distributed between the individual planktonic and aggregated habitats (Van Loosdrecht et al., 1990). Characklis and Marshall (1990) have generally defined biofilms as systems displaying the following four features: 1) cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin, 2) a surface accumulation, which is not necessarily uniform in time or space, 3) a significant fraction of inorganic or abiotic substances held together by the biotic matrix, and 4) transport and transfer processes are rate limiting and play a much more important role than in the suspended growth microbial systems. The fourth characteristic highlights the importance of diffusion and reaction in controlling population structure and associated metabolic processes. As for the previously described layered communities, gradients form in response to the balance between microbial synthesis and consumption of diffusible substances, creating niches distinct from the proximal bulk water. Similar to mat communities, natural biofilm systems provide for a spectrum of stable habitat types. Thus, a biofilm community of thickness less than 1 mm can have a diverse and stable microbial ecology amenable to study. For example, the biofilms produced by oral microbiota colonizing tooth surfaces provide a model system for experimental study and are receiving increasing attention (Kolenbrander, 2000).

The microbial populations colonizing suspended particles demonstrate many attributes of biofilms. Suspended organic particles, marine and lake "snow," conspicuous in aquatic habitats provide hot spots of nutrients and carbon sources for bacterial growth (Alldredge and Silver, 1988; Byung and Azam, 1988; reviewed in Turner, 2002). High densities of multiple microbial populations are embedded in a mucoid extracellular polysaccharide (EPS) matrix, providing opportunity for interaction. Reduced diffusion and high activity serve to create localized concentration gradients. Depletion of oxygen towards the center of larger aggregates supports the coexistence of aerobes and anaerobes (Alldredge and Cohen, 1987), as revealed by the presence of sulfate-reducing bacteria (DeLong et al., 1993) and possibly nitrogen fixation (Paerl and Prufert, 1987). Because microorganisms are the only biota having the capacity to utilize the dilute carbon and energy in many aquatic habitats, the colonization and transformation of these particles by microorganisms represents an important portion of secondary production and may play an important role in food web energetics, atmospheric CO_2 exchange, and flux of nutrients to the deep-sea ecosystem through sedimentation of colonized particles (Decho and Herndl, 1995; Turley et al., 1995). Microbial aggregates provide a link between the dilute dissolved carbon and higher trophic levels as food for larval species and protozoa (Pomeroy, 1974; Karl, 1994) and serve to transfer surface water nutrients to the benthos via sedimentation (Passow, 2002; Turner, 2002).

Interactions among microbial populations in aggregated communities are also modulated by regulatory processes that alter gene expression in relationship to population density. Regulated processes are those that are adaptive only when the population density reaches a critical threshold number (the "quorum"). These sensing mechanisms are addressed in the following section on the metabolic dimension.

The Metabolic Dimension

The previous sections emphasized the role of diffusion and reaction in regulating the chemical structure of a microbial system. The metabolic

dimension underlies the chemical reactions governing the flux of energy and matter through the system. Each microbial population must derive sufficient free energy of reaction for maintenance and growth. The biochemical explanation for how the total free energy available is parceled out among the populations is the metabolic dimension. This dimension encompasses factors regulating the biochemical response and activity of populations in any system. We now have only a sketchy understanding of the metabolic dimension and limit our discussion to selected examples of how quorum sensing, syntrophy, and symbiosis govern the metabolic dimension to shape microbial structure and function.

QUOREM SENSING: A LANGUAGE FOR INTER- AND INTRAPOPULATION COMMUNICATION. It is increasingly apparent that specific signalling molecules modulate reactions within microbial communities (Fuqua et al., 2001; Fuqua and Greenberg, 2003; Xavier and Bassler, 2003). The term "quorum sensing" was introduced to describe signaling systems mediated by diffusible molecules (autoinducers, primarily different forms of peptides and homoserine lactones [HSLs]) that regulate expression of genes that are most beneficial when a critical number of microorganisms (the quorum) is present in a locale. The peptide and HSL types of autoinducers primarily control reactions within a single population, for example, in the production of extracellular hydrolases during tissue invasion or in light generation when colonizing particles or inhabiting specialized light organs of certain marine animals (see the subsection Symbiotic Associations). However, a recently described furanone autoinducer (AI-2) has been implicated in signaling between disparate species of bacteria (Xavier and Bassler, 2003). A role of AI-2 in interspecies communication is receiving experimental support, such as a recent study by McNab et al. (2003) showing that interspecies autoinduction is required for the coordinated development of a biofilm comprised of two species of oral microbes, *Porphyromonas gingivalis* and *Streptococcus gordonii*.

Recognition of the widespread occurrence of quorum sensing among microbial species suggests there is frequently an active and rich dialogue between cells comprising one or more populations (Fuqua et al., 1994; Fuqua et al., 1996; Hastings and Greenberg, 1999). To date, most examples have been defined in the context of disease or symbiosis, and they include such diverse processes as bioluminescence, antibiotic biosynthesis and resistance, production of EPS, swarming, plasmid conjugal transfer, and the production of a variety of virulence determinants. Recent studies have revealed the significance of quorum sensing in regulating the formation of biofilms (Davies et al., 1998; Huber et al., 2001; Fuqua and Greenberg, 2002; Fig. 6). An especially fruitful lab model for understanding the possible role of quorum sensing has been the genetically tractable pathogen, *Pseudomonas aeruginosa*, which forms biofilms in the lungs of cystic fibrosis patients, marking the advancement of a serious and recalcitrant infection of the host. Mutations in the HSL receptors and gene regulation have shown that an active quorum sensing pathway is required for the proper development of the characteristic *P. aeruginosa* biofilm comprised of columns of cells embedded in a polysaccharide matrix with open channels between them. This biofilm structure is likely important for nutrient exchange with the bulk liquid phase. The induction of biofilm formation is also asso-

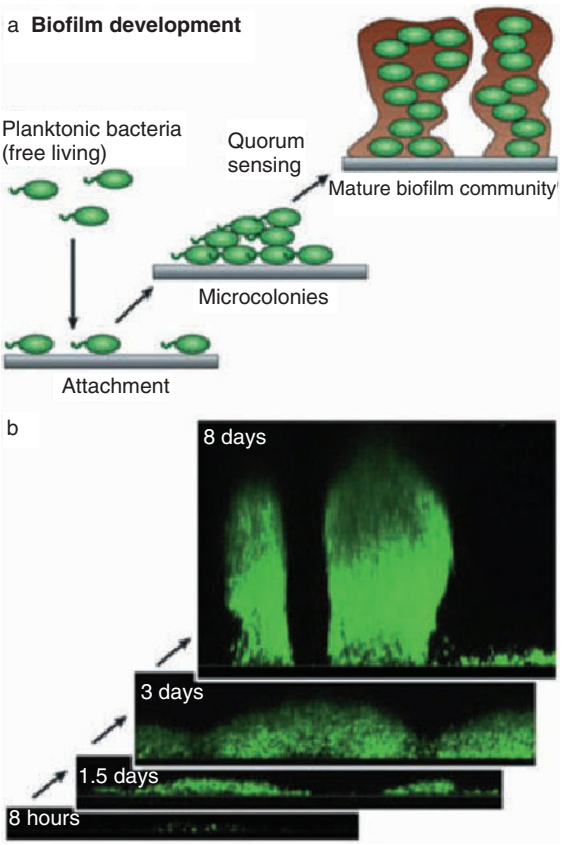

Fig. 6. Biofilm development and quorum sensing. a) The steps involved in a biofilm development. b) Confocal microscope images of a *P. aeruginosa* biofilm developing over time on a microscope slide. The cells are producing green fluorescent protein. The mushroom- and tower-like structures that appear by 8 days are 100 μm high. (The images in b) were kindly provided by M. Welsh, P. Singh, and E. P. Greenberg [University of Iowa, United States].)

ciated with an increase in resistance to antibiotics. Mutants deficient in the production of the specific HSL formed a flat, undifferentiated biofilm.

Although there have been few studies of the possible environmental functions of quorum sensing, there is a growing consensus that this and other chemical signaling systems are a fundamental element of community structure and activity (Fuqua et al., 1996; Fuqua et al., 2001; Hastings and Greenberg, 1999; Xavier and Bassler, 2003). Aggregated microbial communities, regulated in part through quorum sensing, are increasingly viewed as highly adaptive and resilient systems of organization.

SYNTROPHIC ASSOCIATIONS. The metabolic connections among different populations can range from highly specific to generic. An example of a generic association may be simply the use of a vitamin or growth factor released by several other cohabiting populations. This type of interaction would not be expected to exert as much control over the organization of a microbial system or community as connections that specifically link energy recovery between two metabolic pathways. The best examples of systems structured by the parceling of available free energy among interacting populations are found in anaerobic habitats such as the sediment and mat systems described earlier. The anaerobic decomposition of organic material is carried out by the cooperation of several general physiological groups of microorganisms (Schink, 1988). Although a variety of other functional assemblages are present in anaerobic habitats, for the purposes of discussing syntrophic associations in the metabolic dimension, only four main groups are considered here. In the absence of electron acceptors used for anaerobic respiration (e.g., oxyanions of sulfate and nitrate, and oxidized metals), the following four microbial groups are generally recognized to participate synergistically in the anaerobic degradation of organic matter: 1) hydrolytic and fermentative bacteria, which degrade complex biopolymers (e.g., plant cell wall components), and monomers (sugars), and oligomers into acetate, hydrogen, carbon dioxide, and a mixture of short chain fatty acids, alcohols, succinate, and lactate; 2) proton-reducing acetogenic bacteria, which convert fatty acids, alcohols, succinate, and lactate to acetate, hydrogen, and carbon dioxide; 3) hydrogen-oxidizing methanogens, which convert hydrogen and carbon dioxide (as well as other quantitatively less important compounds) to methane and water; and 4) acetoclastic methanogens, which convert acetate into methane and carbon dioxide.

The complete mineralization of organic matter is dependent upon the cooperative growth of all four groups and is sustained by the microbially mediated (via methanogens groups 3 and 4) removal of hydrogen (and acetate). Interdependent growth (syntrophy) was first observed by Bryant and associates (Bryant et al., 1967; Bryant et al., 1977; Wolin and Miller, 1982). Notably, this discovery was a consequence of a failure of the pure culture technique! An organism thought capable of methanogenic growth on ethanol (*Methanobacillus omelianskii*) was found to be a syntrophic association of two species of prokaryotes. One bacterium oxidized ethanol to hydrogen, acetate, and CO_2 (group 2 type). The second was a hydrogenotrophic methanogen (group 3 type). Neither organism was capable of growth on ethanol alone. The energetic basis of this obligate association is the relationship between free energy and hydrogen concentration. The fermentation of ethanol is only favorable at low hydrogen concentrations (negative free energy). Thus, although the ethanol-oxidizing bacterium could initiate fermentation in a closed system (the test tube), hydrogen accumulation soon made this reaction energetically unfavorable. Enter the methanogen—its consumption of hydrogen permitted a continuous fermentation of ethanol. The methanogen was later formally described (*Methanobacterium bryantii*), but its syntrophic bacterial partner has since been lost.

Although interspecies hydrogen transfer was discovered in a closed system, the test tube, it is now recognized to determine higher order structure and activity in both natural and engineered systems. One dramatic example is the granule structure that develops in anaerobic reactors designed to treat industrial waste streams (Sekiguchi et al., 1999). Within upflow anaerobic sludge blanket (UASB) reactors, the four functional assemblages promote the anaerobic degradation of organic industrial waste. These microbiota are retained in the reactor by formation of dense granules of millimetric size, their size controlled by balancing sedimentation with metered upward fluid flow in the reactor. Initial studies of the USAB granules using molecular probes to resolve the distribution revealed fine layering of population types, suggesting tight metabolic connections. The general architecture of each granule consists of a methanogenic core (Archaea), serving to consume hydrogen, CO_2, and acetate, surrounded by outer shells (layers) of fermentative bacteria (groups 1 and 2) that sustain the oxidation of the waste stream components such as propionate, sucrose, and acetate (Fig. 7).

Anaerobic methane oxidation (ANME) is another example of a layered syntrophic association of an inner core of Archaea surrounded by sulfate reducing bacteria (*Desulfosarcina-Desulfococcus* group; Boetius et al., 2000;

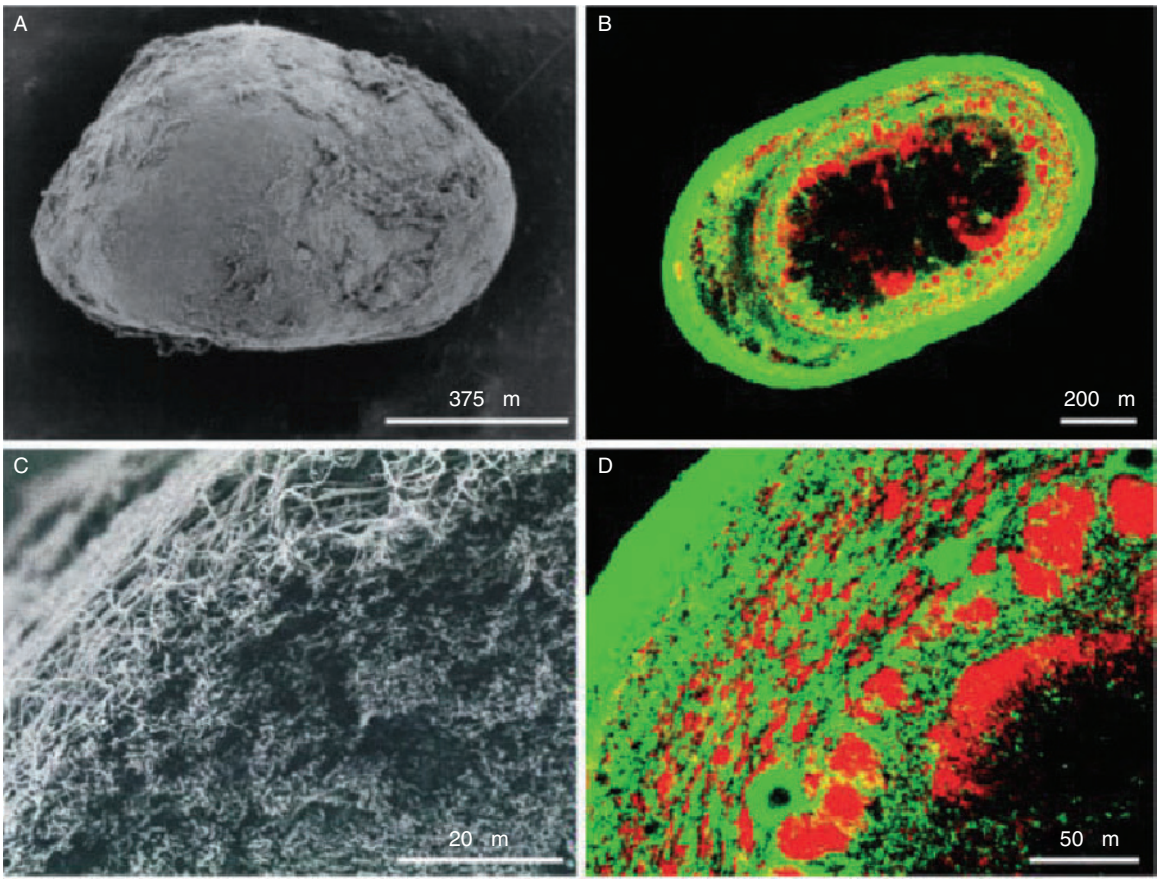

Fig. 7. Sections from mesophilic and thermophilic granules viewed by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). A) SEM of the surface of granules, B) SEM of internal structure illustrating filaments and rods within layers of the granule, C) and D) are sections simultaneously hybridized with Cy-5-labeled bacterial-domain probe (EUB338; green) and rhodamine-labeled archaeal-domain probe (ARC915; red). (From Sekiguchi et al. [1999].)

Orphan et al., 2001; Teske et al., 2001; Michaelis et al., 2003). It has been hypothesized that this association (like the layered granules above) involves methane oxidation to hydrogen and carbon dioxide. If the concentration of hydrogen remains low, this conversion is energetically favorable, although the energy yield is the lowest known to fuel a microbial metabolism. Removal of the hydrogen is accomplished by hydrogen oxidizing sulfate reducers. Kruger et al. (2004) have isolated from microbial mats a nickel-containing protein, which is similar to methyl-coenzyme M reductase from methanogenic Archaea, the enzyme catalyzing the final step in the production of methane. The metabolic reactions between the Archaea and sulfate reducers result in the formation of $CaCO_3$ reefs that measure 1 m wide by 4 m high in the anaerobic regions of the Black Sea (Fig. 8).

SYMBIOTIC ASSOCIATIONS. Multicellular eukaryotes originated in a microbial world. Thus, a complete understanding of the multicellular condition cannot be separated from pervasive and intimate associations with prokaryotes. These associations have influenced the evolution, development, and physiology of all multicellular life forms (reviewed in McFall-Ngai [2001] and McFall-Ngai [2002]). The phenomenon of symbiosis has been known and studied since De Bary (1879) defined it as two dissimilar organisms living in close association and ranges from the beneficial to the pathogenic. Since then the study of prokaryotic contributions to the evolution, development and functioning of plant and animal systems has become a highly active area of investigation (Seckbach, 2002; Bourtzis and Miller, 2003). In medicine there is growing awareness that the so-called "normal flora" is important to both health and disease (Rook and Stanford, 1998; Relman and Falkow, 2001; Hooper et al., 2002; Hooper, 2004). In response to the need for microbiologists and animal and plant biologists to work in concert to enable progress in understanding the details of these interactions, a discipline of Cellular Microbiology was suggested in 1996 to encompass research

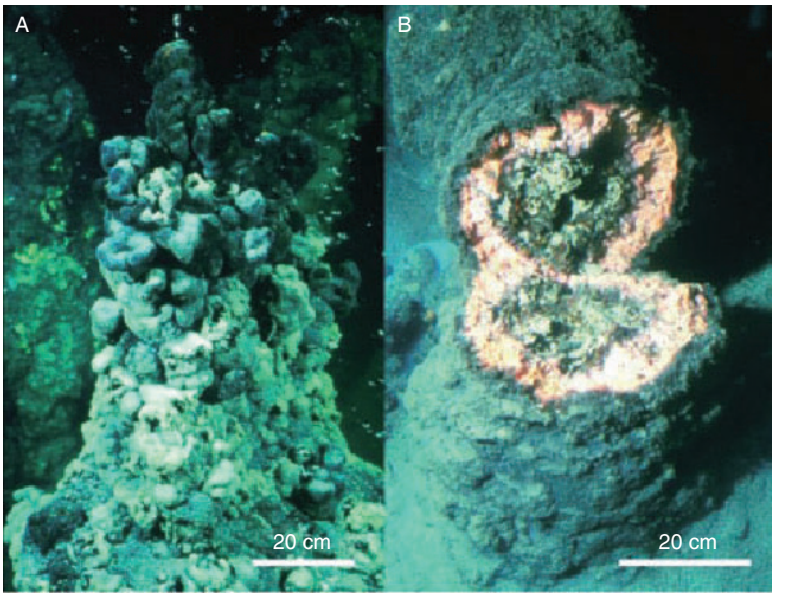

Fig. 8. Image of microbial reef structures. A) Tip of chimney-like structure. B) Broken structure at about 1 m height. The surface of the structure consists of gray-black microbial mat; the interior of the mat is pink. The greenish-gray core consists of porous carbonate. (From Michaelis et al. [2003].)

devoted to characterizing bacterial interactions with eukaryotic cells (Cossart et al., 1996). Although the initial focus was on understanding pathogens, the field has expanded its scope to include the benign and beneficial associations of bacteria with host cells. Here we focus our discussion only on the beneficial bacterial associations and emphasize that examples presented here are by no means the only or the most important associations but are fairly well studied systems that have provided insights into mechanisms.

All multicellular eukaryotic organisms, including humans, harbor bacteria that serve some function and range in complexity from hundreds of species comprising the populations of the mammalian gut to the simplified monospecific cultures in specialized organs such as root nodules of legumes and light organs of marine animals. The potential for cooperative association provides an evolutionary driving force for both the host and the symbiont. Bacterial symbionts of eukaryotes often confer unique abilities that enable their host to survive on resources that would otherwise be unavailable. Examples include: chemoautotrophs (within the large worms, nematodes, bivalves and shrimp) living on sulfide or methane in a variety of marine habitats (Cary et al., 1997; Deming et al., 1997; Streams et al., 1997; Polz et al., 1998b; Fisher, 1990; Fujiwara et al., 2001); the production of toxins and antibiotics by bacteria (Kobayashi and Ishibashi, 1993; Faulkner et al., 1994; Walls et al., 1995; Kaufman et al., 1998; Currie, 1999; Davidson et al., 2001); the production of light (Hastings et al., 1987; Haygood, 1993); bacteria that degrade cellulose and fix nitrogen in shipworm bivalves and terrestrial termites to allow the hosts to live on wood (Breznak, 1982; Waterbury et al., 1983; Lilburn et al., 2001; Distel et al., 2002); and the associations of cyanobacteria with a variety of fungi to fix carbon and nitrogen (lichens). The activities of certain microbe-host associations, such as gut flora of termites and the nitrogen-fixing *Rhizobium* spp. of legumes, influence the structure and function of entire ecosystems.

The host environment in turn impacts the adaptations of the bacterial partner, which may live, divide and grow either extracellularly (usually associated with specific tissue or organ systems) or intracellularly. There is a continuum of dependence on the partnership ranging from bacteria that are free-living and opportunistically colonize the host (i.e., *Vibrio fischeri* and *Rhizobium*) to obligate intracellular symbionts that are unable to live outside of host cells, having lost a variety of genes as a result of their lifestyle (Baumann et al., 1995; Douglas, 1997; Werren, 1997; Tamas et al., 2002). The severe reduction of the genome sizes in *Buchnera* of aphids is a well-studied example (Ochman and Moran, 2001; Wilcox et al., 2003). The smallest genomes (0.6–1.5 megabases [Mb]) among bacteria are found in obligate intracellular parasites and symbionts (Casjens, 1998). Similarly, the host may be able to live without the symbiont under certain conditions (legumes where there is sufficient nitrogen), or they may lose independence altogether. One of the most dramatic examples of symbiotic dependence is the loss of the entire digestive system of hydrothermal vent-associated tubeworms, *Riftia* spp., which rely entirely on their bacteria for sustenance.

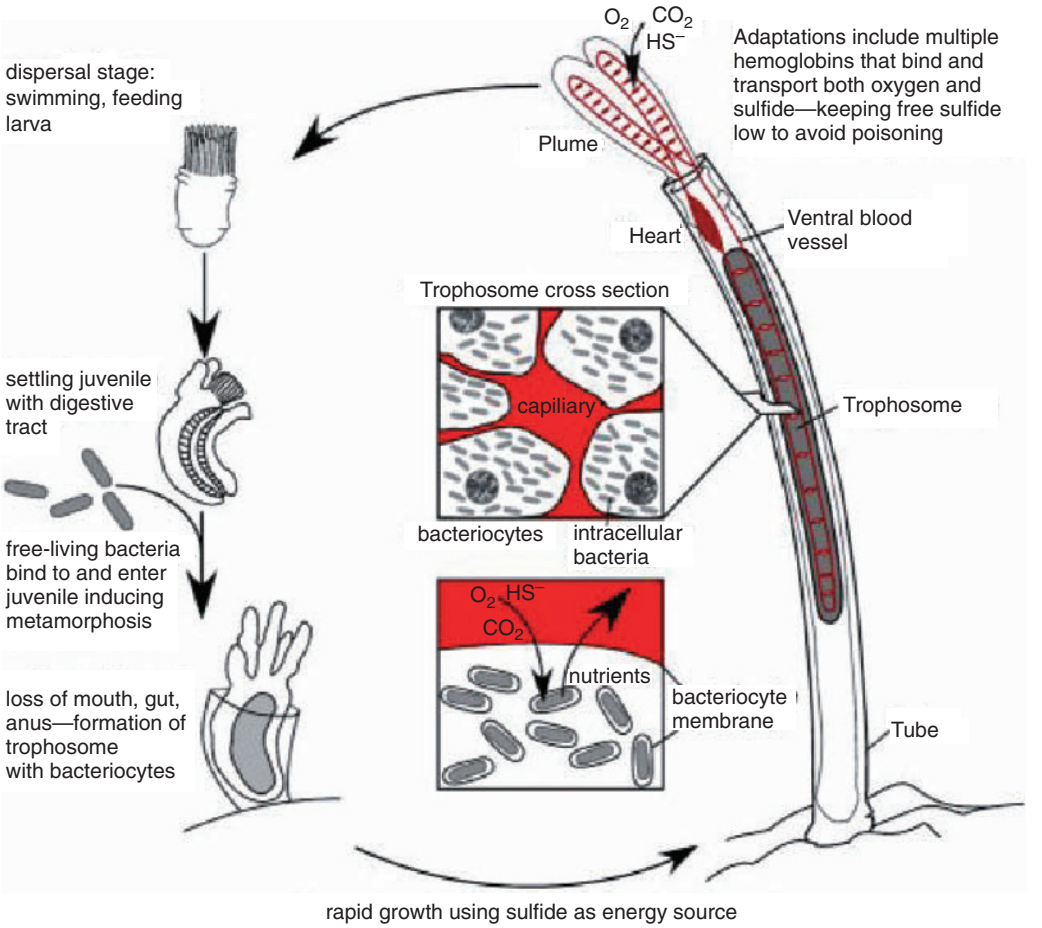

Fig. 9. Bacterial induction of morphological and biochemical adaptations in animals: *Riftia pachyptila* as an example. The dispersal stage resembles other annelid larvae, but upon acquisition of bacterial symbionts, the juvenile loses the digestive tract to form a specialized organ, the trophosome, that houses bacterial cells within host bacteriocytes. In addition to obvious morphological changes, the worm has evolved hemoglobin specialized for transport of both oxygen and sulfide (Childress et al., 1987; Southward, 1999; Bright and Sorgo, 2003; Bright, personal communication).

The vent annelid *Riftia pachyptila* has been profoundly modified from its ancestral form to take advantage of the capabilities of its bacterial partner. This annelid has given up its entire digestive system (no mouth, gut or anus), replacing it with a specialized organ (the trophosome) harboring carbon-fixing sulfide-oxidizing bacteria (reviewed in Fisher [1990] and Fisher [1995]; Fig. 9). Early development of *Riftia* is similar to other marine annelids; a swimming larva locates and settles in an appropriate location near a hydrothermal vent. The developing worm must then recruit appropriate free living bacteria capable of collaborating in an ensuing metamorphosis that results in the loss of the digestive tract to form the trophosome, highly vascularized and packed with intracellular gamma proteobacterial symbionts. A multihemoglobin system found in the red blood and coelomic fluid of these worms accomplishes the delivery of both oxygen and sulfide in a manner that keeps free sulfide levels low but supplies enough to maintain the demands of the symbiont (Fisher et al., 1988; Goffredi et al., 1997a; Zal et al., 1998). The free-living bacteria have not been characterized, and the roles they play in the ecosystem's nutrient cycling outside of the tubeworms, or how they transition to an intracellular lifestyle, are not known.

The early recruitment and colonization of a bacterial symbiont presents a challenge to both partners. The bacteria must evade, or tolerate, host immune defenses and then persist without overgrowth. In turn the host must selectively recruit and encourage growth of its necessary partner without compromising its own health. Several model systems have served to provide detailed understanding of the multiple levels of interaction involved in forming and sustaining a symbiotic association. These systems share the following characteristics that render them more

amenable to study: 1) the bacterial partner can be cultured separately from the host, 2) the host can be studied without the symbiont, 3) genetic manipulation of the bacteria is possible, and 4) the intact lifecycle (or at least early colonization events) can be maintained in the lab (reviewed in McFall-Ngai [2002] and Seckbach [2002]). Notable examples of such systems include the *Vibrio*-squid and *Rhizobium*-legume symbioses (McFall-Ngai, 1999; Ruby, 1999; Stougaerd, 2000; Lum and Hirsch, 2003). These have yielded insight into the biochemistry and genes involved in the initiation, negotiations, colonization, and persistence of the bacterial cells in the host. Key sets of characters that are important for colonization of metazoan and metaphytan hosts include motility, chemotaxis, adhesion, biofilm formation, and quorum sensing (Ruby, 1999; Hirsch and McFall-Ngai, 2000). Many of these are also important in pathogenic associations. In *Euprymna scolopes*, modes of defense against colonization by the wrong bacteria include oxidative stress produced by the host and surface adhesins enabling only specific bacteria to enter and bind to host tissue (Weis et al., 1996; Small et al., 1999; Aeckersberg et al., 2001). Furthermore, to avoid overgrowth, the squid has evolved a behavior of ejecting most of the bacteria at the end of the night, when light is no longer needed, and then allowing the remaining 5% to grow up again during the day while the squid is concealed in the sand. The influence of localized increase in numbers of *Vibrio fischeri* in the water column is not known (Lee and Ruby, 1994; Ruby and Lee, 1998).

We anticipate that this nascent field will be greatly enriched by development of additional model systems, offering comparative framework to study theme and variation in symbiotic systems. Recently developed systems that have proven amenable to dissection of the association in the lab include *Xenorhabdus*-nematode, *Aeromonas*-leech, and *Acidovorax*-earthworm associations. Genetic examination of *Xenorhabdus* spp. and *Photorhabdus* spp. (nematode symbionts; Vivas and Goodrich-Blair, 2001; Heungens et al., 2002; Ffrench-Constant et al., 2003; Martens et al., 2003) and *Aeromonas* sp. (leech symbiont; Graf, 1999; Braschler et al., 2003) has identified genes important for the association. Initial characterization of a specific association between *Acidovorax* spp. bacteria and Lumbricid earthworms suggests that this association is amenable to laboratory study (Fig. 10). Not studied until recently (Schramm et al., 2003), these bacteria have been brought into culture, are vertically transmitted, and likely contribute to nitrogenous waste processing.

Key features that enable bacteria to associate stably with a eukaryotic host are still elusive. The more complete understanding of the complex genetic and metabolic systems of interaction is a central challenge in symbiosis research. With complete genome sequences becoming available, comparisons are possible that may reveal commonalties between bacteria able to associate with hosts and those that do not. To date, the majority of the genomes sequenced are from pathogens of humans or symbionts dependent on close interaction with a eukaryotic host (Ochman and Moran, 2001). Although this is a bias reflective of the human desire to understand disease, it offers comparative opportunities to examine which genes confer the ability to associate stably with a eukaryotic host. Certainly there are pathogenicity islands, sets of genes that confer virulence to normally nonpathogenic bacteria such as *E. coli*. However, there are also examples of symbiosis islands that confer the ability to invade and associate with the host in a positive way, as in the rhizobium-legume interaction (500 kb inserted at a tRNA locus in the plant symbiont *Mesorhizobium loti*; Sullivan and Ronson, 1998). A recent review examining these issues suggests that lineages are either parasitic or symbiotic and do not switch, such that the deep branching pattern of clades indicates one or the other lifestyle (Moran and Wernegreen, 2000a).

The Temporal System

Diel, Seasonal, and Annual Variation in Structure and Activity

The temporal dimension of microbial community structure and activity is determined by a complex interaction of the physical environment on regular daily, seasonal, and annual cycles that influences biology across local, regional and global scales. In aquatic habitats, changes in the stability of the water column related to temperature-mediated changes in water density influence nutrient distribution that ultimately alters microbial activity, community structure, and interactions with higher trophic levels. How the community is shaped by these changes varies and ultimately influences the productivity of the microbial community. Since prokaryotes are the only biota capable of recovering the dilute organic substrates generally present in aquatic systems ("the microbial loop;" Fig. 11), their responses to predictable diel, seasonal, or annual variables are fundamental parameters governing the flux of energy in aquatic habitats (Pomeroy, 1974; Azam et al., 1983; Williams, 2000). The most obvious environmental variables that change on a diel cycle are light (>400 nm) and temperature. Light positively influences primary

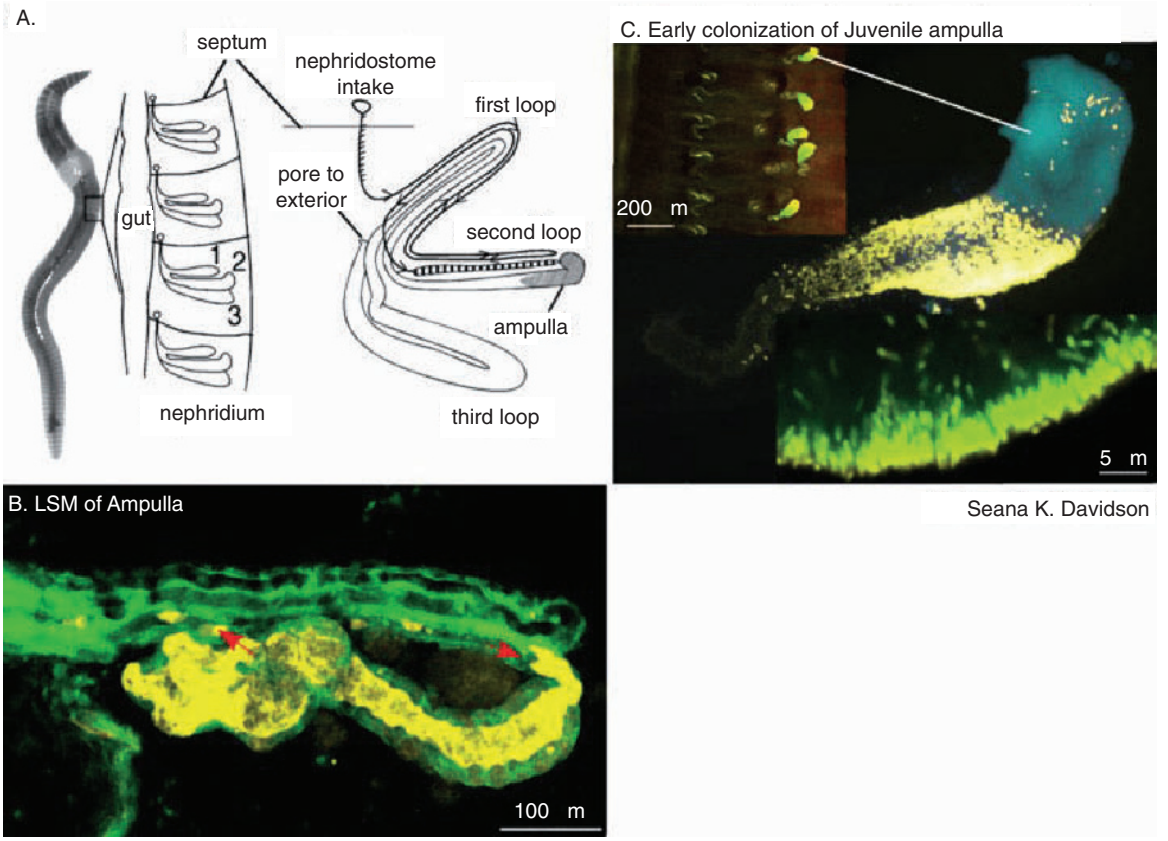

Fig. 10. Earthworm bacterial symbionts. A.) Schematic diagram of the earthworm nephridium showing the directions of flow and the three lobes of the organ. B.) and C.) Laser scanning confocal images of bacterial symbionts of the earthworm nephridia labeled with an *Acidovorax*-specific oligonucleotide probe. B.) Cross section through the ampulla of adult *Eisenia foetida*. C.) Upper inset, low magnification image showing ampulla visible in each of several segments of a juvenile worm, partially colonized ampulla of a juvenile, and cross section showing the binding of the bacterial cells to the tissue.

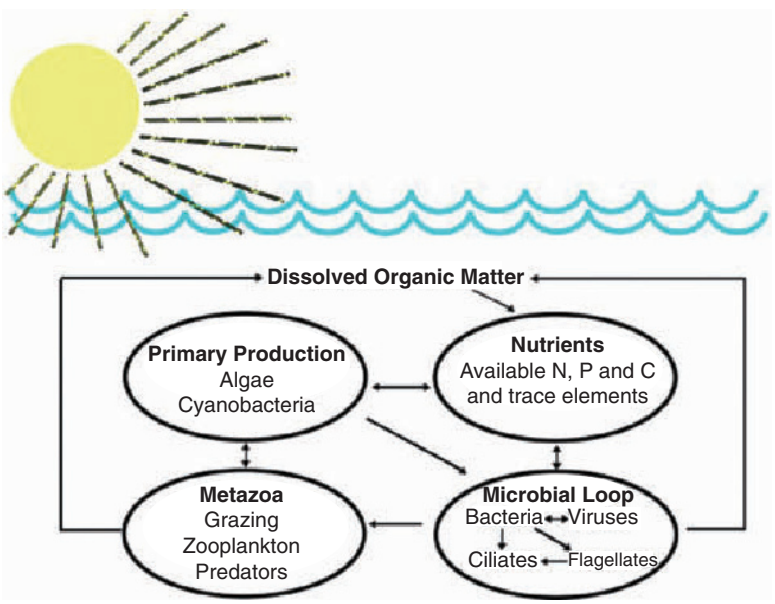

Fig. 11. Microbial loop of the upper water column showing the connections between different trophic levels.

production among cyanobacteria but also inflicts UV radiation damage and creates oxidative stress that reduces both primary and secondary production (Garcia-Pichel et al., 1994; Herndl et al., 1994; Ramsing et al., 1997; Pakulski et al., 1998; Booth et al., 2001b). Examples of variables influencing the seasonal distribution of bacterial species are light (Moore et al., 1998; Casamayor et al., 2002), temperature (Murray et al., 1998; Pernthaler et al., 1998; Crump et al., 2003), nutrients (LeBaron et al., 2001; MacGregor et al., 2001; Ovreas et al., 2003; Simek et al., 2003), changes in organic matter quantity and quality (Van Hannen et al., 1999a; Van Hannen et al., 1999b; Crump et al., 2003), abundance of grazers (Pernthaler et al., 2001; Simek et al., 2001a; Simek et al., 2003), and episodic viral lysis (Van Hannen et al., 1999b; Suttle, 2000; Fuhrman, 2001; Hahn and Hofle, 2001). Interannual climatic changes also impact the physical structure of the oceanic water column, which influences nutrient cycling and leads to shifts in the microbial community structure. We present a few examples from aquatic systems that illustrate the complex interaction of physics and biology on diel, seasonal, and annual time scales that influence microbial community structure and activity.

DIEL FLUCTUATIONS IN MICROBIAL GENE EXPRESSION. Here we present a few examples of regulation of gene expression governing responses to regularly changing conditions. During daily fluctuations in light, temperature and other physical parameters, there is potential for damage as well as the need to take advantage of optimal conditions for nutrient and energy acquisition. The ability to anticipate diel cycles of these parameters and adjust gene expression on a regular schedule may have an adaptive advantage. Recent comparative analyses of complete genomes have revealed that homologues of the key genes involved in circadian regulation (*kai* genes) are widely distributed among bacteria and archaea, implicating a more general adaptive significance among prokaryotes of the ability to anticipate regularly changing environmental circumstance (Dvornyk et al., 2003). Autotrophic and heterotrophic prokaryotes exhibit diel shifts in activity regulated by *kai* genes, which belong to the RecA superfamily of DNA binding proteins (Mori et al., 2002), including nitrogen fixation, cell division, and other metabolic processes, although the clocking mechanism is not yet understood (Dvornyk et al., 2003). Synchrony of the endogenous clock and the environmental temporal cycle has been shown to increase the fitness of cyanobacteria (Ouyang et al., 1998).

Diel changes in expression also occur in direct response to an environmental variable rather than being governed by a circadian gene. For example, RecA expression occurs on a diel cycle but in response to DNA damage in the form of cyclobutane pyrimidine dimers (CPD)resulting from absorbance of light in the ultraviolet wavelength (as reviewed in Holm-Hansen et al. [1993], Mitchell and Karentz [1993], Moran and Zepp [2000b], and Jeffrey et al. [2000]). The number of CPD lesions increases over the course of a day, causing mutations if not repaired. In response, bacterioplankton have coordinated RecA-mediated DNA repair and replication over the diel cycle (Herndl et al., 1994; Pakulski et al., 1998), with RecA protein induction peaking at dusk (Booth et al., 2001b). Since the efficiency of DNA repair varies among bacterial species (Arrieta et al., 2000), diel fluctuation in UV is expected to influence the population structure of microbial communities in the euphotic zone of aquatic systems. These are just two examples of strategies employed by bacteria in response to environmental change on a diel scale, and they emphasize that activities and structure oscillate over time in often predictable patterns.

SEASONAL FLUCTUATIONS IN MICROBIAL POPULATIONS. The potential impact of extreme seasonal changes in chemical and physical variables on a microbial system is well illustrated by a study of the high mountain Lake Gossenkollesee (Pernthaler et al., 1998). Changes in light, temperature, nutrients, and organic matter quantity and quality resulted in bacterial populations that were annually recurrent and seasonally variable. Stratification of the water column occurred from June through September with warming of the surface layers, followed by thermal mixing as temperatures cooled in November and then by ice cover throughout the winter and spring. Algae demonstrated a seasonal peak in productivity from December through February as an under-ice bloom (Fig. 12A). This bloom correlated with bacterial productivity (Sommaruga et al., 1997). During autumnal thermal mixing, the total microbial biomass declined (Fig. 12A) followed by a peak during December, once ice formation had occurred. In contrast, *Alcaligenes* sp. showed an annual maximum in November indicating that total biomass estimates often mask fluxes in individual populations (Fig. 12B). Following ice cover, succession continued to occur beneath the ice at temperatures ranging from 0 to 4.2 °C. For example, *Rhodoferax* spp. decreased at the onset of the under-ice algal bloom and then increased after the under-ice algae declined (Fig. 12B). During the ice cover melt, bacterial populations responded to associated inputs of organic carbon from the melting ice, thermal mixing of the water column, and increased temperature. The tight coupling of seasonal variables and microbial population succession in these lake systems

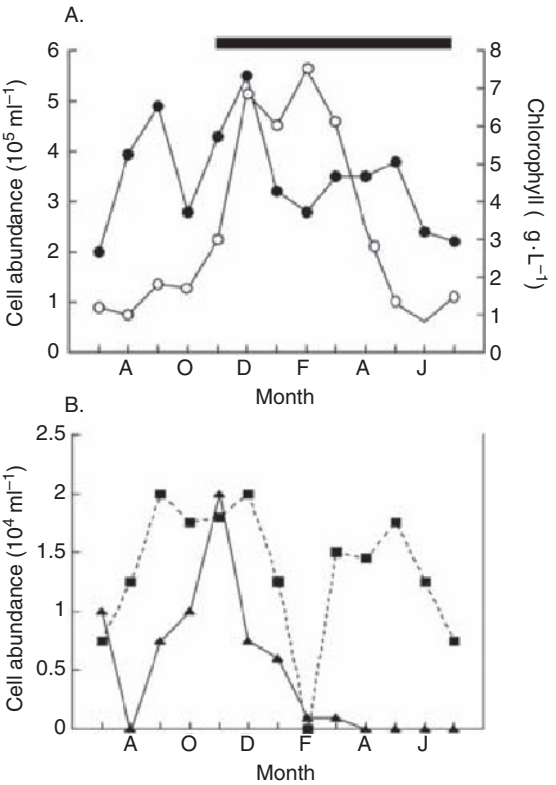

Fig. 12. Seasonal dynamics of primary producers and bacteria at 4 m in Lake Gossenkollesee, an oligotrophic high mountain lake in the Central Alps of Austria. A.) Seasonal fluctuation in chlorophyll a (m) and bacterial cells stained with 4',6'-di-amidino-2-phenylindole (DAPI) (l) at 4 m. The horizontal bar indicates the period of ice cover. B.) Population dynamics of cells hybridizing with probes GKS16 (*Rhodoferax*) (n) and GKS98 (*Alcaligenes*) (s). (From Pernthaler et al. [1998].)

offers opportunities to analyze and better resolve the dynamic interactions between biology, chemistry, and physics in natural systems. Seasonal changes in microbial composition and activity have been investigated and found in other systems including soil, lake sediment, the Mediterranean Sea, and a few oceans (Cannavo et al., 2004; Gallagher et al., 2004; Hasegawa and Okino, 2004; Short et al., 2004; Tanaka and Rassoulzadegan, 2004).

Interaction with higher trophic levels through grazing also influences the seasonal distribution of bacterial species in aquatic systems (as reviewed by Strom, 2000). For example, a seasonal change in nutrient inputs can stimulate a rapid increase of heterotrophic microbial populations which in turn stimulates grazing and growth of protists in aquatic systems (Simek et al., 2003). Grazing by nanoflagellates and protists can be responsible for changes in morphological structure, physiological status of the bacteria (Posch et al., 1999), and taxonomic shifts in the bacterial populations (Hahn, 1999; Simek et al., 2001b), affecting overall cell number and secondary productivity (Simek et al., 2001b; Simek et al., 2003).

Seasonal fluctuations in predation on bacteria by viruses can also alter the distribution of bacteria in a community (as reviewed by Suttle [2000] and Fuhrman [2000]). Since viruses require contact with a host cell, the density of the bacterial cells affects the probability of infection. This implies that since the dominant competitive bacteria is reduced and the rare bacteria are not lysed, viral dynamics in bacterial communities may influence the number of bacterial species that can co-exist in a resource limited environment (Fuhrman, 1999). While viral infections don't lead to extinction, they do shift the relative abundance and growth rates of bacteria on a seasonal timescale. For example, Van Hannen et al. (1999b) showed that the bacterial community structure in freshwater mesocosms was constant until viral lysis of a spring cyanobacterial bloom. The reduction in cyanobacteria was followed by transient blooms of *Cytophaga* and *Actinomycetes* suggesting that these bacterial populations grew on organic matter generated from lysed spring cyanobacterial bloom.

ANNUAL VARIATIONS IN MICROBIAL POPULATIONS. With the awareness that global warming may indeed be altering climate, there is growing interest in how this will influence longer time scale oscillations in climate patterns, and how, in turn, this alters the microbial activities in the oceans and soils globally. An example of a well-studied climatic oscillation, called "El Nino-Southern Oscillation" (ENSO), occurs every 2–7 years (Fiedler, 2002). In non-ENSO years, the trade winds blow in a westerly direction along the equator resulting in increased sea surface height in the west (about 0.5 m). In the eastern equatorial Pacific, water upwells from the deep ocean, causes a decrease in sea surface temperature (about 8°C), and inputs nutrients (NO_3) that fuel algal blooms in an otherwise nutrient limited sea.

During ENSO, the trade winds relax, reducing upwelling, nutrient enrichment (NO_3), and primary productivity (Le Bourgne et al., 2002). These change then influence secondary productivity and food web structure. The warming of the upper surface waters enhances thermal stratification, decreasing the influx of inorganic nutrients (NO_3 and Si) and selecting for N_2-fixing cyanobacteria (as opposed to Si-dependent diatoms). Studies in the North Pacific subtropical gyre suggest that altered nutrient fluxes associated with climate variation have contributed to a shift from eukaryotic primary producers to an array of autotrophic (Karl et al., 2001) and photoheterotrophic prokaryotes (Beja et al., 2000; Beja et

al., 2001; Beja et al., 2002) that now dominates the upper water column in the North Pacific Subtropical Gyre. Thus, the gradual increase in sea and atmospheric temperatures has the potential to alter the time scale and intensity of the ENSO, along with other climatic cycles, influencing the oscillations in microbial ecosystems controlling major biogeochemical cycles (Herbert and Dixon, 2003; Luo et al., 2003; Philander and Fedorov, 2003).

Emerging Technologies Will Enable Characterization of Structure and Function Methodological advancements continue to refine our understanding of microbial communities within an ecological context. Molecular analyses, often based on comparative 16S rRNA sequencing, are now commonly used to explore patterns of diversity and to a lesser extent abundance. These studies have revealed a remarkable diversity of life previously obscured by the limitations of established culture-based descriptions. Continued refinement of methods to enable more comprehensive measurements of microbial populations will begin to illuminate the relationships between population dynamics, physical variables, and the flux of energy and nutrients (carbon, nitrogen, phosphorus, sulfur or other elements).

We anticipate that two analytical developments will greatly enable this research. First, devices or methods capable of making rapid multiple measurements of genotypic and cellular activities (e.g., DNA microarrays) will provide high resolution mapping of temporal and spatial variations in population genotypes and expression patterns. Second, the development of devices or methods capable of measuring the activities at the level of individual cells will define the variability of cellular activity under given conditions and advance culture capabilities. Advances in nanotechnology and microfluidics will likely be key to realizing this second analytical need (Lidstrom and Meldrum, 2003; Hong et al., 2004).

We are now at the leading edge of a revolution in analytical methods that will inform at the level of populations and the single cell. Since this level of resolution will ultimately allow microbiologists to "see the world as microbes see it," this technology will have a profound impact on all aspects of microbiology. Here we highlight only a few promising areas of development for the study of prokaryotes in a more natural context.

MICROAUTORADIOGRAPHY AND FISH. A common indirect measurement of microbial activity has been based on thymidine incorporation into DNA (Fuhrman and Azam, 1980; Fuhrman and Azam, 1982). This and similar approaches (Kirchman et al., 1985) provide an estimate of growth rate averaged over many bacterial groups (see Kirchman and Ducklow, 1993). The contribution of specific populations to bulk processes has been partially revealed by combining the established methods of microautoradiography (MAR) and fluorescence in situ hybridization (FISH; Lee et al., 1999; Ouverney and Fuhrman 1999; Cottrell and Kirchman, 2000; Cottrell and Kirchman, 2003). MAR is based on the incorporation of a specific radioactive substrate added exogenously to an experimental system or environmental sample under controlled conditions. Substrate is generally selected with some understanding of processes intrinsic to a community. Most commonly, cells are fixed to a standard microscope slide, coated with a photographic emulsion, and cells that have assimilated added substrate scored by counting silver grains that develop following an appropriate period of exposure (Brock and Brock, 1966). Currently available imaging devices (e.g., phosphoimagers and beta-emission imagers), although offering wide dynamic ranges, as yet lack the resolution of photographic emulsion (Anderson et al., 1997; Andreasen and Nielsen, 1997; Laniece et al., 1998). FISH using oligonucleotide probes that target varying taxonomic levels from species to higher order phyla are then used to identify which taxa have assimilated added substrate. For example, a general probe for the bacterial domain was used to demonstrate that the capacity to assimilate thymidine is broadly distributed among bacteria inhabiting different ecosystems (Bouvier and del Giorgio, 2001; Cottrell and Kirchman, 2003).

Combined MAR and FISH now provides a method to link novel populations (those identified by rRNA sequence alone) with specific physiological attributes (Nielsen et al., 1998; Lee et al., 1999). Processes and organisms investigated so far include nitrification (Daims et al., 2001a), phosphate accumulation (Nielsen and Nielsen, 2002), sulfate reduction (Ito et al., 2002), and in situ physiology of *Thiothrix* in activated sludge (Nielsen et al., 2000). Recently MAR-FISH has been combined with the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to identify actively respiring cells in filaments of *Thiothrix* (Nielsen, 2003). An important caveat of interpretation is that the method detects uptake of a substrate but does not necessarily imply growth. Microorganisms accumulating certain substrates or compounds (such as phosphate) for storage can be mistaken for actively growing populations.

ISOTOPIC ANALYSES. Stable isotope analysis of microbial communities has been used to identify sources of organic carbon that support bacterial growth in a variety of aquatic systems (Coffin et al., 1989; Hullar et al., 1996). The use

of molecular methods together with stable isotope analysis has served to develop links between phylogenetic affiliation and metabolism of specific populations (Coffin et al., 1989; Kelley et al. 1998; Boetius et al., 2000; Radajewski et al., 2000; Radajewski et al., 2002; Radajewski et al., 2003; Orphan et al., 2001; Whitby et al., 2001; Boschker and Middleburg, 2002; MacGregor et al., 2002; Michaelis et al., 2003). An exemplary method is stable isotope probing (SIP) of nucleic acids (Radajewski et al., 2000). As described for MAR-FISH, these analyses are constrained by the requirement to incubate an environmental sample with substrate (labeled with a heavy isotope) in an enclosed system. Following incubation, DNA (or RNA) is extracted and separated on a density gradient, the heavy fraction derived from populations having incorporated the labeled substrate into their nucleic acids. Functional and taxonomic gene markers are then used to characterize those populations contributing to the heavy (or light) DNA fractions. Recent studies have used 16S rRNA sequence analyses of the heavy DNA fraction to demonstrate consumption of ^{13}C-labeled methanol by members of the *Acidobacterium* division and Alphaproteobacteria (Radajewski et al., 2000), incorporation of ^{13}C -labeled CO_2 by autotrophic nitrifiers (Whitby et al., 2001), and incorporation of ^{13}C -labeled phenol by populations metabolizing phenol in bioreactors (Manefield et al., 2002). SIP has also been used in conjunction with other biomarkers, such as fatty acids, to identify general assemblages of microorganisms contributing to specific activities in the environment (as reviewed in Boschker and Middleburg, 2002). The method has primary utility in assessing assimilatory reactions in contained environmental samples that allow for significant enrichment with the labeled substrate and require relatively short incubation periods. Substrates for dissimilatory reactions (e.g., denitrification of nitrate) cannot be directly measured via incorporation into nucleic acids, and long incubations may contribute to indirect labeling of populations via leakage of labeled metabolites from primary consumers (cross feeding). Genomic studies combined with SIP have the potential to elucidate functional diversity in relation to microbial community structure (Wellington et al., 2003).

More recently, FISH has been combined with secondary ion mass spectrometry (SIMS) to measure isotopic composition of individual cells (Orphan et al., 2001) involved in anaerobic methane oxidation. Another recently described rRNA-based approach used biotin-labeled DNA probes to selectively recover specific rRNA populations for isotopic characterization (MacGregor et al., 2002). In combination with radiotracer approaches, this technique can be used to measure biogeochemical fluxes through specific microbial populations.

DNA MICROARRAYS. DNA-based methods are now commonly used for more direct analysis of community structure. Even so, no study has yet to fully resolve the population structure of a natural microbial community. This is primarily because available formats for nucleic acid-based characterization are not suitable for extensive or intensive analyses. Fingerprinting methods such as denaturing gradient gel electrophoresis (Muyzer et al., 1996) and terminal restriction fragment length polymorphisms analysis (Liu et al., 1997) provide a relatively rapid overview of changing community structure but do not serve to either resolve or identify all contributing populations, and interpretations suffer from the limitations of polymerase chain reaction (PCR) biases (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998a). An alternative format based on hybridization of complex nucleic acid mixtures to dense arrays of DNA probes (DNA microarrays) offers the potential for rapid and high-resolution analysis of sequence composition. Each array element is comprised of a specific DNA probe (an oligomer or PCR amplification product) designed to hybridize to a specific target sequence. Microarrays were initially developed for expression analysis as a natural outgrowth of genomics—the DNA probes designed to target all genes in a specific target organism. Microarrays also provide a format suited to more holistic analyses of microbial communities (Guschin et al., 1997; Gibson et al., 2002).

Initial applications of microarrays in microbial ecology have been primarily demonstration studies, using probes targeting a selected set of conserved genes to evaluate population structure (e.g., ribosomal RNA genes) or potential activity (e.g., nitrification, denitrification, and methanotrophy; Loy et al., 2002; Bodrossy et al., 2003; El Fantroussi et al., 2003; Taroncher-Oldenburg et al., 2003). Most have relied on PCR amplification to generate sufficient target for hybridization and therefore suffer from the recognized limitations of PCR amplification of mixed environmental gene targets—not all genes are equally amplified. However, the greater challenge is interpretation of hybridization events. Unlike expression analysis of a single organism, characterization of an environmental sample may be confounded by far greater and undefined genetic complexity (Torsvik et al., 1990). Environmental studies have therefore relied upon independent assessments, generally by limited sequence analysis of the amplification products applied to the microarray, to confirm hybridization results (Cho and Tiedje, 2001; Koizumi et al., 2002; Bodrossy et al., 2003). Since different envi-

ronments present different compositions of nontarget populations, independent validation would be required for each application. Thus, before DNA microarrays will become robust tools for analysis of environmental systems, validation must become an integrated design feature. For example, on-chip thermal dissociation analysis (El Fantroussi et al., 2003) provided an additional criterion for assessing whether hybridization originates from a target or closely related nontarget sequence. We anticipate that future developments will allow hybridization of DNA and RNA isolated directly from environmental samples, and thus avoid the biases of PCR amplifications.

The Future

This is only a selected sampling of the rapidly changing technology landscape that will enable high-resolution analyses of microbial community structure and function. In discussing these few technical advances, we have also omitted many important developments in high throughput methods for culturing environmental organisms (Connon and Giovannoni, 2002; Zengler et al., 2002). Recent development here suggests that the isolation of microorganisms that have "resisted" cultivation in the past will soon be commonplace. This exciting development will place tremendous demands on microbial physiologists, and we anticipate that high throughput methods of physiological and genetic characterization will naturally develop as the number of microorganisms in culture dramatically expands. The development of nanotechnology promises to provide tools to study the physiology of single cells and the measurement of fluxes on scales that are relevant to the microbial cell. Although applications of current methods are associated with many limitations of format and interpretation, continued advances in technology will largely determine the future of environmental microbiology as we continue to resolve the structure and function of complex communities that sustain our biosphere.

Literature Cited

Aeckersberg, F., C. Lupp, B. Feliciano, and E. G. Ruby. 2001. Vibrio fischeri outer membrane protein OmpU plays a role in normal symbiotic colonization. J. Bacteriol. 183:6590–6597.

Alldredge, A. L., and Y. Cohen. 1987. Can microscale chemical patches persist in the sea? Microelectrode study of marine snow, fecal pellets. Science 235:689–691.

Alldredge, A. L., and M. W. Silver. 1988. Characteristics, dynamics and significance of marine snow. Progr. Oceanogr. 20:41–82.

Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.

Amann, R. 2000a. Who is out there? Microbial aspects of biodiversity. System. Appl. Microbiol. 23:1–8.

Amann, R., and W. Ludwig. 2000b. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol. Rev. 24:555–565.

Anderson, K. M., P. H. Cheung, and M. D. Kelly. 1997. Rapid generation of homologous internal standards and evaluation of data for quantitation of messenger RNA by competitive polymerase chain reaction. J. Pharmacol. Toxicol. 38:133–140.

Andreasen, K., and P. H. Nielsen. 1997. Application of microautoradiography to study substrate uptake by filamentous microorganisms in activated sludge. Appl. Environ. Microbiol. 63:3632–3668.

Arrieta, J. M., M. G. Weinbauer, and G. J. Herndl. 2000. Interspecific variability in sensitivity to UV radiation and subsequent recovery in selected isolates of marine bacteria. Appl. Environ. Microbiol. 66:1468–1473.

Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad. 1983. The ecological role of water column microbes in the sea. Mar. Ecol. Progr. Ser. 10:257–263.

Bahr, M., J. E. Hobbie, and M. L. Sogin. 1996. Bacterial diversity in an Arctic lake: A freshwater SAR11 cluster. Aquat. Microb. Ecol. 11:271–277.

Barns, S. M., C. F. Delwiche, J. D. Palmer, and N. R. Pace. 1996. Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc. Natl. Acad. Sci. 93:9188–9193.

Baumann, P., L. Baumann, C. Y. Lai, D. Roubakhsh, N. A. Moran, and M. A. Clark. 1995. Genetics, physiology, and evolutionary relationships of the genus Buchnera intracellular symbionts of aphids. Ann. Rev Microbiol. 49:55–94.

Beijerinck, M. W. 1913. De infusies en de ontdekking der backteriën. *In:* Jaarboek van de Koninklijke Akademie van Wetenschappen. Müller. Amsterdam, The Netherlands.

Beja, O., L. Aravind, and E. V. Koonin. 2000. Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. Science 289:1902–1906.

Beja, O., E. N. Spudich, and J. L. Spudich. 2001. Proteorhodopsin phototrophy in the ocean. Nature 411:786–789.

Beja, O., M. T. Suzuki; J. F. Heidelberg, W. C. Nelson, C. M. Preston, T. Hamada, J. A. Eisen, C. M. Fraser, and E. F. Delong. 2002. Unsuspected diversity among marine aerobic anoxygenic phototrophs. Nature 415: 630–633.

Blackburn, N., T. Fenchel, and J. Mitchell. 1998. Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. Science 282:2254–2256.

Blackburn, N., and T. Fenchel. 1999. Influence of bacteria, diffusion and sheer on microscale nutrient patches, and implications for bacterial chemotaxis. Mar. Ecol. Progr. Ser. 189:1–7.

Bodrossy, L., N. Stralis-Pavese, J. C. Murrell, S. Radajewski, A. Weilharter, and A. Sessitsch. 2003. Development and validation of a diagnostic microbial microarray for methanotrophs. Environ. Microbiol. 5:566–582.

Boetius, A., K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Gieseke, R. Amann, B. B. Jørgensen, U.

Witte, and O. Pfannkuche. 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407:623–626.

Booth, M. G., L. Hutchinson, M. Brumsted, P. Aas, R. B. Coffin, R. C. Downer, C. A. Kelley, M. M. Lyons, J. D. Pakulski, S. L. H. Sandvik, W. H. Jeffrey, and R. V. Miller. 2001a. Quantification of recA gene expression as an indicator of repair potential in marine bacterioplankton communities of Antarctica. Aquat. Microb. Ecol. 24:51–59.

Booth, M. G., W. H. Jeffrey, and R. V. Miller. 2001b. RecA expression in response to solar UVR in the marine bacterium Vibrio natriegens. Microb. Ecol. 42:531–539.

Boschker, H. T. S., and J. J. Middelburg. 2002. Stable isotopes and biomarkers in microbial ecology. FEMS Microbiol. Ecol. 40:85–95.

Bourtzis, K., and T. A. Miller (Eds.). 2003. Insect Symbiosis. CRC Press. Boca Raton, FL.

Bouvier, T., and P. A. del Giorgio. 2003. Factors influencing the detection of bacterial cells using fluorescence in situ hybridization (FISH): A quantitative review of published reports. FEMS Microbiol. Ecol. 44:3–15.

Braschler, T. R., S. Merino, J. M. Tomas, and J. Graf. 2003. Complement resistance is essential for colonization of the digestive tract of Hirudo medicinalis by Aeromonas strains. Appl. Environ. Microbiol. 69:4268–4271.

Breznak, J. A. 1982. Intestinal microbiota of termites and other xylophagous insects. Ann. Rev Microbiol. 36:323–343.

Brock, T. D., and M. L. Brock. 1966. Autoradiography as a tool in microbial ecology. Nature 209:734–736.

Brusseau, G. A., B. E. Rittmann, and D. A. Stahl. 1998. Addressing the microbial ecology of marine biofilms. *In:* Molecular Approaches to the Study of the Ocean. Chapman and Hall. New York, NY.

Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe. 1967. Methanobacillus omelianskii: A symbiotic association of two species of bacteria. Arch. Microbiol. 59:20–31.

Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill. 1977. Growth of Desulfovibrio in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. 33:1162–1169.

Buttner, M., D. L. Xie, H. Nelson, W. Pinther, G. Hauska, and N. Nelson. 1992. Photosynthetic reaction center genes in green sulfur bacteria and in photosystem-1 are related. Proc. Natl. Acad. Sci. 89:8135–8139.

Button, D. K. 1994. The physical base of marine bacterial ecology. Microb. Ecol. 28:273–285.

Canfield, D. E., and D. J. DesMarais. 1991. Aerobic sulfate reduction in microbial mats. Science 251:1471–1473.

Canfield, D. E., and D. J. D. Marais. 1993. Biogeochemical cycles of carbon, sulfur, and free oxygen in a microbial mat. Geochim. Cosmochim. Act. 57:3971–3984.

Cary, S. C., M. T. Cottrell, J. L. Stein, F. Camacho, and D. Desbruyeres. 1997. Molecular identification and localization of filamentous symbiotic bacteria associated with the hydrothermal vent annelid Alvinella pompejana. Appl. Environ. Microbiol. 63:1124–1130.

Casamayor, E. O., C. Pedros-Alio, G. Muyzer, and R. Amann. 2002. Microheterogeneity in 16S ribosomal DNA defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations. Appl. Environ. Microbiol. 68(4):1706–1714.

Casjens, S. 1998. The diverse and dynamic structure of bacterial genomes. Ann. Rev. Genet. 32:339–445.

Characklis, W. G., and K. C. Marshall. 1990. Biofilms. Wiley-Liss. New York, NY. 796.

Childress, J. J., H. Felbeck, and G. N. Somero. 1987. Symbiosis in the deep-sea. Sci. Am. 256(5):114–130.

Cho, J. C., and J. M. Tiedje. 2001. Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. Appl. Environ. Microbiol. 67:3677–3682.

Coffin, R. B., B. Fry, B. J. Peterson, and R. T. Wright. 1989. Carbon isotopic compositions of estuarine bacteria. Limnol. Oceanogr. 34:1305–1310.

Coffin, R. B., D. J. Velinsky, R. Devereux, W. A. Price, and L. A. Cifuentes. 1990. Stable carbon isotope analysis of nucleic acids to trace sources of dissolved substrates used by estuarine bacteria. Appl. Environ. Microbiol. 56:2012–2020.

Cohen, Y. 1989. Microbial Mats: Physiological Ecology of Benthic Microbial Communities. ASM Press. Washington, DC.

Connon, S. A., and S. J. Giovannoni. 2002. High throughput methods for culturing microorganisms in very low nutrient media yield diverse new marine isolates. Appl. Environ. Microbiol. 68:3878–3885.

Cossart, P., P. Boquet, S. Normark, and R. Rappuoli. 1996. Cellular microbiology emerging. Science 271:315–316.

Cottrell, M. T., D. N. Wood, L. Y. Yu, and D. L. Kirchman. 2000. Selected chitinase genes in cultured and uncultured marine bacteria in the alpha- and gamma-subclasses of the Proteobacteria. Appl. Environ. Microbiol. 66:1195–1201.

Cottrell, M. T., and D. L. Kirchman. 2003. Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware Estuary. Limnol. Oceanogr. 48:168–178.

Crump, B. C., G.W. Kling, M. Bahr, and J. E. Hobbie. 2003. Bacterioplankton community shifts in an arctic lake correlate with seasonal changes in organic matter source. Appl. Environ. Microbiol. 69:2253–2268.

Currie, C. R., J. A. Scott, R. C. Summerbell, and D. Malloch. 1999. Fungus growing ants use antibiotic producing bacteria to control garden parasites. Nature 398:701–704.

Daims, H., J. L. Nielsen, P. H. Nielsen, K.-H. Schleifer, and M. Wagner. 2001. In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. Appl. Environ. Microbiol. 67:5273–5284.

Daubin, V., N. A. Moran, and H. Ochman. 2003. Phylogenetics and the cohesion of bacterial genomes. Science 301:829–832.

Davidson, S. K., S. W. Allen, G. E. Lim, C. M. Anderson, and M. G. Haygood. 2001. Evidence for the biosynthesis of bryostatins by the bacterial symbiont "Candidatus Endobugula sertula" of the bryozoan Bugula neritina. Appl. Environ. Microbiol. 67:4531–4537.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298.

De Bary, A. 1879. Die Erscheinung der Symbiose. Naturforsch. Bersamml. 1–121.

Decho, A. W., and G. J. Herndl. 1995. Microbial activities and the transformation of organic matter within mucilaginous material. Sci. Total Environ. 165:33–42.

Delong, E. F., R. B. Frankel, and D. A. Bazylinski. 1993. Multiple evolutionary origins of magnetotaxis in bacteria. Science 259:803–806.

Delong, E. F., K. Y. Wu, B. B. Prézelin, and R. V. M. Jovine. 1994. High abundance of archaea in Antarctic marine picoplankton. Nature 371:695–697.

Delong, E. F., and N. R. Pace. 2001. Environmental diversity of Bacteria and Archaea. System. Biol. 50:470–481.

Deming, J. W., A. L. Reysenbach, S. A. Macko, and C. R. Smith. 1997. Evidence for the microbial basis of a chemoautotrophic invertebrate community at a whale fall on the deep seafloor: Bone colonizing bacteria and invertebrate endosymbionts. Microsci. Res. Techn. 37:162–170.

Devereux, R., M. Delaney, F. Widdel, and D. A. Stahl. 1989. Natural relationships among sulfate reducing eubacteria. J. Bacteriol. 171:6689–6695.

Devereux, R., S. H. He, C. L. Doyle, S. Orkland, D. A. Stahl, J. Legall, and W. B. Whitman. 1990. Diversity and origin of Desulfovibrio specie: Phylogenetic definition of a family. J. Bacteriol. 172:3609–3619.

Distel, D. L., D. J. Beaudoin, and W. Morrill. 2002. Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve Lyrodus pedicellatus (Bivalvia:Teredinidae). Appl. Environ. Microbiol. 68:6292–6299.

Dobrindt, U., F. Agerer, K. Michaelis, A. Janka, C. Buchrieser, M. Samuelson, C. Svanborg, G. Gottschalk, H. Karch, and J. Hacker. 2003. Analysis of genome plasticity in pathogenic and commensal Escherichia coli isolates by use of DNA arrays. J. Bacteriol. 185:1831–1840.

Doolittle, W. F. 1999. Phylogenetic classification and the universal tree. Science 284:2124–2128.

Douglas, A. E. 1997. Parallels and contrasts between symbiotic bacteria and bacterial-derived organelles: Evidence from Buchnera, the bacterial symbiont of aphids. FEMS Microbiol. Ecol. 24:1–9.

Dvornyk, V., O. Vinogradova, and E. Nevo. 2003. Origin and evolution of circadian clock genes in prokaryotes. Proc. Natl. Acad. Sci. 100:2495–2500.

Elena, S. F., and R. E. Lenski. 2003. Evolution experiments with microorganisms: The dynamics and genetic bases of adaptation. Nature Rev. Genet. 4:457–469.

El Fantroussi, S., H. Urakawa, A. E. Bernhard, J. J. Kelly, P. A. Noble, H. Smidt, G. M. Yershov, and D. A. Stahl. 2003. Direct profiling of environmental microbial populations by thermal dissociation analysis of native rRNAs hybridized to oligonucleotide microarrays. Appl. Environ. Microbiol. 69:2377–2382.

Faulkner, J., M. D. Unson, and C. A. Bewley. 1994. The chemistry of some sponges and their symbionts. Pure Appl. Chem. 66:1983–1990.

Fenchel, T., and R. N. Glud. 1998. Veil architecture in a sulphide-oxidizing bacterium enhances countercurrent flux. Nature 394:367–369.

Fenchel, T. 2002. Microbial behavior in a heterogeneous world. Science 296:1068–1071.

Ferdelman, T. G., C. Lee, S. Pantoja, J. Harder, B. M. Bebout, and H. Fossing. 1997. Sulfate reduction and methanogenesis in a Thioploca dominated sediment off the coast of Chile. Geochim. Cosmochim. Act. 61:3065–3079.

Ffrench-Constant, R., N. Waterfield, P. Daborn, S. Joyce, H. Bennett, C. Au, A. Dowling, S. Boundy, S. Reynolds, and D. Clarke. 2003. Photorhabdus: Towards a functional genomic analysis of a symbiont and pathogen. FEMS Microbiol. Rev. 26:433–456.

Fiedler, P. C. 2002. Environmental change in the eastern tropical Pacific Ocean: Review of ENSO and decadal variability. Mar. Ecol. Progr. Ser. 244:265–283.

Field, K.G., D. Gordon, T. Wright, M. Rappe, E. Urbach, K. Vergin, and S. J. Giovannoni. 1997. Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. Appl. Environ. Microbiol. 63:63–70.

Fisher, C. R., J. J. Childress, and N. K. Sanders. 1988. The role of Vestimentiferan hemoglobin in providing an environment suitable for chemoautotrophic sulfide-oxidizing endosymbionts. Symbiosis 5:229–246.

Fisher, C. R. 1990. Chemoautotrophic and methanotrophic symbioses in marine invertebrates. Rev. Aquat. Sci. 2:399–436.

Fisher, C. R. 1995. Towards and appreciation of hydrothermal-vent animals: Their environment, physiological ecology, and tissue stable isotope values. *In:* S. E. Humphris, R. A. Zierenberg, L. S. Multineaux, and R. E. Thompson (Eds.) Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions. Washington, DC. AGU Monograph Series:297–316.

Fossing, H., V. A. Gallardo, B. B. Jørgensen, M. Huttel, L. P. Nielsen, H. Schulz, D. E. Canfield, S. Forster, R. N. Glud, J. K. Gundersen, J. Kuver, N. B. Ramsing, A. Teske, B. Thamdrup, and O. Ulloa. 1995. Concentration and transport of nitrate by the mat-forming sulfur bacterium Thioploca. Nature 374:713–715.

Fründ, C., and Y. Cohen. 1992. Diurnal cycles of sulfate reduction under oxic conditions in microbial mats. Appl. Environ. Microbiol. 58:70–77.

Fuhrman, J. A., and F. Azam. 1980. Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl. Environ. Microbiol. 39:1085–1095.

Fuhrman, J. A., and F. Azam. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: Evaluation and field results. Mar. Biol. 66:109–120.

Fuhrman, J. A., K. McCallum, and A. A. Davis. 1992. Novel major Archaebacterial group from marine plankton. Nature 356:148–149.

Fuhrman, J. A., K. Mccallum, and A. A. Davis. 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl. Environ. Microbiol. 59:1294–1302.

Fuhrman, J. A. 1999. Marine viruses and their biogeochemical and ecological effects. Nature 399:541–548.

Fuhrman, J. A. 2000. Impact of viruses on bacterial processes. *In:* D. L. Kirchman (Ed.) Microbial Ecology of the Sea. Wiley-Liss. New York, NY. 327–350.

Fujiwara, Y., C. Kato, N. Masui, K. Fujikura, and S. Kojima. 2001. Dual symbiosis in the cold-seep thyasirid clam Maorithyas hadalis from the hadal zone in the Japan Trench, western Pacific. Mar. Ecol. Progr. Ser. 214:151–159.

Fukui, M., A. Teske, and B. Assmus. 1999. Physiology, phylogenetic relationships, and ecology of filamentous sulfate-reducing bacteria (genus Desulfonema). Arch. Microbiol. 172(4):193–203.

Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1994. Quorum sensing in bacteria: The LuxR-LuxI family of cell density responsive transcriptional regulators. J. Bacteriol. 176:269–275.

Fuqua, C., S. C. Winans, and E. P. Greenberg. 1996. Census and consensus in bacterial ecosystems: The LuxR-LuxI

family of quorum-sensing transcriptional regulators. Ann. Rev Microbiol. 50:727–751.

Fuqua, C., M. R. Parsek, and E. P. Greenberg. 2001. Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum sensing. Ann. Rev. Genet. 35:439–468.

Fuqua, C., and E. P. Greenberg. 2002. Listening in on bacteria: Acyl-homoserine lactone signaling. Nature Rev. Molec. Cell. Biol. 3:685–695.

Gallagher, J. M., M. W. Carton, D. Eardly, and J. W. Patching. 2004. Spatio-temporal variability and diversity of water column prokaryotic communities in the eastern North Atlantic. FEMS Microbiol. Ecol. 47:249–262.

Gallardo, C. S. 1977. Two modes of development in morph-species Crepidula dilatata (Gastropoda Calyptraeidae) from Southern Chile. Mar. Biol. 39:241–251.

Garcia-Pichel, F., M. Mechling, and R.W. Castenholz. 1994. Diel migrations of microorganisms within a benthic, hypersaline mat community. Appl. Environ. Microbiol. 60:1500–1511.

Garnier, T., K. Eiglmeier, J. C. Camus, N. Medina, H. Mansoor, M. Pryor, S. Duthoy, S. Grondin, C. Lacroix, C. Monsempe, S. Simon, B. Harris, R. Atkin, J. Doggett, R. Mayes, L. Keating, P. R. Wheeler, J. Parkhill, B. G. Barrell, S. T. Cole, S. V. Gordon, and R. G. Hewinson. 2003. The complete genome sequence of Mycobacterium bovis. Proc. Natl. Acad. Sci. USA 100:7877–7882.

Gibson, G. 2002. Microarrays in ecology and evolution: A preview. Molec. Ecol. 11(1):17–24.

Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field. 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63.

Giovannoni, S. J., M. S. Rappe, K. L. Vergin, and N. L. Adair. 1996. 16S rRNA genes reveal stratified open ocean bacterioplankton populations related to the Green Non-sulfur Bacteria. Proc. Natl. Acad. Sci. USA. 93:7979–7984.

Goffredi, S. K., J. J. Childress, N. T. Desaulniers, and F. H. Lallier. 1997. Sulfide acquisition by the vent worm Riftia pachyptila appears to be via uptake of HS-, rather than H_2S. J. Exp. Biol. 200:2609–2616.

Graf, J. 1999. Symbiosis of Aeromonas veronii biovar sobria and Hirudo medicinalis, the medicinal leech: A novel model for digestive tract associations. Infect. Immun. 67:1–7.

Grotzinger, J. P., and A. H. Knoll. 1999. Stromatolites in precambrian carbonates: Evolutionary mileposts or environmental dipsticks? Ann. Rev. Earth Pl. Sci. 27:313–358.

Guschin, D. Y., B. K. Mobarry, D. Proudnikov, D. A. Stahl, B. E. Rittmann, and A. D. Mirzabekov. 1997. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl. Environ. Microbiol. 63:2397–2402.

Hahn, M. W., and M. G. Hofle. 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiol. Ecol. 35:113–121.

Hasegawa, T., and T. Okino. 2004. Seasonal variation of denitrification rate in Lake Suwa sediment. Limnology 5:33–39.

Hastings, J. W., J. Makemson, and P. V. Dunlap. 1987. Are growth and luminescence regulated independently in light organ symbionts? Symbiosis 4:3–24.

Hastings, J. W., and E. P. Greenberg. 1999. Quorum sensing: The explanation of a curious phenomenon reveals a common characteristic of bacteria. J. Bacteriol. 181: 2667–2668.

Hayashi, T., K. Makino, M. Ohnishi, K. Kurokawa, K. Ishii, K. Yokoyama, C. G. Han, E. Ohtsubo, K. Nakayama, T. Murata, M. Tanaka, T. Tobe, T. Iida, H. Takami, T. Honda, C. Sasakawa, N. Ogasawara, T. Yasunaga, S. Kuhara, T. Shiba, M. Hattori, and H. Shinagawa. 2001. Complete genome sequence of enterohemorrhagic Escherichia coli O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res. 8:11–22.

Haygood, M. G. 1993. Lightorgan symbioses in fishes. Crit. Rev. Microbiol. 19:191–216.

Head, I. M., W. D. Hiorns, T. M. Embley, A. J. McCarthy, and J. R. Saunders. 1993. The phylogeny of autotrophic ammonia oxidizing bacteria as determined by analysis of 16s ribosomal-RNA gene sequences. J. Gen. Microbiol. 139:1147–1153.

Herbert, J. M., and R. W. Dixon. 2003. Is the ENSO phenomenon changing as a result of global warming? Phys. Geogr. 23:196–211.

Herndl, G. J., G. Mullerniklas, and J. Frick. 1993. Major role of ultraviolet in controlling bacterioplankton growth in the surface layer of the ocean. Nature 361:717–719.

Heungens, K., C. E. Cowles, and H. Goodrich-Blair. 2002. Identification of Xenorhabdus nematophila genes required for mutualistic colonization of Steinernema carpocapsae nematodes. Molec. Microbiol. 45:1337–1353.

Hirsch, A. M., and M. J. McFall-Ngai. 2000. Fundamental concepts in symbiotic interactions: Light and dark, day and night, squid and legume. J. Plant Growth Reg. 19:113–130.

Holm-Hansen, O., D. Lubin, and E. W. Hebling. 1993. Ultraviolet radiation and its effect on organisms in aquatic environments. *In:* A. R. Young, L. Bjorn, J. Moan, and W. Nultsch (Eds.) Environmental UV Photobiology. Plenum Press. New York, NY. 379–425.

Hong, J. W., V. Studer, G. Hang, W. F. Anderson, and S. R. Quake. 2004. A nanoliter scale nucleic acid processor with parallel architecture. Nature Biotechnol. 22:435–439.

Hooper, L. V., T. Midtvedt, and J. I. Gordon. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Ann. Rev. Nutr. 22:283–307.

Hooper, L. V. 2004. Bacterial contributions to mammalian gut development. Trends Microbiol. 12:129–134.

Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and Eberl. 2001. The cep quorum sensing system of Burkholderia cepacia H111 controls biofilm formation and swarming motility. Microbiology 47:2517–2528.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180:6793–6793.

Hullar, M. A. J., B. Fry, B. J. Peterson, and R. T. Wright. 1996. Microbial utilization of estuarine dissolved organic carbon: A stable isotope tracer approach tested by mass balance. Appl. Environ. Microbiol. 62:2489–2493.

Ito, T., J. L. Nielsen, S. Okabe, Y. Watanabe, and P. H. Nielsen. 2002. Phylogenetic identification and substrate uptake patterns of sulfate-reducing bacteria inhabiting an oxic-anoxic sewer biofilm determined by combining microautoradiography and fluorescent in situ hybridization. Appl. Environ. Microbiol. 68:356–364.

Jannasch, H. W., D. C. Nelson, and C. O. Wirsen. 1989. Massive natural occurrence of unusually large bacteria (Beggiatoa sp) at a hydrothermal deep-sea vent site. Nature 342:834–836.

Jeffrey, W., J. P. Kase, and S. W. Wilhelm. 2000. UV radiation effects on heterotrophic bacterioplankton and viruses in marine ecosystems. *In:* S. D. Mora (Ed.) The Effects of UV Radiation in the Marine Environment. Cambridge University Press. New York, NY.

Jørgensen, B. B., and Y. Cohen. 1977. Solar Lake (Sinai) sulfur cycle of benthic cyanobacterial mats. Limnol. Oceanogr. 22:657–666.

Jörgensen, B. B., and N. P. Revsbech. 1983. Colorless sulfur bacteria, Beggiatoa Spp and Thiovulum Spp in O_2 and H_2S microgradients. Appl. Environ. Microbiol. 45:1261–1270.

Jørgensen B. B. 1994. Sulfate reduction and thiosulfate transformations in a cyanobacterial mat during a diel oxygen cycle. FEMS Microbiol. Ecol. 13(4):303–312.

Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules. *In:* H. N. Munro (Ed.) Mammalian Protein Metabolism. Academic Press. New York, NY.

Karl, D. M. 1994. Accurate estimation of microbial loop processes and rates. Microb. Ecol. 28:147–150.

Karl, D. M., R. R. Bidigare, and R. M. Letelier. 2001. Long-term changes in plankton community structure and productivity in the North Pacific Subtropical Gyre: The domain shift hypothesis. Deep Sea Res. Part II, 48:1449–1470.

Karner, M. B., E. F. Delong, and D. M. Karl. 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature 409:507–510.

Kaufman, M. R., Y. Ikeda, C. Patton, G. Van Dykhuizen, and D. Epel. 1998. Bacterial symbionts colonize the accessory nidamental gland of the squid Loligo opalescens via horizontal transmission. Biol. Bull. US 194:36–43.

Kelley, C. A., R. B. Coffin, and L. A. Cifuentes. 1998. Stable isotope evidence for alternative bacterial carbon sources in the Gulf of Mexico. Limnol. Oceanogr. 43: 1962–1969.

Kirchman, D. L., E. Knees, and R. Hodson. 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl. Environ. Microbiol. 49:599–607.

Kirchman, D. L., and H. W. Ducklow. 1993. Estimating conversion factors for the thymidine and leucine methods for measuring bacterial production. *In:* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (Eds.) Handbook of Methods in Aquatic Microbial Ecology. Lewis Publishers. New York, NY. 513–518.

Kobayashi, J., and M. Ishibashi. 1993. Bioactive metabolites of symbiotic marine microorganisms. Chem. Rev. 93: 1753–1769.

Koizumi, Y., J. J. Kelly, T. Nakagawa, H. Urakawa, S. El-Fantroussi, S. Al-Muzaini, M. Fukui, Y. Urushigawa, and D. A. Stahl. 2002. Parallel characterization of anaerobic toluene- and ethylbenzene-degrading microbial consortia by PCR-denaturing gradient gel electrophoresis, RNA-DNA membrane hybridization, and DNA microarray technology. Appl. Environ. Microbiol. 68: 3215–3225.

Kolenbrander, P. E. 2000. Oral microbial communities: Biofilms, interactions, and genetic systems. Ann. Rev Microbiol. 54:413–437.

Kruger, M., A. Meyerdierks, F. O. Glockner. R. Amann, F. Widdel, M. Kube, R. Reinhardt, J. Kahnt, R. Bocher, R. K., Thauer, and S. Shima. 2003. A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. Nature 426:878–881.

Lane, D. J., A. P. Harrison, D. Stahl, B. Pace, S. J. Giovannoni, G. J. Olsen, and N. R. Pace. 1992. Evolutionary relationships among sulfur-oxidizing and iron-oxidizing eubacteria. J. Bacteriol. 174:269–278.

Laniece, P., Y. Charon, A. Cardona, L. Pinot, S. Maitrejean, R. Mastrippolito, B. Sandkamp, and L. Valentin. 1998. A new high resolution radioimager for the quantitative analysis of radiolabelled molecules in tissue section. J. Neurosci. Meth. 86:1–5.

Lebaron, P., P. Servais, M. Troussellier, C. Courties, G. Muyzer, L. Bernard, H. Schafer, R. Pukall, E. Stackebrandt, T. Guindulain, and J. Vives-Rego. 2001. Microbial community dynamics in Mediterranean nutrient enriched seawater mesocosms: Changes in abundances, activity and composition. FEMS Microbiol. Ecol. 34:255–266.

Lebourgne, R., R. T. Barber, T. Delacroix, H. Y. Inoue, D. J. Mackey, and M. Rodier. 2002. Pacific warm pool and divergence: Temporal and zonal variations on the equator and their effects on the biological pump. Deep. Sea. Res. II Top. Stud. Oceanogr. 49:2471–2512.

Lee, K. H., and E. G. Ruby. 1994. Effect of the squid host on the abundance and distribution of symbiotic Vibrio fischeri in nature. Appl. Environ. Microbiol. 60:1565–1571.

Lee, N., P. H. Nielsen, K. H. Andreasen, S. Juretschko, J. L. Nielsen, K.-H. Schleifer, and M. Wagner. 1999. Combination of fluorescent in situ hybridization and microautoradiography: A new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. 65:1289–1297.

Lidstrom, M. E., and D. R. Meldrum. 2003. Life on a chip. Nature Rev. Microbiol. 1:158–164.

Lilburn, T. C., K. S. Kim, N. E. Ostrom, K. R. Byzek, J. R. Leadbetter, and J. A. Breznak. 2001. Nitrogen fixation by symbiotic and free-living spirochetes. Science 292: 2495–2498.

Liu, W. T., T. L. Marsh, H. Cheng, and L. J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516–4522.

Loy, A., A. Lehner, N. Lee, J. Adamczyk, H. Meier, J. Ernst, K.-H. Schleifer, and M. Wagner. 2002. Oligonucleotide microarray for 16S rRNA gene based detection of all recognized lineages of sulfate reducing prokaryotes in the environment. Appl. Environ. Microbiol. 68:5064–5081.

Lum, M. R., and A. M. Hirsch. 2003. Roots and their symbiotic microbes: Strategies to obtain nitrogen and phosphorus in a nutrient limiting environment. Am. J. Plant Growth Reg. 21:368–382.

Luo, J. J., S. Masson, S. Behera, P. Delecluse, S. Gualdi, A. Navarra, and T. Yamagata. 2003. South Pacific origin of the decadal ENSO-like variation as simulated by a coupled GCM. Geophys. Res. Lett. 30:2250.

MacGregor, B. J., D. P. Moser, B. J. Baker, E. W. Alm, M. Maurer, K. H. Nealson, and D. A. Stahl. 2001. Seasonal and spatial variability in Lake Michigan sediment small-subunit rRNA concentrations. Appl. Environ. Microbiol. 67:3908–3922.

MacGregor, B. J., V. Bruchert, S. Fleischer, and R. Amann. 2002. Isolation of small-subunit rRNA for stable isotopic characterization. Environ. Microbiol. 4:451–464.

Maier, S., H. Volker, M. Beese, and V. A. Gallardo. 1990. The fine structure of Thioploca aruacae and Thioploca chileae. Can. J. Microbiol. 36:438–448.

Manefield, M., A. S. Whiteley, R. I. Griffiths, and M. J. Bailey. 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl. Environ. Microbiol. 68:5367–5373.

Martens, E. C., K. Heungens, and H. Goodrich-Blair. 2003. Early colonization events in the mutualistic association between Steinernema carpocapsae nematodes and Xenorhabdus nematophila bacteria. J. Bacteriol. 185:3147–3154.

Massana, R., A. E. Murray, C. M. Preston, and E. F. Delong. 1997. Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. Appl. Environ. Microbiol. 63:50–56.

Massana, R., L. J. Taylor, A. E. Murray, K. Y. Wu, W. H. Jeffrey, and E. F. Delong. 1998. Vertical distribution and temporal variation of marine planktonic archaea in the Gerlache Strait, Antarctica, during early spring. Limnol. Oceanogr. 43:607–617.

Massana, R., E. F. Delong, and C. Pedros-Alio. 2000. A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces. Appl. Environ. Microbiol. 66:1777–1787.

Mayr, E. 1982. The Growth of Biological Thought: Diversity, Evolution, and Inheritance. Belknap Press of Harvard University Press. Cambridge, MA.

McFall-Ngai, M. J., and E. G. Ruby. 1998. Sepiolids and vibrios: When first they meet—reciprocal interactions between host and symbiont lead to the creation of a complex light emitting organ. Bioscience 48:257–265.

McFall-Ngai, M. J. 1999. Consequences of evolving with bacterial symbionts: Insights from the Squid-Vibrio associations. Ann. Rev. Ecol. Syst. 30:235–256.

McFall-Ngai, M. J. 2001. Identifying "prime suspects": Symbioses and the evolution of multicellularity. Comp. Biochem. Phys. B 129:711–723.

McFall-Ngai, M. J. 2002. Unseen forces: The influence of bacteria on animal development. Dev. Biol. 242:1–14.

McNab, R., S. K. Ford, A. El-Sabaeny, B. Barbieri, G. S. Cook, and R. J. Lamont. 2003. LuxS-based signaling in Streptococcus gordonii: Autoinducer 2 controls carbohydrate metabolism and biofilm formation with Porphyromonas gingivalis. J. Bacteriol. 185:274–284.

Michaelis, W., R. Seifert, K. Nauhaus, T. Treude, V. Thiel, M. Blumenberg, K. Knittel, A. Gieseke, K. Peterknecht, T. Pape, A. Boetius, R. Amann, B. B. Jørgensen, F. Widdel, J. R. Peckmann, N. V. Pimenov, and M. B. Gulin. 2002. Microbial reefs in the Black Sea fueled by anaerobic oxidation of methane. Science 297:1013–1015.

Minz, D., S. Fishbain, S. J. Green, G. Muyzer, Y. Cohen, B. E. Rittmann, and D. A. Stahl. 1999a. Unexpected population distribution in a microbial mat community: Sulfate reducing bacteria localized to the highly oxic chemocline in contrast to an eukaryotic preference for anoxia. Appl. Environ. Microbiol. 65:4659–4665.

Minz, D., J. L. Flax, S. J. Green, G. Muyzer, Y. Cohen, M. Wagner, B. E. Rittmann, and D. A. Stahl. 1999b. Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. Appl. Environ. Microbiol. 65:4666–4671.

Mitchell, D. L., and D. Karentz. 1993. The induction and repair of DNA photodamage in the environment. *In:* A. R. Young, L. Bjorn, J. Moan, and W. Nultsch (Eds.) Environmental UV Photobiology. Plenum Press. New York, NY. 379–425.

Moore, L. R., G. Rocap, and S. W. Chisholm. 1998. Physiology and molecular phylogeny of coexisting Prochlorococcus ecotypes. Nature 393:464–467.

Moran, N. A., and J. J. Wernegreen. 2000a. Lifestyle evolution in symbiotic bacteria: Insights from genomics. Trends Ecol. Evol. 15:321–326.

Moran, M. A., and R. G. Zepp. 2000b. UV radiation effects on microbes and microbial processes. *In:* D. L. Kirchman (Ed.) Microbial Ecology of the Oceans. Wiley-Liss. New York, NY. 201–228.

Mori, T., S. V. Saveliev, and Y. Xu. 2002. Circadian clock protein KaiC forms ATP-dependent hexameric rings and binds DNAP. Proc. Natl. Acad. Sci. 99:17203–17208.

Morris, R. M., M. S. Rappe, S. A. Connon, K. L. Vergin, W. A. Siebold, C. A. Carlson, and S. J. Giovannoni. 2002. SAR11 clade dominates ocean surface bacterioplankton communities. Nature 420:806–810.

Murray, A. E.; C. M. Preston, R. Massana, L. T. Taylor, W. K. Blakis, and E. F. Delong. 1998. Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. Appl. Environ. Microbiol. 64:2585–2595.

Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1996. Profiling of complex microbial populations by denaturing gradient gel electrophoresis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59:695–700.

Nealson, K. H., and D. Stahl. 1997. Biogeochemical cycling and bacterial metabolism: What can we learn from layered microbial communities? *In:* J. Banfield (Ed.) Geomicrobiology: Interactions Between Microbes and Minerals. Mineralogical Society of America. New York, NY.

Nielsen, P. H., K. Andreasen, M. Wagner, L. L. Blackall, H. Lemmer, and R. J. Seviour. 1998. Variability of type 021N in activated sludge as determined by in situ substrate uptake pattern and in situ hybridization with fluorescent rRNA targeted probes. Water Sci. Technol. 37:423–440.

Nielsen, P. H., M. A. de Muro, and J. L. Nielsen. 2000. Studies on the in situ physiology of Thiothrix spp. present in activated sludge. Environ. Microbiol. 2:389–398.

Nielsen, J. L., and P. H. Nielsen. 2002. Quantification of functional groups in activated sludge by microautoradiography. Water Sci. Technol. 46:389–395.

Nielsen, J. L., M. A. de Muro, and P. H. Nielsen. 2003. Evaluation of the redox dye 5-cyano-2,3-tolyl-tetrazolium chloride for activity studies by simultaneous use of microautoradiography and fluorescence in situ hybridization. Appl. Environ. Microbiol. 69:641–643.

Nyholm, S. V., B. Deplancke, H. R. Gaskins, M. A. Apicella, and M. J. McFall-Ngai. 2002. Roles of Vibrio fischeri and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the Euprymna scolopes light organ. Appl. Environ. Microbiol. 68:5113–5122.

Ochman, H., J. G. Lawrence, and E. A. Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405:299–304.

Ochman, H., and N. A. Moran. 2001. Genes lost and genes found: Evolution of bacterial pathogenesis and symbiosis. Science 292:1096–1098.

Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl. 1986. Microbial ecology and evolution: A ribosomal RNA approach. Ann. Rev Microbiol. 40:337–365.

Orphan, V. J., C. H. House, K. U. Hinrichs, K. D. McKeegan, and E. F. Delong. 2001. Methane consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. Science 293:484–487.

Ouverney, C. C., and J. A. Fuhrman. 1999. Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol. 65:3264–3264.

Ouyang, Y., C. R. Andersson, T. Kondo, S. S. Golden, and C. H. Johnson. 1998. Resonating circadian clocks enhance fitness in cyanobacteria. Proc. Natl. Acad. Sci. 95:8660–8664.

Ovreas, L., D. Bourne, R. A. Sandaa, E. O. Casamayor, S. Benlloch, V. Goddard, G. Smerdon, M. Heldal, and T. F. Thingstad. 2003. Response of bacterial and viral communities to nutrient manipulations in seawater mesocosms. Aquat. Microb. Ecol. 31:109–121.

Paerl, H. W., and L. E. Prufert. 1987. Oxygen-poor microzones as potential sites of microbial N_2 fixation in nitrogen depleted aerobic marine waters. Appl. Environ. Microbiol. 53:1078–1087.

Pakulski, J. D., P. Aas, W. Jeffrey, M. Lyons, L. G. van Waasbergen, D. Mitchell, and R. Coffin. 1998. Influence of light on bacterioplankton production and respiration in a subtropical coral reef. Aquat. Microb. Ecol. 15:315–315.

Papke, R. T., N. B. Ramsing, M. M. Bateson, and D. M. Ward. 2003. Geographical isolation in hot spring cyanobacteria. Environ. Microbiol. 5:650–659.

Passow, U. 2002. Transparent exopolymer particles (TEP) in aquatic environments. Progr. Oceanogr. 55:287–333.

Pennisi, E. 2001. Microbial genomes: Sequences reveal borrowed genes. Science 294:1634–1635.

Perna, N. T., G. Plunkett, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Postal, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grotbeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamousis, J. Apodaca, T. S. Anantharaman, J. Y. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner. 2001. Genome sequence of enterohaemorrhagic Escherichia coli O157:H. Nature 410:240–245.

Pernthaler, J., F. O. Glockner, S. Unterholzner, A. Alfreider, R. Psenner, and R. Amann. 1998. Seasonal community and population dynamics of pelagic bacteria and archaea in a high mountain lake. Appl. Environ. Microbiol. 64:4299–4306.

Pernthaler, J., T. Posch, K. Simek, J. Vrba, A. Pernthaler, F. O. Glockner, U. Nubel, R. Psenner, and R. Amann. 2001. Predator-specific enrichment of actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture. Appl. Environ. Microbiol. 67:2145–2155.

Pfenning, N. 1967. Photosynthetic bacteria. Ann. Rev. Microbiol. 221–285.

Philander, S. G., and A. Fedorov. 2003. Is El Nino sporadic or cyclic? Ann. Rev. Earth Planet. Sci. 31:579–594.

Polz, M. F., and C. M. Cavanaugh. 1998a. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64:3724–3730.

Polz, M. F., J. J. Robinson, C. M. Cavanaugh, and C. L. Van Dover. 1998b. Trophic ecology of massive shrimp aggregations at a mid-Atlantic ridge hydrothermal vent site. Limnol. Oceanogr. 43:1631–1638.

Pomeroy, L. R. 1974. The ocean's foodweb: A changing paradigm. Bioscience 24:499–504.

Posch, T., K. Simek, J. Vrba, S. Pernthaler, J. Nedoma, B. Sattler, B. Sonntag, and R. Psenner. 1999. Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. Aquat. Microb. Ecol. 18:235–246.

Purcell, E. M. 1977. Life at low Reynolds-Number. Am. J. Phys. 45:3–11.

Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murrell. 2000. Stable-isotope probing as a tool in microbial ecology. Nature 403:646–649.

Radajewski, S., G. Webster, D. S. Reay, S. A. Morris, P. Ineson, D. B. Nedwell, J. I. Prosser, and J. C. Murrell. 2002. Identification of active methylotroph populations in an acidic forest soil by stable isotope probing. Microbiology 148:2331–2342.

Radajewski, S., I. R. McDonald, and J. C. Murrell. 2003. Stable-isotope probing of nucleic acids: A window to the function of uncultured microorganisms. Curr. Opin. Biotechnol. 14:296–302.

Ramsing, N. B., M. J. Ferris, and D. M. Ward. 1997. Light-induced motility of thermophilic Synechococcus isolates from Octopus Spring, Yellowstone National Park. Appl. Environ. Microbiol. 63:2347–2354.

Rappe, M. S., S. A. Connon, K. L. Vergin, and S. J. Giovannoni. 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418:630–633.

Raskin, L., J. M. Stromley, B. E. Rittman, and D. A. Stahl. 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl Environ Microbiol. 60(4):1232–1240.

Relman, D. A., and S. Falkow. 2001. The meaning and impact of the human genome sequence for microbiology. Trends Microbiol. 9:206–208.

Revsbech, N. P., and B. B. Jørgensen. 1983. Photosynthesis of benthic microflora measured with high spatial-resolution by the oxygen microprofile method: Capabilities and limitations of the method. Limnol. Oceanogr. 28:749–756.

Risatti, J. B., W. C. Capman, and D. A. Stahl. 1994. Community structure of a microbial mat: The phylogenetic dimension. Proc. Natl. Acad. Sci. USA 91:10173–10177.

Rocap, G., D. L. Distel, J. B. Waterbury, and S. W. Chisholm. 2002. Resolution of Prochlorococcus and Synechococcus ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. Appl. Environ. Microbiol. 68:1180–1191.

Rocap, G., F. W. Larimer, J. Lamerdin, S. Malfatti, P. Chain, N. A. Ahlgren, A. Arellano, M. Coleman, L. Hauser, W. R. Hess, Z. I. Johnson, M. Land, D. Lindell, A. F. Post, W. Regala, M. Shah, S. L. Shaw, C. Steglich, M. B. Sullivan, C. S. Ting, A. Tolonen, E. A. Webb, E. R. Zinser, and S. W. Chisholm. 2003. Genome divergence in two Prochlorococcus ecotypes reflects oceanic niche differentiation. Nature 424:1042–1047.

Rook, G. A. W., and J. L. Stanford. 1998. Give us this day our daily germs. Immunol Today 19:113–116.

Ruby, E.G., and K. H. Lee. 1998. The Vibrio fischeri Euprymna scolopes light organ association: Current ecological paradigms. Appl. Environ. Microbiol. 64:805–812.

Ruby, E. G. 1999. The Euprymna scolopes-Vibrio fischeri symbiosis: A biomedical model for the study of bacterial colonization of animal tissue. J. Molec. Microbiol. Biotechnol. 1(1):13–21.

Sassen, R., H. H. Roberts, P. Aharon, J. Larkin, E. W. Chinn, and R. Carney. 1993. Chemosynthetic bacterial mats at

cold hydrocarbon seeps, Gulf of Mexico continental slope. Organ. Geochem. 20:77–85.

Schaechter, M. 2003. Integrative microbiology: The third golden age. J. Biosci. 28:149–154.

Schink, B. 1988. Principles and limits of anaerobic degradation: Environmental and technological aspects. *In:* A. J. B. Zehnder (Ed.) Biology of Anaerobic Microorganisms. John Wiley. New York, NY. 771–846.

Schramm, A., S. K. Davidson, J. A. Dodsworth, H. L. Drake, D. A. Stahl, and N. Dubilier. 2003. Acidovorax-like symbionts in the nephridia of earthworms. Environ. Microbiol. 5:804–809.

Schulz, H. N., and B. B. Jørgensen. 2001. Big bacteria. Ann. Rev Microbiol. 55:105–137.

Seckbach, J. 2002. Symbiosis: Mechanisms and model systems. *In:* J. Seckbach (Ed.) Cellular Origin and Life in Extreme Habitats. Kluwer Academic Publishers. Norwell, MA.

Sekiguchi, Y., Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada. 1999. Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl. Environ. Microbiol. 65:1280–1288.

Short, S. M., B. D. Jenkins, and J. P. Zehr. 2004. Spatial and temporal distribution of two diazotrophic bacteria in Chesapeake Bay. Appl. Environ. Microbiol. 70:2186–2192.

Simek, K., P. Kojecka, J. Nedoma, P. Hartman, J. Vrba, and J. R. Dolan. 1999. Shifts in bacterial community composition associated with different microzooplankton size fractions in a eutrophic reservoir. Limnol. Oceanogr. 44:1634–1644.

Simek, K., J. Armengol, M. Comerma, J. C. Garcia, P. Kojecka, J. Nedoma, and J. Hejzlar. 2001a. Changes in the epilimnetic bacterial community composition, production, and protist induced mortality along the longitudinal axis of a highly eutrophic reservoir. Microb. Ecol. 42:359–371.

Simek, K., J. Pernthaler, M. G. Weinbauer, K. Hornak, J. R. Dolan, J. Nedoma, M. Masin, and R. Amann. 2001b. Changes in bacterial community composition and dynamics and viral mortality rates associated with enhanced flagellate grazing in a mesoeutrophic reservoir. Appl. Environ. Microbiol. 67:2723–2733.

Simek, K., K. Hornak, M. Masin, U. Christaki, J. Nedoma, M. G. Weinbauer, and J. R. Dolan. 2003. Comparing the effects of resource enrichment and grazing on a bacterioplankton community of a mesoeutrophic reservoir. Aquat. Microb. Ecol. 31:123–135.

Small, A. L., and M. J. McFall-Ngai. 1999. Halide peroxidase in tissues that interact with bacteria in the host squid Euprymna scolopes. J. Cell. Biochem. 72:445–457.

Sommaruga, R., I. Obernosterer, G. J. Herndl, and R. Psenner. 1997. Inhibitory effect of solar radiation on thymidine and leucine incorporation by freshwater and marine bacterioplankton. Appl. Environ. Microbiol. 63:4178–4184.

Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace. 1984. Analysis of hydrothermal vent-associated symbionts by ribosomal RNA sequences. Science 224:409–411.

Stahl, D. 1993. The natural history of microorganisms. ASM News 59:609–613.

Staley, J. T., and J. J. Gosink. 1999. Poles apart: Biodiversity and biogeography of sea ice bacteria. Ann. Rev. Microbiol. 53:189–215.

Stougaerd, J. 2000. Regulators and regulation of legume root nodule development. Plant Physiol. 124:531–539.

Streams, M. E., C. R. Fisher, and A. FialaMedioni. 1997. Methanotrophic symbiont location and fate of carbon incorporated from methane in a hydrocarbon seep mussel. Mar. Biol. 129:465–476.

Strom, S. L. 2000. Bactivory: Interactions between bacteria and their grazers. *In:* D. L. Kirchman (Ed.) Microbial Ecology of the Oceans. Wiley-Liss. New York, NY. 351–386.

Sullivan, J. T., and C. W. Ronson. 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Natl. Acad. Sci. USA 95:9059–9059.

Suttle, C. A. 2002. Community structure: Viruses. *In:* C. J. Hurst, R. L. Crawford, G. R. Knudsen, M. J. McInerney, and L. D. Stetzenbach (Eds.) Manual of Environmental Microbiology. ASM Press. Washington, DC. 364–370.

Suzuki, M. T., and S. J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62:625–630.

Taillefert, M., B. J. MacGregor, J. F. Gaillard, C. P. Lienemann, D. Perret, and D. A. Stahl. 2002. Evidence for a dynamic cycle between Mn and Co in the water column of a stratified lake. Environ. Sci. Technol. 36:468–476.

Tamas, I., L. Klasson, B. Canback, A. K. Naslund, A. S. Eriksson, J. J. Wernegreen, J. P. Sandstrom, N. A. Moran, and S. G. E. Andersson. 2002. 50 million years of genomic stasis in endosymbiotic bacteria. Science 296:2376–2379.

Tanaka, T., and F. Rassoulzadegen. 2004. Vertical and seasonal variations of bacterial abundance and production in the mesopelagic layer of the NW Mediterranean Sea: Bottom-up and top-down controls. Deep Sea Res. I. Oceanogr. Res. Pap. 51:521–544.

Taroncher-Oldenburg, G., E. M. Griner, C. A. Francis, and B. B. Ward. 2003. Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl. Environ. Microbiol. 69:1159–1171.

Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittmann, and D. A. Stahl. 1994. Evolutionary relationships among ammonia-oxidizing and nitrite-oxidizing bacteria. J. Bacteriol. 176:6623–6630.

Teske, A., N. B. Ramsing, K. Habicht et al. 1998. Sulfate-reducing bacteria and their activities in cyanobacterial mats of Solar Lake (Sinai, Egypt). Appl. Environ. Microbiol. 64:2943–2951 .

Teske, A., K. U. Hinrichs, V. Edgcomb, A. D. V. Gomex, D. Kysela, S. P. Sylva, M. L. Sogin, and H. W. Jannasch. 2001. Microbial diversity of hydrothermal sediments in the Guaymas Basin: Evidence for anaerobic methotrophic communities. Appl. Environ. Microbiol. 68:1994–2007.

Thamdrup, B., and D. E. Canfield. 1996. Fate of elemental sulfur in an intertidal sediment. FEMS Microbiol. Ecol. 19:95–103.

Thar, R., and T. Fenchel. 2001. True chemotaxis in oxygen gradients of the sulfur-oxidizing bacterium Thiovulum majus. Appl. Environ. Microbiol. 67:3299–3303.

Torsvik, V., J. Goksoyr, and F. L. Daae. 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. 56:782–787.

Torsvik, V., and L. Ovreas. 2002a. Microbial diversity and function in soil: From genes to ecosystems. Curr. Opin. Microbiol. 5:240–245.

Torsvik, V., L. Ovreas, and T. F. Thingstad. 2002b. Prokaryotic diversity: Magnitude, dynamics, and controlling factors. Science 296:1064–1066.

Turley, C. M., and P. J. Mackie. 1995. Bacterial and cyanobacterial flux to the deep NE Atlantic on sediment particles. Deep Sea Res. I Oceanogr. Res. Pap. 42:1453–1474.

Turner, J. T. 2002. Zooplankton fecal pellets, marine snow and sinking phytoplankton blooms. Aquat. Microb. Ecol. 27:57–102.

Van Hannen, E. J., W. Mooij, M. P. van Agterveld, H. J. Gons, and H. J. Laanbroek. 1999a. Detritus-dependent development of the microbial community in an experimental system: Qualitative analysis by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 65:2478–2484.

Van Hannen, E. J., G. Zwart, M. P. van Agterveld, H. J. Gons, J. Ebert, and H. J. Laanbroek. 1999b. Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. Appl. Environ. Microbiol. 65:795–801.

Van Loosdrecht, M. C. M., J. Lyklema, W. Norde, and A. J. B. Zehnder. 1990. Influence of interfaces on microbial activity. Microbiol. Rev. 54:75–87.

Vivas, E. I., and H. Goodrich-Blair. 2001. Xenorhabdus nematophilus as a model for host-bacterium interactions: rpoS is necessary for mutualism with nematodes. J. Bacteriol. 183:4687–4693.

Walls, J. T., A. J. Blackman, and D. A. Ritz. 1995. Localization of the amathamide alkaloids in surface bacteria of Amathia-Wilsoni Kirkpatrick, 1888 (Bryozoa, Ctenostomata). Hydrobiologia 297:163–172.

Ward, D. M., R. Weller, and M. M. Bateson. 1990. 16S ribosomal RNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345:63–65.

Waterbury, J. B., C. B. Calloway, and R. D. Turner. 1983. A cellulolytic nitrogen-fixing bacterium cultured from the gland of Deshayes in shipworms (Bivalvia, Teredinidae). Science 221:1401–1403.

Weis, V. M., A. L. Small, and M. J. McFall-Ngai. 1996. A peroxidase related to the mammalian antimicrobial protein myeloperoxidase in the Euprymna-Vibrio mutualism. Proc. Natl. Acad. Sci. USA 93:13683–13688.

Wellington, E. M. H., A. Berry, and M. Krsek. 2003. Resolving functional diversity in relation to microbial community structure in soil: Exploiting genomics and stable isotope probing. Curr. Opin. Microbiol. 6:295–301.

Werren, J. H. 1997. Biology of Wolbachia. Ann. Rev. Entomol. 42:587–609.

West, N. J., and D. J. Scanlan. 1999. Niche-partitioning of Prochlorococcus populations in a stratified water column in the eastern North Atlantic Ocean. Appl. Environ. Microbiol. 65:2585–2591.

Whitaker, R. J., D. W. Grogan, and J. W. Taylor. 2003. Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301:976–978.

Whitby, C. B., G. Hall, R. Pickup, J. R. Saunders, P. Ineson, N. R. Parekh, and A. McCarthy. 2001. ^{13}C incorporation into DNA as a means of identifying the active components of ammonia-oxidizer populations. Lett. Appl. Microbiol. 32:398–401.

Whiteley, M., M. G. Bangera, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg. 2001. Gene expression in Pseudomonas aeruginosa biofilms. Nature 413:860–864.

Wilcox, J. L., H. E. Dunbar, R. D. Wolfinger, and N. A. Moran. 2003. Consequences of reductive evolution for gene expression in an obligate endosymbiont. Molec. Microbiol. 48:1491–1500.

Williams, Le B. 2000. Heterotrophic bacteria and the dynamics of dissolved organic material. *In:* D. L. Kirchman (Ed.) Microbial Ecology of the Oceans. Wiley-Liss. New York, NY. 153–200.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Woese, C. R., O. Kandler, and M. L. Wheelis. 1990. Towards a natural system of organisms: Proposal for the domains archaea, bacteria, and eucarya. Proc. Natl. Acad. Sci. USA 87:4576–4579.

Woese, C. R. 2000. Interpreting the universal phylogenetic tree. Proc. Natl. Acad. Sci. USA 97:8392–8396.

Wolin, M. J., and T. L. Miller. 1982. Interspecies hydrogen transfer: 15 years later. ASM News 48:561–565.

Wren, B. W. 2000. Microbial genome analysis: Insights into virulence, host adaptation and evolution. Nature Rev. Genet. 1:30–39.

Wright, T. D., K. L. Vergin, P. W. Boyd, and S. J. Giovannoni. 1997. A novel delta-subdivision proteobacterial lineage from the lower ocean surface layer. Appl. Environ. Microbiol. 63:1441–1448.

Xavier, K. B., and B. L. Bassler. 2003. LuxS quorum sensing: More than just a numbers game. Curr. Opin. Microbiol. 6:191–197.

Zal, F., E. Leize, F. H. Lallier, A. Toulmond, A. Van Dorsselaer, and J. J. Childress. 1998. S-sulfohemoglobin and disulfide exchange: The mechanisms of sulfide binding by Riftia pachyptila hemoglobins. Proc. Natl. Acad. Sci. USA 95:8997–9002.

Zengler, K., G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller. 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA 99:15681–15686.

Zuckerkandl, E., and L. Pauling. 1965. Molecules as documents of evolutionary history. J. Theoret. Biol. 8:357–366.

PART 2

Symbiotic Associations

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CHAPTER 2.1

Cyanobacterial-Plant Symbioses

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Introduction

Cyanobacteria are unique in the wide range of symbiotic associations they form with eukaryotic hosts including plants, fungi, sponges and protists (for reviews, see Adams [2000], Rai et al. [2000], Rai et al. [2002b], and Bergman et al. [2003]). Cyanobacteria are photoautotrophs, and in many cases facultative heterotrophs and nitrogen fixers, and can provide nonphotosynthetic hosts with both nitrogen and carbon. Even if the benefit to the host is clear, that to the cyanobacteria is less obvious. They often receive carbon from photosynthetic hosts, but they are capable of carbon fixation themselves. Perhaps a more likely advantage is protection from predation and from environmental extremes, such as high light intensity and desiccation, in the enclosed environment provided by the host.

The cyanobacterial symbionts of plants all possess at least two essential characteristics—the ability to differentiate heterocysts, which are specialized nitrogen-fixing cells (for a review, see Adams and Duggan, 1999), and hormogonia, which are short, gliding filaments that lack heterocysts and provide a means of dispersal (Campbell and Meeks, 1989; Meeks, 1990; Meeks, 1998; Johansson and Bergman, 1994; Bergman et al., 1996). The hormogonia serve as the infective agents in most plant symbioses; some plants enhance their chances of infection by producing chemical signals that stimulate hormogonia formation and also chemoattractants that direct hormogonia into the plant tissue. Cyanobacteria are not restricted to the roots of plants but can infect thalli, stems and leaves. The major hosts are bryophytes (see the section Cyanobacteria in Symbiosis with Hornworts and Liverworts in this Chapter), the angiosperm *Gunnera* (see the section Interactions in the Nostoc-Gunnera Symbiosis in this Chapter), the aquatic fern *Azolla* (see the section The Azolla-Anabaena Symbiosis in this Chapter), fungi (forming lichens; see the section Cyanolichens in this Chapter), the fungus *Geosiphon* (see the section The Geosiphon Pyriformis—Nostoc Endocyanosis and its Relationship to the Arbuscular Mycorrhiza (AM) in this Chapter), and cycads (for a review of cycad symbioses, see Costa and Lindblad, 2002).

SECTION I: Cyanobacteria in Symbiosis with Hornworts and Liverworts

Bryophyte Symbioses

The division Bryophyta consists of the Hepaticae (liverworts), the Anthocerotae (hornworts), and the Musci (mosses), all of which are small, nonvascular terrestrial plants, some of which form epiphytic or endophytic associations with cyanobacteria, primarily of the genus *Nostoc*. Only the liverwort and hornwort associations will be dealt with here, but the cyanobacteria-moss associations have been well reviewed recently (Solheim and Zielke, 2002). In their natural habitat the liverworts and hornworts grow as a prostrate gametophyte thallus a few centimeters in length, attached to the substratum by primitive roots known as "rhizoids." Mature symbiotic colonies can be seen as dark spots 0.5–1.0 mm in diameter within the plant tissue (Fig. 1). Of the more than 340 liverwort genera, only four are known to develop associations with cyanobacteria: two (*Marchantia* and *Porella*) forming epiphytic associations and two (*Blasia* and *Cavicularia*) forming endophytic associations (Meeks, 1990). Four of the six hornwort genera (*Anthoceros*, *Phaeoceros*, *Notothylas* and *Dendroceros*) form endophytic associations (Meeks, 1990). Although possibly more common than once thought, the epiphytic associations are poorly understood (Dalton and Chatfield, 1985; Brasell et al., 1986), whereas the endophytic associations have been well studied because of their ease of growth in the laboratory. *Anthoceros*, *Phaeoceros* and *Blasia* can all be grown conveniently in shaken liquid culture (Fig. 2), with or without their symbiotic partners, and can be readily reinfected with their original partner or with cyanobionts

Fig. 1. The liverwort *Blasia pusilla* showing the prominent midribs of the thallus surrounded by the dark spots of *Nostoc* colonies. From Adams (2000), with permission.

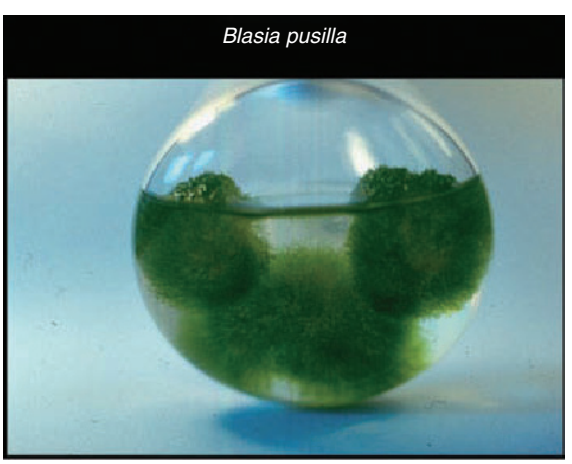

Fig. 2. *Blasia pusilla* grown in liquid medium and viewed from beneath a 250-ml Erlenmeyer flask. Three large masses of thallus are shown here, but for experimental purposes the thallus would be periodically fragmented to maintain a large number of smaller pieces. From Adams (2002), with permission.

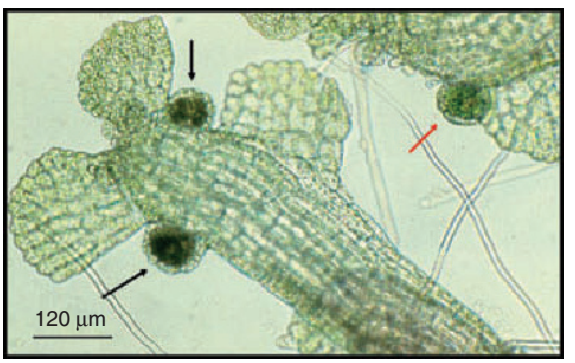

Fig. 3. Part of a *Blasia pusilla* thallus infected in the laboratory with two different cyanobacteria. The two darker colonies (black arrows) contain a different *Nostoc* strain from the paler one (red arrow). Photo courtesy of S. Babic. From Adams and Duggan (1999), with permission.

from *Gunnera*, cycads, lichens and even some free-living strains (Enderlin and Meeks, 1983; Meeks, 1988; Kimura and Nakano, 1990; Meeks, 1990; Babic, 1996; West and Adams, 1997; Adams, 2002; Fig. 3).

The Symbionts

For a cyanobacterium to establish a successful plant symbiosis it must possess at least two essential characteristics—the ability to differentiate both heterocysts, which are specialized nitrogen-fixing cells (for a review, see Adams and Duggan, 1999), and hormogonia, which are short, gliding filaments that lack heterocysts and provide a means of dispersal (Campbell and Meeks, 1989; Campbell and Meeks, 1990; Campbell and Meeks, 1998; Johansson and Bergman, 1994; Bergman et al., 1996). Heterocysts fix dinitrogen for both partners, and the motile hormogonia, which are a transient phase of the lifecycle, enable the otherwise immotile cyanobacterial filaments to gain entry to the plant host (see the section Bryophyte Structures and Their Infection in this Chapter). The symbiotically competent cyanobacteria are hormogonia-forming strains of mostly the genus *Nostoc*, although *Calothrix* and *Chlorogloeopsis* strains have been shown to reconstitute the symbiosis with *Blasia* and *Phaeoceros* (West and Adams, 1997). In the field, a single liverwort or hornwort thallus can become infected by many different *Nostoc* strains (West and Adams, 1997; West et al., 1999; Costa et al., 2001).

Hormogonia differentiation is triggered by environmental stimuli, including the dilution of liquid cultures, or their transfer to solid medium, or exposure to red light (Herdman and Rippka, 1988; Tandeau de Marsac, 1994). Their formation can also be triggered by exudates from plants

such as *Anthoceros* (Campbell and Meeks, 1989), *Blasia* (Knight and Adams, 1996), *Gunnera* (Rasmussen et al., 1994), and wheat roots (Gantar et al., 1993; Knight and Adams, 1996).

Bryophyte Structures and Their Infection

In the bryophyte-cyanobacteria symbioses, the symbionts infect existing plant structures. In the liverwort *Blasia*, the cyanobacteria occupy roughly spherical structures, known as auricles, on the underside of the thallus (Figs. 4 and 5). The thallus of the hornworts *Anthoceros* and *Phaeoceros* is much thicker than that of *Blasia* and the cyanobacteria are found in slime cavities, within the thallus, that open to the ventral surface via slit-like pores (Fig. 6). *Blasia* auricles have two slime papillae, one of which (the inner slime papilla) partly fills the auricle cavity, and the other (the outer slime papilla) arises from the thallus adjacent to the auricle (Figs. 4 and 5). The cyanobacteria enter *Blasia* auricles, and presumably hornwort slime cavities, as hormogonia (see the section on The Symbionts in this Chapter), whereupon they lose motility and differentiate heterocysts (Kimura and Nakano, 1990; Babic, 1996).

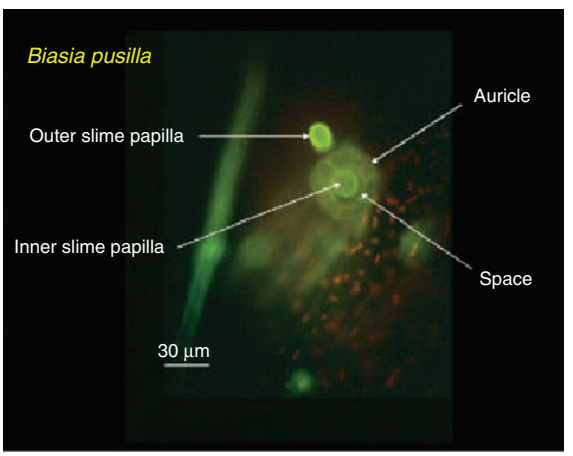

Fig. 4. Fluorescence photomicrograph of a *Blasia pusilla* auricle showing the inner and outer papillae. Sample stained with calcofluor white. Photo courtesy of S. Babic. From Adams (2000), with permission.

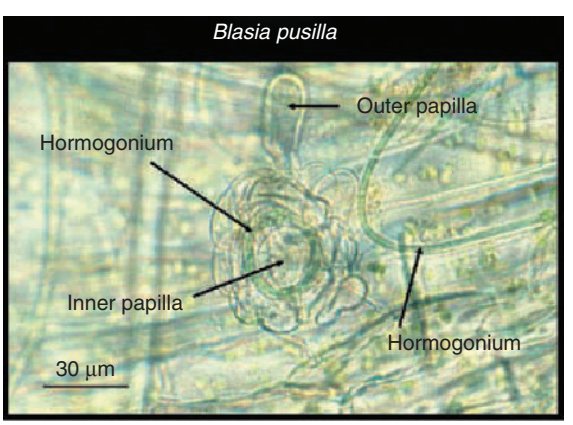

Fig. 5. Photomicrographs of a *Blasia pusilla* auricle infected with cyanobacteria in the laboratory. This auricle has just been infected by a hormogonium which is still motile (and hence out of focus) within the space between the inner papilla and the auricle wall. Another motile hormogonium is present just outside the auricle. Photo courtesy of S. Babic. From Adams (2002), with permission.

Bryophyte-Cyanobacterium Signal Exchange

Anthoceros punctatus releases an unidentified, low molecular mass, heat-labile product that stimulates hormogonia formation in *Nostoc* strains (Campbell and Meeks, 1989). This hormogonia-inducing factor (HIF) seems to be produced as a result of nitrogen starvation, as it is not present when the hornwort is cultured in medium containing excess NH_4^+. Compounds with similar activity to HIF are found in *Gunnera* stem gland mucilage (Rasmussen et al., 1994), wheat root exudates (Gantar et al., 1993), and *Blasia* exudates (Babic, 1996; Watts et al., 1999; Watts, 2001). Combined nitrogen-starved *Blasia* releases factors that trigger hormogonia formation (Babic, 1996) and serve as very effective chemoattractants (Knight and Adams, 1996; Watts et al., 1999; Watts, 2001).

As a symbiotic colony develops, filamentous protrusions grow from the host plant into the colony, possibly to enhance nutrient exchange between host and symbiont (see the section Morphological Modifications to Bryophyte and

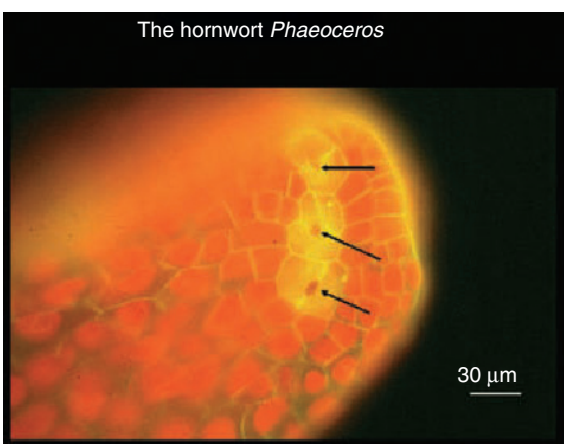

Fig. 6. Fluorescence photomicrograph of the hornwort *Phaeoceros* sp. showing the slit-like entrance (arrow) to the slime cavity. Sample stained with calcofluor white. Photo courtesy of S. Babic. From Adams (2000), with permission.

Symbiont in this Chapter). What signal induces these changes in the host is not known; however, arabinogalactan proteins (AGPs) are released by many cyanobacteria (Bergman et al., 1996), and such AGPs are thought to have important roles in plant growth and development (Pennell, 1992). Liverworts also produce AGPs (Basile, 1990), the inner and outer slime papillae of *Blasia* and the slime cavity of *Phaeoceros* stain with Yariv reagent, which is specific for AGPs, and with anti-AGP monoclonal antibodies (Watts, 2001).

Another group of potential signalling molecules in cyanobacteria-plant symbioses is the flavonoids; these are secreted by legumes and are involved in the initial signalling in the symbiosis with *Rhizobium*, by binding to the transcriptional activator NodD (Fisher and Long, 1992). Seed rinse from *Gunnera*, an angiosperm that forms symbiosis with *Nostoc*, can induce expression of *nod* genes in *Rhizobium* (Bergman et al., 1996; Rasmussen et al., 1996; Rai et al., 2000), and the flavonoid naringin induces expression of *hrmA* (see the sections Cell Division Control and Hormogonia Formation and The Hrm Operon in this Chapter) in *Nostoc punctiforme* (Cohen and Yamasaki, 2000). Expression of the *N. punctiforme hrmA* gene is also induced by a combination of components, including deoxyanthocyanins, found in extracts of the water-fern *Azolla* which forms symbioses with *Anabaena* (Cohen et al., 2002).

Host-Cyanobiont Interactions Post-Infection

Cell Division Control and Hormogonia Formation In symbiosis with *Anthoceros* the doubling time of *Nostoc* can be 240 h, compared with 45 h in the free-living state (Meeks, 1990). This slowed growth of the cyanobiont ensures that its growth rate matches that of the host plant. The mechanism of this growth control is unknown, but it seems not to be nitrogen limitation, even though the host takes most of the nitrogen fixed by its partner (see the section Nitrogen Fixation and Transfer of Fixed Nitrogen in this Chapter).

As well as controlling the growth rate of the cyanobiont, the host must control hormogonia formation. Prior to infection the host plant stimulates the development of hormogonia in potential partners by releasing HIF (see the section Bryophyte-Cyanobacterium Signal Exchange in this Chapter). However, once infection has occurred, the plant must prevent hormogonia differentiation within the developing colony because hormogonia lack heterocysts and so cannot form a viable, nitrogen-fixing colony. A hormogonia repressing factor (HRF), found in aqueous extracts of *Anthoceros* tissue (Cohen and Meeks, 1997; Meeks, 1998), inhibits HIF-induced hormogonia formation in wild-type *N. punctiforme*. The expression of two genes, *hrmA* and *hrmU*, is induced by HRF but not by HIF. These observations imply that the gene products of the *hrmUA* operon block hormogonium formation, perhaps by the production of an inhibitor or by the catabolism of an activator (Cohen and Meeks, 1997; see the section Genetic Analysis of the Nostoc-Anthoceros Association in this Chapter).

Morphological Modifications to Bryophyte and Symbiont In free-living cyanobacteria, heterocyst frequency is typically 4–10% of cells, whereas in symbiosis with hornworts and liverworts frequencies are usually considerably higher (Adams, 2000; Table 1). Although, in at least *Anthoceros*, some heterocysts seem to be senescent or dead (Meeks, 1990), increase in heterocyst frequency is still correlated with elevated rates of nitrogen fixation. Because heterocysts are unable to fix CO_2, this elevated heterocyst

Table 1. Summary of morphological and physiological changes in cyanobacteria symbiotically associated with hornworts and liverworts.

Characteristic	Hornworts	Liverworts
Plant structure infected	Slime cavities	Auricles
Cyanobiont	*Nostoc*	*Nostoc*[a]
Location of cyanobiont	Intercellular	Intercellular
Heterocyst frequency (%)[b]	30–50	30–50
Nitrogenase specific activity[d]	443	n.d.
Glutamine synthetase		
Amount of protein[c]	~86	n.d.
Specific activity[c]	~38	n.d.
Form of combined N released	NH_4^+	NH_4^+
Light-dependent CO_2 fixation (%)[d]	12	n.d.
Rubisco		
Amount of protein[d]	100	n.d.
Specific activity[d]	12	n.d.

Abbreviations: Rubisco, ribulose bisphosphate carboxylase/oxygenase; and n.d., not determined, though likely to be similar to hornwort data.

[a]The symbionts are *Nostoc* spp. in almost all cases; there have been rare reports of *Calothrix* spp. as symbionts (The Symbionts).

[b]Heterocyst frequencies are expressed as a percentage of total cells. Typical values for free-living cyanobacteria are 4–10%.

[c]Values are for the symbiont as a percentage of the same cyanobacterium in the free-living state.

[d]Values are expressed as a percentage of those for the free-living cyanobacteria.

From Steinberg and Meeks (1989, 1991), Meeks (1990), Rai (1990), and Bergman et al. (1992).

frequency results in a loss of CO_2-fixing capacity, which can be compensated by the supply of carbon skeletons by the host. In *Anthoceros*, and presumably all endophytic bryophyte associations, nitrogenase gene expression and heterocyst development in the symbiotically associated *Nostoc* appear to be controlled by plant signals (Campbell and Meeks, 1992).

Morphological changes are also observed in the bryophyte following infection. In both *Blasia* and *Anthoceros*, branched, multicellular filaments grow from the wall of the symbiotic cavity and invade the colony, increasing the surface area of contact between the cyanobacteria and the bryophyte (Rodgers and Stewart, 1974; Rodgers and Stewart, 1977; Duckett et al., 1977; Renzaglia, 1982; Kimura and Nakano, 1990; Gorelova et al., 1996). In *Blasia* these filaments are derived from the inner slime papilla and possess transfer cell morphology, implying an involvement in nutrient exchange.

Nitrogen Fixation and Transfer of Fixed Nitrogen The elevated rate of nitrogen (N_2) fixation in bryophyte-associated cyanobacteria broadly correlates with the increased heterocyst frequency in symbiosis (Table 1). The N_2 fixation rate of the *Anthoceros-Nostoc* association is 4- to 35-fold higher than that of free-living *Nostoc* (Steinberg and Meeks, 1991). Such a high rate of N_2 fixation cannot be supported by the reduced photosynthetic capacity of the cyanobiont and must rely on reduced carbon derived from the plant.

Nitrogen fixed by the cyanobiont is released to the plant as ammonia (Table 1) in both *Anthoceros* (Rodgers and Stewart, 1974; Stewart and Rodgers, 1977a; Meeks et al., 1985a; Meeks et al., 1985b) and *Blasia* (Rodgers and Stewart, 1974; Stewart and Rodgers, 1977a), and initial uptake of the ammonia occurs via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway of the host (Meeks et al., 1983; Meeks et al., 1985b; Meeks, 1990; Rai, 1990). In *Anthoceros* the cyanobiont retains as little as 20% of the nitrogen it fixes (Meeks et al., 1985a) yet shows no signs of nitrogen deprivation. Ammonia is released by the cyanobiont as a consequence of decreased activity of glutamine synthetase, the first enzyme in the GS-GOGAT pathway, which is the primary route of ammonia assimilation in cyanobacteria. In *Anthoceros* the decreased activity of GS appears to be the result of an undetermined posttranslational modification of the enzyme, because the amount of GS protein differs little in filaments of free-living and symbiotically-associated *Nostoc* (Joseph and Meeks, 1987; Lee et al., 1988; Meeks, 1990; Table 1).

Carbon Dioxide Assimilation and Transfer of Carbon The Calvin cycle is the primary route of CO_2 fixation in free-living and symbiotically-associated cyanobacteria, with ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) as the primary carboxylating enzyme (Tabita, 1994). The rate of light-dependent CO_2 fixation in the *Nostoc* symbiont of *Anthoceros*, immediately after its separation from symbiosis, is eightfold lower than that of the same cyanobacterium in the free-living state (Steinberg and Meeks, 1989; Meeks, 1990; Table 1). However, the level of Rubisco protein is similar in the two cases (Rai et al., 1989; Steinberg and Meeks, 1989; Meeks, 1990), implying that activity is regulated by an unidentified posttranslational modification of the enzyme (Steinberg and Meeks, 1989; Meeks, 1990). The cyanobiont therefore grows photoheterotrophically, receiving fixed carbon from its photosynthetic host, probably in the form of sucrose (Stewart and Rodgers, 1977a; Steinberg and Meeks, 1991). In at least *Anthoceros*, the presence of glycogen granules in the cells of symbiotically associated *Nostoc* implies that the symbiont is not starved of carbon (Meeks, 1990).

Genetic Analysis of the Nostoc-Anthoceros Association

Meeks and coworkers have developed genetic techniques, including transposon mutagenesis, for the analysis of the symbiotically competent cyanobacterium *Nostoc punctiforme* strain ATCC 29133 (Cohen et al., 1994; Cohen et al., 1998), and have used these techniques to identify a number of genes involved in the initial infection of *Anthoceros*. This has been aided by the availability of the complete genome sequence of *Nostoc punctiforme* {DOE Joint Genome Institute website} (see http://www.jgi.doe.gov).

The Hrm Operon In a transposon mutant of *Nostoc* 29133, characterized by an increased rate of initial infection of *Anthoceros* (Cohen and Meeks, 1997; Table 2), Meeks et al. (1999) identified two open reading frames (ORFs), *hrmU* and *hrmA*, flanking the site of transposition (Fig. 7). *hrmA* has no significant similarity to sequences in major databases, whereas *hrmU* has similarity to the sequences of mannonate oxidoreductase genes and 2-keto-3-deoxygluconate dehydrogenase genes. Expression of *hrmUA* is induced by an aqueous extract of *A. punctatus* but not by the hormogonium inducing factor, HIF. The aqueous extract appears to contain a hormogonium repressing factor (HRF) because it suppresses HIF-induced hormogonia forma-

Table 2. Effect of insertion mutations on the symbiotic infectiveness and effectiveness of *Nostoc* 29133 strains in association with *Anthoceros punctatus*.

Strain (gene)	nmol C_2H_2 reduced per min per: Colonies per mg dry wt per µg Chl *a*	g of fresh weight	Colony ($\times 10^{-3}$)	Gene induction factor(s)
ATCC 29133 (WT)	0.21	6.3	12.4	n.d.
UCD 328 (*hrmA*)	1.6	6.1	8.6	HRF
UCD 398 (*sigH*)	1.2	8.0	10.1	HIF
UCD 400 (*tprN*)	0.49	10.4	6.7	HIF and HRF

Abbreviations: Chl *a*, chlorophyll *a*; HRF, aqueous extract of *A. punctatus* containing hormogonium repressing factor identified as inducing the *hrm* operon; WT, wild type; HIF, exudate of *A. punctatus* containing hormogonium inducing factor; and n.d., not determined.
From Meeks et al. (1999). The standard deviations and number of replicates have been omitted for simplicity.

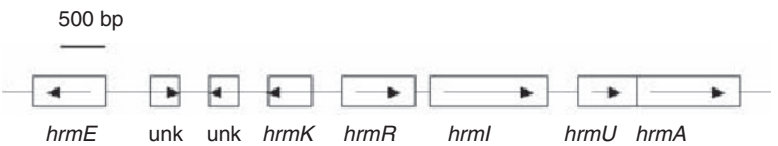

Fig. 7. Map of the open reading frames in the *hrm* locus of *Nostoc punctiforme*. The direction of transcription is indicated by the arrows. Unk are unknown proteins. Sizes are approximate. Adapted from Campbell et al. (2003).

tion in the wildtype but not the mutant. Whereas HIF is released into the growth medium, HRF is probably released into the symbiotic cavity, suppressing further hormogonium formation and permitting heterocyst differentiation.

At the 5′ end of *hrmUA*, three other ORFs (*hrmI*, *hrmR* and *hrmK*) are followed by two ORFs coding for unknown proteins, followed by *hrmE*, which has similarity to an aldehyde reductase (Fig. 7). HrmI shows similarity to uronate isomerase, HrmR to the LacI/GalR family of transcriptional repressors, and HrmK to gluconate kinases. HrmR is a DNA binding protein that binds sugar ligands and represses transcription of *hrmR* and *hrmE* (Campbell et al., 2003). Galacturonate abolishes in vitro binding of HrmR to DNA, implying that the in vivo inducer may be a sugar molecule similar to, or containing, galacturonate. These observations led Meeks and coworkers to propose the following model for the way in which the HRF external signal is transduced into *Nostoc*. HRF enters the *Nostoc* cell and it, or a derivative similar to galacturonate, binds to HrmR, rendering it incapable of binding to the *hrmR* and *hrmE* operator regions; this derepresses transcription of these genes, leading to inhibition of hormogonia formation (Campbell et al., 2003).

sigH AND *ctpH* Mutation of the *Nostoc* 29133 *sigH* gene, which encodes an alternative RNA polymerase sigma subunit, produces no obvious phenotype in filaments grown in medium with or without combined nitrogen but results in an increased infection phenotype when they are cocultured with *A. punctatus* (Campbell et al., 1998; Meeks et al., 1999; Table 2). Transcription of *sigH* is induced by *Anthoceros* HIF, but not by HRF, and *hrmA* transcription is not altered in a *sigH*$^-$ mutant. Thus, although the *hrmA*$^-$ and *sigH*$^-$ mutants both have an increased infection phenotype, it seems likely that increased infection has a different basis in the two strains (Meeks et al., 1999).

The gene *ctpH* lies immediately 5′ of *sigH* and encodes a protein with significant similarity to carboxy-terminal proteases of the cyanobacterium *Synechocystis* PCC 6803 (Meeks et al., 1999). In *Synechocystis* 6803, this gene is required for processing the carboxy-terminal portion of the photosystem II D1 protein in the thylakoid lumen (Anbudurai et al., 1994). However, in *Nostoc* 29133, *ctpH* seems to have a different physiological role because it is not transcribed under vegetative growth conditions, but transcription is induced by *Anthoceros* HIF. The significance of this is not understood.

tprN Lying 3′ of the gene *devR*, expression of which is essential for heterocyst maturation, is the gene *tprN*, which encodes a protein with similarity to tetratricopeptide repeat proteins (Campbell et al., 1996). These proteins have been studied primarily in eukaryotes in which they are required for a variety of functions from cell cycle control to transcription repression and protein transport (Lamb et al., 1995). Inactivation of *tprN* in *Nostoc* 29133 has no apparent phenotypic effect in the free-living growth state, but the mutant infects *Anthoceros* at about twice

the level of the wildtype (Table 2). Transcription of *tprN* occurs during vegetative growth but increases in the presence of both HIF and HRF (Meeks et al., 1999). The significance of this in the infection process is not known.

ntcA, *hetR*, AND *hetF* *Nostoc punctiforme* (*Nostoc* 29133) strains unable to develop heterocysts because of mutations in either *hetR* or *hetF* can still infect *Anthoceros* at a rate similar to that of the wildtype, despite being incapable of forming a functional nitrogen-fixing symbiosis (Wong and Meeks, 2002). *hetR* is thought to be the primary activator of heterocyst development (Wolk, 2000), and the HetF protein seems to be a positive activator of heterocyst differentiation, enhancing transcription of *hetR* and ensuring that HetR is localized to developing heterocysts (Wong and Meeks, 2001).

In cyanobacteria, NtcA functions as a nitrogen-dependent global regulator and controls the transcription of a number of genes, including *hetR* (Fiedler et al., 2001; Herrero et al., 2001). The *Nostoc punctiforme ntcA* mutant, UCD 444, forms motile hormogonia with wildtype morphology but at only 5–15% of the wildtype frequency (Wong and Meeks, 2002). However, rather than infecting *Anthoceros* at a reduced frequency, as might be expected, the *ntcA* mutant fails to infect at all. This noninfective phenotype can be complemented with copies of *ntcA*.

SECTION II: Interactions in the Nostoc-Gunnera Symbiosis

The Nostoc-Gunnera Symbiosis

Although cyanobacterial-plant symbioses are the most widespread of the nitrogen-fixing symbioses, with hosts throughout the plant kingdom, those symbioses with angiosperms (flowering plants) are presently restricted to one monogeneric family, the Gunneraceae. This contrasts with the more recently evolved rhizobia- or *Frankia*-angiosperm symbioses, which involve a considerably wider angiosperm host range. The scarcity is also unexpected as angiosperms form the ecologically most successful plant division on earth. In addition, cyanobacteria are globally widespread with a morphological variation surpassing most other prokaryotes. In spite of this, the cyanobacterial range is narrow, with only one cyanobacterial genus, *Nostoc*, functioning as microsymbiont in *Gunnera*. However, as the Gunneraceae is one of the oldest angiosperm families and with *Gunnera* and cyanobacterial fossils dating back some 90 million years ago (Ma) and 3 billion years ago (Ba), respectively, this symbiosis may have persisted for a long time. Prior to that, however, the same cyanobacterial genus may also have given rise to chloroplasts by entering other ancestral eukaryotic cells. The chloroplast genome of *Arabidopsis* is indeed more similar to that of *Nostoc* than to the unicellular cyanobacteria tested (Martin et al., 2002). As this ca. 400 million-year-old endosymbiotic event (or series of events) was the origin of all plants and algae, such endosymbiosis has, at least once, totally revolutionized our biosphere.

A UNIQUE ENDOSYMBIOSIS Although the symbiosis was first described by Reinke in 1873 (Reinke, 1873), understanding of the infection mechanism in this unique angiosperm symbiosis is incomplete. In contrast to the other cyanobacterial-plant symbioses, the *Gunnera* symbiosis is exclusively intracellular. Still, being a facultative symbiosis, the cyanobiont is easily separated from the plant and may be grown independently, and the symbiosis can be reconstituted under laboratory conditions. This makes the *Nostoc-Gunnera* symbiosis an excellent model for identifying mechanisms involved in plant endosymbioses and indirectly in plastid evolution. Also, since it is the only plant endosymbiosis, the symbiotic development in *Gunnera* may have evolved further than that in the other plant symbioses in which the cyanobacterium remains extracellular.

The Symbionts

The genus *Gunnera* was named by C. von Linné in honor of the Norwegian bishop Gunnérus, a person Linné admired. The approximately 30–50 *Gunnera* species are mostly subtropical to tropical perennial herbs, the exception being the smallest, *G. herteri*, which is annual (Wanntorp et al., 2001; Osborne and Sprent, 2002b). The *Gunnera* plants are composed of large compound spikes and are rhizomatous, or more seldom stoloniferous, and have rhubarb-like leaves. Plant sizes vary considerably; some are gigantic and may be the largest herbs on earth, such as species in South America, Hawaii and Asia, whereas others are small and creeping, such as the stoloniferous species in New Zealand. In nature, *Gunnera* spp. seem to be invariably infected by cyanobacteria (Wanntorp et al., 2001; Osborne and Sprent, 2002b).

Ever since the discovery of this peculiar symbiosis (Reinke, 1873), cyanobacteria of the genus *Nostoc*, which are filamentous and differentiate heterocysts, have been identified as the most frequent cyanobionts (see Meeks et al. [2001], Meeks and Elhai [2002], and Bergman et al. [2003]). The phenotypic range of the cyanobiont

of *Gunnera* is wide in terms of morphology, pigmentation, and colony shape and size, which is obvious when isolates are cultivated (Bergman et al., 1992; Rasmussen and Nilsson, 2002). A genotypic variation has also recently been verified using genetic fingerprinting of 45 cultured isolates originating from 11 *Gunnera* species (Nilsson et al., 2000; Rasmussen and Svenning, 2001) and natural cyanobacteria freshly collected from different *Gunnera* growing in Chile (Guevara et al., 2002). One specific *Gunnera* plant may also occasionally be infected with more than one *Nostoc* strain (Nilsson et al., 2000), while no variation within one plant was found in cyanobionts of *Gunnera* spp. sampled from the natural stands in Chile (Guevara et al., 2002) using the same fingerprinting technique. Recent 16S rRNA analyses also demonstrate that all *Gunnera* isolates examined belong to the genus *Nostoc* (Rasmussen and Svenning, 2001).

Specificity and Recognition Although all *Nostoc* strains form hormogonia per definition, still only certain strains of *Nostoc* are accepted as symbionts, which suggests the existence of other selective recognition mechanisms (see Rasmussen and Nilsson, 2002). The intracellular position of the cyanobiont in *Gunnera* may also impose more severe restrictions on the symbiotic partners than in the other intercellular, and possibly less intimate, plant symbioses. On the other hand, cyanobacterial isolates from cycads and bryophytes readily invade *Gunnera* cells and vice versa.

Host Structures and the Infection Process

The Site of Gunnera Infection—the Gland Infection occurs via a peculiar bright red gland (Fig. 8), already clearly visible at the developing cotyledon (see Bergman [2002a], Bergman and Osborne [2002b], and Bergman et al. [2003]).

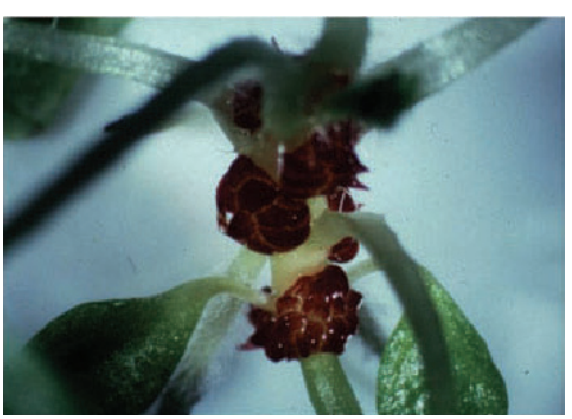

Fig. 8. *Gunnera* seedling with red stem glands.

The glands secrete a carbohydrate rich mucilage (Fig. 9) when noninfected, and new glands continuously develop at the base of each new leaf petiole, i.e., near the growing stem apices, which also become covered by the mucilage. Cyanobacterial infected glands are closed and do not release mucilage. Although root primordia were earlier suggested to be the point of entry (Schaede, 1951), the present consensus is that glands are the sole cyanobacterial entry point (Silvester and McNamara, 1976b; Bonnett and Silvester, 1981; Towata, 1985; Johansson and Bergman, 1992). It has been proposed that these modified glands should be termed "nodules" (Silvester and McNamara, 1976b), and indeed a distinct and well-functioning symbiotic "organ," however restricted in time and space, develops below the gland surface on infection. Each gland, and possibly also each channel, functions as an independent infection unit, which would explain why several cyanobionts may be found inside one individual gland (Johansson and Bergman, 1992; Nilsson et al., 2000).

The involvement of other microorganisms in the establishment of the *Gunnera* symbiosis, as proposed by Towata (1985), is not likely; this can be demonstrated by, for instance, reconstitution experiments under sterile laboratory conditions (Silvester and McNamara, 1976b; Johansson and Bergman, 1992). In addition, some cells of the gland have heavy tannin depositions, which have been suggested to prevent the invasion of noncompatible or unwanted microorganisms (fungi and bacteria), which often reside together with cyanobacteria in the channel mucilage (Towata, 1985).

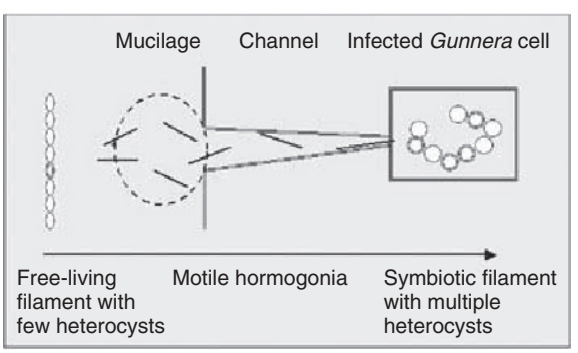

Fig. 9. Schematic illustration of the *Nostoc* infection process in *Gunnera*. Vegetative cells of *Nostoc* with a low frequency of heterocysts are attracted to the mucilage pouring out of the *Gunnera* stem and stolon gland. Motile hormogonia are induced by the mucilage and the cyanobacterium proceeds towards the interior of the gland. At the bottom of the channel the cyanobacterium penetrates *Gunnera* cells. After internalization, a cyanobacterial phenotype with larger cells and supernumerous heterocysts (up to 80%) develop. The arrow indicates the direction of infection.

THE INFECTION PROCESS The focus has so far primarily been on morphological and adaptive changes in the cyanobiont. The plasticity held by *Nostoc* in these respects, utilized by the plant throughout the infection process, is likely a key factor contributing to its success as a *Gunnera* symbiont. A typical feature of the *Nostoc-Gunnera* symbioses is the tight regulation by the plant of cyanobacterial cell division, cell differentiation, and physiological performance.

Hormogonium Differentiation A terrestrial cyanobacterium like *Nostoc* would (under normal free-living conditions) primarily occur as nonmotile, vegetative filaments with heterocysts at regular intervals (about 5–10% of the total cell number; Fig. 9). On contact with *Gunnera*, the gland and the plant apex are, however, soon covered by a cyanobacterial "biofilm" composed of tightly packed hormogonia (Osborne et al., 1991; Johansson and Bergman, 1992; Johansson and Bergman, 1994). Differentiation of these small-celled motile hormogonia is essential for the whole *Gunnera* infection process; they act as a means for the cyanobacterium both to reach and to invade the *Gunnera* organ (the gland; Fig. 9). The mucilage has a pivotal role during this process (Rasmussen et al., 1994). It is composed of highly glycosylated arabinogalactan proteins (AGPs; Rasmussen et al., 1996) and stimulates not only growth, but also hormogonium differentiation. A low molecular weight (<12 kDa), heat-labile protein, not yet characterized, which acts as hormogonium inducing factor (HIF), has been identified in the mucilage (Rasmussen et al., 1994). Molecular mechanisms behind the induction and differentiation are still largely unexplored.

Preliminary studies, using subtractive hybridization and proteomics (two-dimensional [2-D] gel electrophoresis coupled to mass spectrometry) of soluble *Nostoc* proteins treated with *Gunnera* mucilage, show that the induction of hormogonium differentiation is also reflected in a differential expression of genes and proteins, whose expression is either up- or down-shifted or both. For instance, three mucilage induced *hie* (host induced expression) genes have been identified, including a putative precursor of a pheromone-like signaling peptide (HieA), an outer membrane or secreted glycoprotein (HieB), and a protein probably involved in adaptation to acidity (HieC; Liaimer et al., 2001). The latter may be important as the *Gunnera* mucilage has a pH of 4–5 (Rasmussen et al., 1994). Another set of proteins was also identified as being differentially expressed in hormogonia (Klint et al., 2003). These proteins, which were predominantly surface associated, may have roles in motility, recognition, adhesion, as well as in communication with host plants. The mucilage therefore appears to have important functions at earlier stages of the *Gunnera* infection process.

Entrance and Penetration The *Gunnera* glands are composed of a set of up to nine papillae surrounding a central papilla (Johansson and Bergman, 1992; Uheda and Silvester, 2001). Between the papillae, deep invaginations lead into the stem tissue, through which the mucilage is released. The hormogonia use these narrow channels to enter the dark interior of the *Gunnera* stems (Fig. 9). As this is against the normal positive phototactic behavior of *Nostoc*, a potent attractant must be released by the plant, possibly carried by the mucilage. Motility is crucial at this stage, as the direction of infection (Fig. 9) is opposite to that of the flow of mucilage. Upon reaching the bottom of the gland channels, the cyanobacterium penetrates the thin walls of smaller meristematic and dividing cells lining the channel (Silvester and McNamara, 1976b; Johansson and Bergman, 1992; Johansson and Bergman, 1994; Uheda and Silvester, 2001). A delimited tissue of *Nostoc*-infected *Gunnera* cells is formed within a few days of inoculation. The mechanism(s) involved in the actual host cell penetration is still unknown, although Towata (1985) suggested the occurrence of pectolytic or cellulolytic activities in the mucilage of *G. kaalensis*. Also lining the channel are the thick-walled secretory cells releasing the mucilage (Towata, 1985).

Signal Exchange Between the Cyanobacterium and the Host

Besides HIFs, the plant signals involved in hormogonium differentiation still await genetic identification and chemical characterization, as do the cellular response signaling cascades in *Nostoc*. In this context, a highly interesting question is whether the differentiation of hormogonia resulting from a biotic stimulus (such as *Gunnera* mucilage) triggers specific genes (such as those involved in "symbiotic competence") but not those triggered by any abiotic stimulus (such as red light). Also interesting are mechanisms involved in the initial rapid cell division and the machinery behind motility. All studies do, however, verify that the plant influences cyanobacterial morphology and behavior at all stages of the infection process, and that this includes several fundamental cyanobacterial processes such as growth, cell division, cell differentiation, ammonia assimilation, and phototactic behavior. The question is whether this is triggered by plant compounds or by the environment within the plant. For instance, the symbiotic tissue is low in

oxygen and light, which may have consequences for gene expression.

Another open question is to what extent the release of the auxin IAA (indole-3-acetic acid) by *Nostoc* (Sergeeva et al., 2000) acts as a signal or influences the development of the symbiotic *Gunnera* tissues. Indeed, cyanobacteria seem to have the potential to produce all major phytohormones (Liaimer and Bergman, 2003) and also to release "AGP-like" proteoglucans, which may also influence plant development (Bergman et al., 1996).

Host-Cyanobacterial Interactions Post Infection

Internalization of the cyanobiont elicits novel, dramatic modifications of cyanobacterial morphology and function (Fig. 9). Because hormogonia lack heterocysts, they are unable to fix nitrogen. Thus, the hormogonium stage is lethal under free-living conditions (unless combined nitrogen is available), and hence is, of necessity, transient. Redifferentiation into vegetative filaments with heterocysts occurs after 1–2 days. The maintenance of a continuous vegetative stage with heterocysts is a prerequisite for the symbiosis to persist as an efficient provider of combined nitrogen. Repression of hormogonium differentiation in *Gunnera* may be achieved by homologues to the hormogonium repressing factor(s) (HRFs) identified in the bryophyte symbiosis (see Meeks and Elhai, 2002).

When inside the *Gunnera* cells, the cyanobacterial cells enlarge and cell division is restricted (Söderbäck and Bergman, 1992b). In addition, the often shorter filaments remain surrounded by the host cell plasmalemma through the pinocytosis process. This membrane, like the peribacteroid membrane in *Rhizobium*-legume symbioses, acts as the barrier between the symbionts but allows exchange of metabolites. The *Gunnera* cells eventually become filled with cyanobacterial filaments, which soon start to differentiate an abnormally high frequency of heterocysts (Fig. 9). Once infection is complete, the host must tightly control cyanobiont growth to avoid being outgrown, and this may explain the enlargement of cyanobacterial cells so typical for the endosymbiotic stage (see Rai et al. [2000] and Bergman [2002a]).

The dramatic morphological transitions seen in *Nostoc* on entering *Gunnera* cells are also reflected in the transcription of genes (and the corresponding proteins) related to heterocyst differentiation and nitrogen fixation (see Table 1 in Bergman, 2002a). For instance, the expression of the *hetR* gene (the master gene for heterocyst differentiation) correlates positively with the increase in heterocyst frequency, as does the expression of the nitrogen-responsive transcription factor encoded by *ntcA*, whereas *nifH* expression is (as expected) already high close to the growing apex. By contrast, the expression of the *glnB* gene, encoding the signal transduction protein P_{II}, decreases along the same symbiotic profile (Wang et al., 2004). Such overexpression of both *hetR* and *ntcA* and the contrasting downregulation of *glnB* are features indicating important regulatory differences between the symbiotic and free-living life stages. The significance of these findings in relation to cyanobacterial cell differentiation and the establishment and maintenance of an efficient nitrogen-fixing cyanobacterial-plant symbiosis now needs to be explored further. Akinetes have never been detected, not even in older parts of the symbiosis (glands at the base of the rhizome or stolon), though *hetR* is suggested to be involved in akinete differentiation (Zhou and Wolk, 2002). This suggests that conditions in *Gunnera* are never harsh enough or that *hetR* expression for some reason is solely devoted to heterocyst differentiation in symbiosis.

Cross-sectioning of rhizomes of mature plants reveals the final outcome of the symbiosis (i.e., distinct and bright blue-green pigmented but restricted and delimited cyanobacterial colonies seen scattered in the rhizome or along the stolons; Osborne et al., 1991). However, the sites of infection comprise only a small proportion of the total plant biomass, particularly in the large species.

Nitrogen Fixation and the Transfer of Nitrogen

As with most other plant symbioses, the main function of the cyanobacterium in *Gunnera* is to cover the total combined nitrogen requirement of the host via nitrogen fixation (Silvester and Smith, 1969; Silvester, 1976a; Bonnett and Silvester, 1981; Osborne et al., 1992). The heterocysts act as the nitrogen-producing entities, holding all the nitrogenase (Söderbäck et al., 1990; Söderbäck, 1992a), and are capable of supporting the entire symbiosis with combined nitrogen. In addition, the cyanobacterium attains enhanced nitrogen fixation capacities compared to its free-living relatives (Silvester, 1976a; Bonnett and Silvester, 1981). This may be due to the high heterocyst frequency, or to an enhanced nitrogen starvation caused by the continuous N-drainage from the cyanobiont.

Up to 90% of the nitrogen fixed is exported from the cyanobacterium to the host (Silvester et al., 1996). This is likely to be due to downregulation of the glutamine synthetase protein levels, specifically in heterocysts, as well as activities

in symbiosis (Söderbäck, 1992a). As in most nitrogen-fixing plant symbioses, the nitrogen fixed is primarily released as NH_4^+ (Silvester et al., 1996). The *Nostoc*-infected *Gunnera* tissues are always well invested with vascular strands that facilitate exchange of metabolites such as nitrogen (see Fig. 8 in Bergman et al., 1992). Multiple vascular strands persist (polystele) in *Gunnera*, which may be reminiscent of an aquatic ancestry (Osborne et al., 1991). Stock and Silvester (1994) showed, using pulse chase labeling with ^{15}N, that the nitrogen fixed was efficiently transported from mature to young parts (with lower heterocyst frequencies) of *G. monoica* stolons, and that N-translocation occurs via the phloem.

Carbon Assimilation and the Transfer of Fixed Carbon As *Nostoc* inside the *Gunnera* cells is excluded from light, the host must supply the cyanobiont with fixed carbon via its photosynthesis. Hence, the cyanobiont must adapt to a heterotrophic mode of life to generate enough reductant and ATP to support the demanding nitrogen fixation process (Söderbäck and Bergman, 1992b; Söderbäck and Bergman, 1993; Black et al., 2002). Nevertheless, total pigment and ribulose-1,5-bisphosphate carboxylase levels remain constant along the developmental sequence, from young to old parts, although their values show a decrease if related to cell volume because cell volume increases considerably in older cells (Söderbäck and Bergman, 1992b). The high frequency of heterocysts also drastically diminishes the number of vegetative cells, but the use of gas chromatography with mass spectrometry (GC-MS) has shown that still only the vegetative cells are actively taking up C (Black et al., 2002). Following ^{14}C translocation in *Nostoc*-infected *Gunnera* stolons reveals that the *Nostoc*-infected tissues at the apex of *G. magellanica* stolons are particularly efficient sinks for newly fixed plant carbon (Söderbäck and Bergman, 1993). The phloem of *Gunnera* has the unusual capacity to contemporaneously transport N outwards and C inward towards the symbiotic tissue (Stock and Silvester, 1994). A tight interaction of nitrogen and carbon metabolism in the *Gunnera* symbioses is also suggested. *hetR* expression in symbiotically competent *Nostoc* (PCC 9229) is negligible in the absence of a carbon source in darkness but pronounced in the presence of exogenous fructose (Wouters et al., 2000).

Ecological Importance

Our understanding of the ecology and significance (e.g., as a nitrogen-fixer) of this ancient plant and its cyanobiont is still rudimentary. For a detailed review of the ecology of *Gunnera*, the reader is referred to Osborne and Sprent (2002b). The geographic range of *Gunnera* was considerably wider in the past when the climate was more favorable (Osborne et al., 1991; Osborne and Sprent, 2002b). Today, *Gunnera* typically grows in super-humid habitats and often at high elevations or on steep cliffs. The genus is found in all continents, except in Europe and polar regions (see Wanntorp et al. [2001] and Osborne and Sprent [2002b]). Some large *Gunnera* species were introduced into Europe as ornamental plants at the end of the ninteenth century, and eventually some plants escaped and became invasive, e.g., in western Ireland, the Channel Islands, and the Azores (Osborne et al., 1991; Osborne and Sprent, 2002b).

The genetics (*rbcL* and *rps*16 introns) of *Gunnera* plant species have recently been analyzed (Fig. 10). The large species in South America and

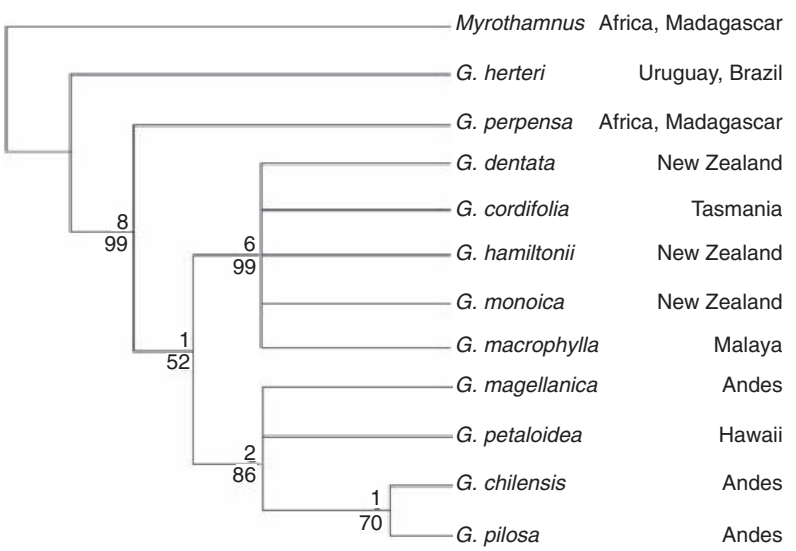

Fig. 10. Phylogram of 11 *Gunnera* species representing varying sizes and geographical origin. *Myrothamnus* is an African shrub-like plant growing in dry areas, as opposed to *Gunnera* species that prefer wet environments characterized by high humidity and high rainfall. From Wanntorp et al. (2001).

Hawaii distinctly group together in one clade, the often smaller species of New Zealand and Southeast Asia group in another, while *G. perpensa* (the first *Gunnera* to be described by Linné) and *G. herteri* (with the smallest size) are sister groups, representing Africa and Brazil, respectively (Wanntorp et al., 2001).

Conclusions

From a cyanobacterial perspective the *Nostoc-Gunnera* symbiosis may on the one hand seem wasteful; the cyanobiont merely functions as a N-producing entity with highly suppressed growth, and is possibly deprived of generating a new generation of cyanobionts. On the other hand, it may be beneficial; the cyanobiont no doubt extends its ecological niche to also include symbiotically competent cells of an angiosperm. In this way, the cyanobacterium not only gains access to plant leaves and roots and their nutrient acquisition capacities, but it also finds shelter from all possible predators, being the sole organism in this "golden cage."

The data obtained so far clearly show that cyanobacterial morphology and protein and gene expression patterns are drastically affected prior to, during, and after the establishment of the *Nostoc-Gunnera* symbiosis, although no symbiosis-specific genes and proteins, equivalent to the *nod* genes and Nod-factors in the *Rhizobium*-legume symbioses, have yet been discovered. However, it seems logical to assume that equally advanced molecular mechanisms must persist in a cyanobacterial-angiosperm endosymbiosis to generate this well coordinated and successful interaction.

SECTION III: The *Azolla-Anabaena* Symbiosis

Introduction

Taxonomy and Distribution The *Azolla-Anabaena* symbiosis is a mutualistic association among the aquatic fern *Azolla*, the filamentous, heterocystous, nitrogen-fixing cyanobacterium *Anabaena*, and endosymbiotic bacteria. The genus *Azolla* contains seven extant species that are divided into two sections on the basis of spore morphology. Section *Azolla* (New World species) includes *A. caroliniana*, *A. mexicana*, *A. filiculoides*, *A. microphylla* and *A. rubra*. Section *Rhizosperma* (Old World species) includes *A. pinnata* and *A. nilotica*. Geographically, *A. pinnata* is found in Australia, New Zealand, Japan, Asia and Africa, and *A. nilotica* is primarily found in Africa (Saunders and Fowler, 1993). Species from the section *Azolla* are more widely distributed around the world and are found in Europe, Asia, Africa, Australia and America. However, the distribution of *A. rubra* is restricted to New Zealand and Australia (Large and Braggins, 1993). The distribution of some species has been impacted by human effects (Janes, 1998a).

Morphology The plant's shape, color and size change significantly under different growth conditions (Janes, 1998b). The rhizome is branched, bearing alternate leaves that are bilobed. The ventral lobe is transparent and serves to float the plant on the surface of the water, whereas the dorsal photosynthetic lobe contains a leaf cavity in which the symbionts are found. The roots are adventitious. The shedding of roots and branches is related to environmental and physiological factors and enables the plant to reproduce via vegetative fragmentation. Factors affecting the growth of *Azolla* include genotype, temperature, light (intensity, quality and photoperiod), water chemistry (including pH, salinity and nutrients), and influence of pests and diseases (see Singh and Singh, 1997).

General Characteristics The association has been most frequently used as an alternative nitrogen fertilizer in rice fields, as well as a supplemental animal fodder. *Azolla* provides the cyanobiont with nutrients, including fixed carbon, and the cyanobiont provides the host with combined nitrogen (via nitrogen fixation). The exact role of the endosymbiotic bacteria in the association remains unclear; however, some possible functions have been suggested.

The Symbionts

Cyanobacterial Symbionts

Identification The filamentous, heterocystous, nitrogen-fixing cyanobacterial symbionts in the *Azolla* association have been extensively studied using both traditional and modern molecular techniques. In addition to characterization of the cyanobionts after they have been directly extracted from the association, there have been a number of studies in which cyanobacteria were isolated and cultured, in attempts to study the cyanobacterial symbionts in a free-living state. While numerous researchers have reported success in isolation and cultivation of the symbiotically associated cyanobacteria (Newton and Herman, 1979; Tel-Or et al., 1983; Gebhardt and Nierzwicki-Bauer, 1991; see Braun-Howland and Nierzwicki-Bauer, 1990), molecular studies (primarily based on restriction fragment length polymorphism [RFLP] analyses) have indicated

that none of the isolates represent the major cyanobacterial symbionts in the association (Gebhardt and Nierzwicki-Bauer, 1991). Though not conclusively demonstrated, major as well as some minor cyanobacteria may be present in the association, with the more readily cultured cyanobacteria representing minor symbionts (Gebhardt and Nierzwicki-Bauer, 1991). The other possible explanation is that the isolates presumably obtained from the association are actually epiphytes. Regardless, based on molecular studies it has not been conclusively demonstrated that the major cyanobacterial symbiont from the association can be cultured in a free-living state.

Given the challenge of studying the cyanobacterial symbionts in a free-living state, direct molecular studies have been used for accurate identification. Restriction fragment length polymorphism (RFLP) analyses (Gebhardt and Nierzwicki-Bauer, 1991), polymerase chain reaction (PCR) fingerprinting (Zheng et al., 1999), random amplified polymorphic DNAs (RAPDs; Van Coppenolle et al., 1995), as well as fluorescence in situ hybridizations (FISH) (Bushnell, 1998) have been used to examine the identity of the symbiotic cyanobacteria (Fig. 11). Regardless of the approach used, the cyanobiont referred to as "*Anabaena azollae*" has in most instances been described as being somewhat related to *Anabaena* or *Nostoc* (Plazinski et al., 1990c; Gebhardt and Nierzwicki-Bauer, 1991). A recent study (Baker et al., 2003) using comparisons of sequences of the phycocyanin intergenic spacer and a fragment of the 16S rRNA gene, places the *Azolla* cyanobiont in the order Nostocales but in a separate group from *Anabaena* or *Nostoc*. Additionally, near full-length (1500 bp) 16S rRNA sequencing and phylogenetic analysis of major cyanobionts from a variety of *Azolla* species yielded similar results (Milano, 2004). In 1989, Komarek and Anagnostidis placed the *Azolla* cyanobiont in a revised genus named "*Trichormus*," on the basis of morphology (Komarek and Anagnostidis). This is not inconsistent with the most recent molecular based findings.

The taxonomy of the cyanobionts is generally in agreement with the taxonomy of the host plant (Plazinski, 1990a; Van Coppenolle et al., 1993; Zheng et al., 1999). These findings, taken in conjunction with the continuous maintenance of the symbiosis throughout the lifecycle of the plant (see the section The Infection Process in this Chapter), suggest co-evolution of the cyanobionts and the host plant.

Developmental Profile Along the Main Stem Axis The growth of the endophyte is coordinated with the growth of the plant. In the apical meristem and younger leaves, the cyanobacterial vegetative cells are smaller than in older leaves and undergo frequent cell divisions. Increases in leaf age are accompanied by a decrease in cell division and increased size of the cyanobacterial vegetative cells, as well as increased heterocyst frequencies (Hill, 1975). The number of heterocysts and nitrogen fixation rates varies in leaves of different ages, as well as in different *Azolla* species (Hill, 1977). Heterocyst frequencies can reach up to 20–30% of the cells within a filament in the symbiotically associated *Anabaena*. These are much higher than the typical 10% heterocyst frequency in free-living *Anabaena* species.

Bacterial Symbionts The presence of bacteria residing within the leaf cavity of *Azolla* has been recognized for many years (Carrapico, 1991), yet still unclear is the specific function(s) of most of the bacteria in this symbiosis. Initial attempts to

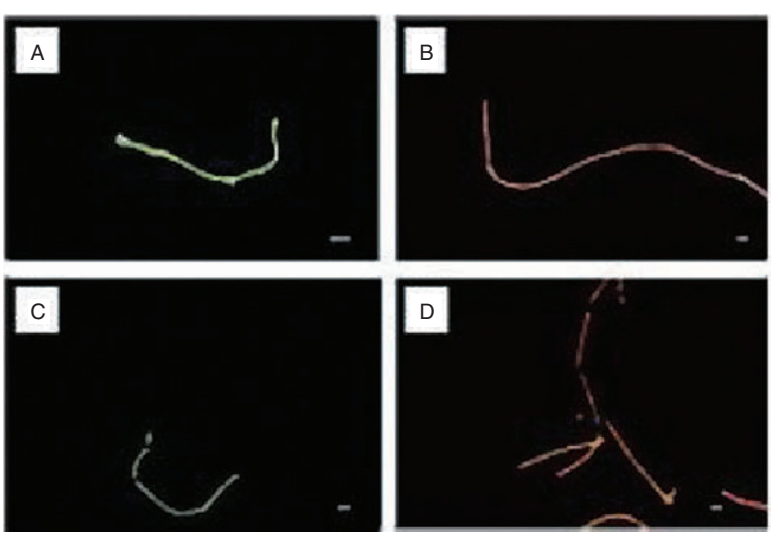

Fig 11. Fluorescent in situ hybridization (FISH) results, using Newton's isolate and different fluorescein isothiocyanate (FITC)-labeled 16S rRNA oligonucleotide probes, demonstrate that this isolate is not the same as the major cyanobiont of *A. caroliniana*. A) 16S rRNA universal probe, B) cyanobacterial group-specific probe (cells treated first with RNAse), C) Newton's isolate-specific 16S rRNA probe, and D) *A. caroliniana* major cyanobiont species-specific 16S rRNA probe. Bars represent approximately 25 μm (Bushnell, 1998).

study the symbiotic bacteria employed traditional microbiological, biochemical and physiological techniques for the identification of bacteria isolated from a variety of *Azolla* species. Utilizing these approaches there were many reports of *Arthrobacter* spp. (most frequently *A. globiformis*) occurring in symbiotic association with *Azolla* (Gates et al., 1980; Wallace and Gates, 1986; Forni et al., 1989; Forni et al., 1990; Nierzwicki-Bauer and Aulfinger, 1991; Shannon et al., 1993). *Agrobacterium* has also been reported to be isolated from different *Azolla* species (Plazinski et al., 1990b; Shannon et al., 1993; Serrano et al., 1999). Other bacteria, such as *Staphylococcus* sp., *Rhodococcus* spp., *Corynebacterium jeikeium* and *Weeksella zoohelcum* were identified by BIOLOG and API tests as being in association with *Azolla* (Serrano et al., 1999). A detailed review of the identification of bacteria isolated from *Azolla* species is provided in Lechno-Yossef and Nierzwicki-Bauer (2002b). Molecular techniques, in particular 16S rDNA gene amplification, cloning, screening, sequencing and phylogenetic analysis, have recently provided more detailed information on the identity of the symbiotic bacteria (Lechno-Yossef, 2002a; Milano, 2003). In the accessions studied, sequence similarity found that the most abundant bacterial symbionts in *A. caroliniana* and *A. filiculoides* were *Frateuria aurantia* and *Agrobacterium albertimagni*, and in *A. mexicana*, *Agrobacterium tumefaciens* (Lechno-Yossef, 2002a).

Host Structures and the Infection Process

The Leaf Cavity In the association, the symbionts reside in a leaf cavity, an extracellular compartment in the dorsal lobe of the leaf. In mature leaves, the symbionts (cyanobacteria and bacteria) are located in the periphery of the leaf cavity in mucilaginous material between internal (Nierzwicki-Bauer et al., 1989) and external envelopes (Uheda and Kitoch, 1991). Electron microscopic analysis combined with specific staining showed that the inner envelope does not have a tripartite structure typical of a membrane and is rich in lipids (Nierzwicki-Bauer et al., 1989). The external three-layered envelope is believed to contain cutinic and suberic substances, as revealed by response to chemical treatments of degradation using hot alkali methanol (de Roissart et al., 1994).

The adaxial epidermis of the leaf cavity contains a pore that is surrounded by two cell layers (Veys et al., 1999; Veys et al., 2000). One layer inside the pore is composed of teat-shaped cells that are extended from the adaxial epidermis. The other layer corresponds to the inner epidermis, which lines the inside of the cavity. Three to four tiers of teat cells form a cone-like pore with an average diameter at the base of 80 μm. The pore opening is larger in younger leaves, and the morphology of the teat cells suggests that their function is as a physical barrier to prevent particles and organisms from entering the cavity and the symbionts from exiting (Veys et al., 2002).

The Infection Process *Azolla* is a heterosporous water fern that is capable of both sexual and asexual reproduction. Unlike any of the other cyanobacterial symbioses, the host is in continual association with the symbionts. Thus, rather than reinfect *Azolla*, the symbionts retain coordinated growth in association with the host throughout its lifecycle. Descriptions of the processes involved in maintaining the continual association during sexual and asexual reproduction are described briefly below.

Sexual Reproduction Sporulation is the sexual reproduction process in *Azolla*. During sexual reproduction the host produces both mega- and microsporocarps. The partitioning of the cyanobacterial filaments into the developing sporocarps and the re-establishment of the symbiosis following embryogenesis in *A. mexicana* have been described (Perkins and Peters, 1993; Peters and Perkins, 1993). The symbionts that are used as inoculum to the developing sporocarps come from the dorsal lobe of the same leaf in which the sporocarps are developing.

The cyanobiont akinetes (which function as spores) and the bacterial symbionts (which do not always show ultrastructural characteristics of spore envelopes; Aulfinger et al., 1991) found in the megasporocarps are transferred to the developing spores and sporelings. After separation of the megasporocarp from the plant, part of the indusium is shed, and the proximal half becomes the indusium cap. The symbionts reside in a space called "the inoculation chamber" (Peters and Perkins, 1993), located between the indusium cap and the apical membrane of the megasporocarp. Following fertilization and the beginning of embryogenesis, the symbionts resume metabolic activity. With the assistance of cotyledonary hairs, the symbionts are introduced into the embryonic leaf, before it displaces the indusium cap (Peters and Perkins, 1993). Leaves, which grow from the meristem, are initially unlobed but contain a structure similar to the leaf cavity that contains the symbionts. As the frondling continues to grow, the symbionts are distributed into the developing leaf cavities by a mechanism similar to the transfer mechanism used during asexual reproduction via vegetative fragmentation (see the section Asexual Reproduction in this Chapter).

Asexual Reproduction The main form of reproduction in *Azolla* is vegetative fragmentation. The apical meristem of each branch contains a colony of undifferentiated cyanobacterial cells. Cyanobacterial filaments from the apical colony are introduced into the leaf primordium before the development of the leaf and leaf cavity are complete. The partitioning of the endophytes into the developing leaves is facilitated by entanglement around primary branched hair (PBH) cells of *Azolla* (Calvert and Peters, 1981). The leaf cavity starts to develop and engulf the cyanobacterial colony in the fourth or fifth leaf along the stem axis. In this way symbionts are inoculated into every leaf cavity that is formed. The development of the leaf cavity is also accompanied by the formation of simple hair cells by *Azolla* (Peters and Calvert, 1983).

"Artificial" In Vitro Infection of Cyanobacteria In sporulating *Azolla*, sexual hybridization between different *Azolla* species, as well as the formation of new combinations of *Azolla* and *Anabaena*, has been somewhat successful (Watanabe, 1994; Watanabe and Van Hove, 1996). For example, *Anabaena* from *A. microphylla* (MI4031) was successfully introduced into *A. filiculoides* (FI1034) by exchange of the indusium cap of the megaspore (Lin et al., 1989). Successful sexual hybridizations between *A. microphylla* (megasporocarp) and *A. filiculoides* (microsporocarp; Wei et al., 1988; Do et al., 1989), between *A. filiculoides* (megasporocarp) and *A. microphylla* (microsporocarp; Watanabe et al., 1993), and between *A. mexicana* and *A. microphylla* (Zimmerman et al., 1991) have also been reported. The key to these successes has been having the cyanobacteria at the appropriate stage of development (during akinete germination and vegetative cell growth) that mimics what naturally occurs in situ.

Host-Symbiont Signal Exchange

The recognition between *Azolla* and *Anabaena azollae* is facilitated by lectins in both the plant (Mellor et al., 1981) and the cyanobionts (Kobiler et al., 1981; Kobiler et al., 1982). Additionally, bacteria isolated from *A. pinnata* and *A. filiculoides* have been shown to contain lectins (Serrano et al., 1999). The presence of Rhizobiaceae symbionts in association with different *Azolla* species and cultures examined would suggest that this group of bacteria has a role in the symbiosis. Plazinski et al. (1991) showed that the *nodL* and *nodABC* genes gave hybridization signals to a plasmid and the chromosome of the isolate AFSR-1 from *A. filiculoides*. These authors suggest that the *nod* genes, if active in the bacterial symbionts of *Azolla*, play a regulatory role in the development of the symbiosis or in the maintenance of bacterial association with the plant.

Host-Cyanobiont Interactions Post-Infection

Morphological Modifications to Host and Cyanobacteria The leaf cavity and inner and external envelopes of *Azolla* do not appear to be present only when it is symbiotically associated with the cyanobionts. These structures are present in both *Anabaena*-free and *Anabaena*-containing *Azolla* (Nierzwicki-Bauer et al., 1989). This evidence excludes the involvement of the cyanobionts in the formation of these structures. However, given that the cyanobiont-free plants examined still contained symbiotic bacteria, a possible role of bacteria in the synthesis of the leaf cavity envelopes cannot yet be excluded.

Nitrogen Fixation and Transfer of Fixed Nitrogen Nitrogen fixation is carried out by the heterocysts of *Anabaena azollae*. In leaf cavities of different ages along the stem axis of *Azolla*, the heterocyst frequencies and nitrogen fixation rates vary. Nitrogen fixation, as determined by the acetylene reduction assay, occurs in the apical (younger) leaves but not in the stem apex, increases and reaches a peak in leaves of middle age, and then decreases in the older leaves (Canini et al., 1990). Ammonium, the product of nitrogen fixation, is released from heterocysts and assimilated by *Azolla* into glutamate using the glutamine synthetase (GS)-glutamate synthase (GOGAT) system (Peters and Calvert, 1983). Nitrogenous compounds in the form of glutamate, glutamine, ammonia and other glutamate derivatives are transferred from the mature leaf cavities to the stem apex (Peters et al., 1985). In *Anabaena azollae*, the activity and protein content of GS are only 5–10% of that of free-living *Anabaena* (Orr and Haselkorn, 1982). However, the nitrogen fixation activity is much higher because of the increased number of heterocysts. Some of the bacteria found in this association can fix nitrogen. Immunoelectron microscopy studies using antibodies against the Fe and FeMo protein subunits of nitrogenase revealed that a subset of the bacteria in the *A. caroliniana* and *A. filiculoides* associations contained these nitrogenase subunits (Lindblad et al., 1991). The potential nitrogen-fixing contribution of the bacteria in the association separate from that of cyanobacterial symbionts could not be measured because they coexist in the leaf cavities and, once removed, are likely to have altered capabilities.

Carbon Assimilation and Transfer of Fixed Carbon The cyanobiont, *Anabaena azollae*, has photosynthetic capabilities; however, in the symbiotic state it is believed to contribute less than 5% of the total CO_2 fixed in the association (Kaplan and Peters, 1988). Pulse-chase studies have shown that sucrose from the plant is supplied to and accumulated by the cyanobiont (Peters et al., 1985). Simple hair cells of *Azolla* are involved in the transport of sugars from the photosynthetic mesophyll cells to the leaf cavity. Simple hair cells have ATPase activity in their plasmalemma and some accumulation of starch in their chloroplasts (which do not possess ribulose 1,5 bisphosphate carboxylase, RuBisCO), suggesting active transfer of sugars from the simple hairs to the leaf cavity (Carrapico and Tavares, 1989). Additionally, primary branched hair cells, having the morphology of a transfer cell, are believed to be involved in nutrient transfer from the plant to *Anabaena* (Peters et al., 1985).

Ecological Importance—Friend or Foe?

The *Azolla-Anabaena* symbiosis is of tremendous ecological importance, having both positive and negative impacts. On the positive side, the association has been extensively used as a biofertilizer, providing a source of combined nitrogen in the form of ammonium, thereby reducing or eliminating the need for the addition of chemical fertilizers. This role has been most extensively used in conjunction with rice paddies or fertilization of fields. The growth of *Azolla* into thick mats also makes it effective in suppressing weed growth. Owing to its high protein content, *Azolla* is used as a fodder for sheep, pigs, ducks, etc. The ability of *Azolla* to remove nitrates and phosphorous from water has resulted in improvement of water quality. Additionally, *Azolla* has been used to remove heavy metals from water. Ten useful characteristics attributed to this association have been described (Van Hove and Lejeune, 1996; Lejeune et al., 1999), with the capacity to fix atmospheric nitrogen, high productivity, high protein content, and a depressive influence on both aquatic weeds and NH_3 volatilization, being considered unquestionably useful.

The same characteristic feature that makes *Azolla* useful for weed suppression and biofertilization of fields (namely, the ability to grow in thick mats) also results in a number of negative ecological impacts. For example, growth of *Azolla* mats in streams in Zimbabwe has been shown to have a negative impact on animal biodiversity (Gratwicke and Marshall, 2001). In many regions where *Azolla* is an invasive species it has overgrown many native species. In efforts to control *Azolla* growth, biological controls such as the introduction of a frond-feeding weevil (McConnachie et al., 2004) or the flea beetle (Hill and Oberholzer, 2002) are being explored. Thus, the overall ecological impact of the *Azolla-Anabaena* association continues to expand and may reach even to Mars, since *Azolla* is currently being used in studies examining possible bioregenerative life support on Mars (http://www.highmars.org/niac/niac04.html, The Caves of Mars Project website).

SECTION IV: Cyanolichens

Introduction

Lichens are associations of symbiotic fungi and green algae (bipartite lichens) or symbiotic fungi, green algae, and cyanobacteria (tripartite lichens). A lichen thallus is quite distinct in appearance from either of its symbionts, and its name refers to the dominating fungal partner (the mycobiont). Lichen thalli represent an integration of the mycobiont's heterotrophic metabolism and the autotrophic metabolism of the photosynthetic partners (the photobionts: green algae and cyanobacteria). In tripartite lichens, the cyanobacterial partner (the cyanobiont) is also referred to as the "secondary photobiont," whereas the green algal partner is referred to as the "primary photobiont." All lichens having a cyanobiont, either as the sole photobiont or as a secondary photobiont, are called "cyanolichens." For lack of space, this review on cyanolichens is brief. The reader can find further details in books and reviews elsewhere (Galun, 1988; Ahmadjian, 1993; Nash, 1996; Rai et al., 2002b; Rikkinen, 2002a). The journal *Bryologist* regularly lists recent literature on lichens, and a literature search is also possible at Mattick's Literature Index website (http://www.toyen.uio.no/botanisk/bot-mus/lav/sok_rll.htm).

There are approximately 1550 known species of cyanolichens, representing roughly 12–13% of all known lichens. Among these, two thirds are bipartite and the rest tripartite species. Lichen symbioses are thought to have arisen independently on several occasions. An estimated 100 lichenization events have occurred during diversification of extant fungi (Aptroot, 1998; see also Rikkinen, 2002a).

The Symbionts

Mycobionts The current classification of fungi is in transition, and molecular approaches are being used to fine-tune it (Tehler et al., 2000; see also Rikkinen, 2002a). Approximately 13,500

species of lichen-forming fungi presently belong mostly to the Ascomycetes (98%) and very few to the Basidiomycetes (1.6%) and Fungi Imperfecti (0.4%). About 15–18 Orders of Ascomycetes (nearly 130 genera from 50 families) include lichen-forming taxa (see Rikkinen, 2002a). Most are from two Orders, the Lecanorales and Lichinales. Nearly 1700 species of fungi associate with different types of cyanobacteria. A fairly comprehensive list of these has been provided earlier (Rikkinen, 2002a).

Cyanobionts A variety of heterocyst-producing and unicellular cyanobacteria occur as cyanobionts in cyanolichens where the mycobiont is an ascomycete. Among heterocystous forms, *Nostoc* is the most common. Others are *Scytonema*, *Calothrix*, *Dichothrix* and *Fischerella* (including *Hypomorpha*, *Stigonema* and *Mastigocladus*). Unicellular forms that occur as cyanobionts in cyanolichens include *Gloeocapsa* (also *Chroococcus*), *Gloeothece*, *Synechocystis* (also *Aphanocapsa*), *Chroococcidiopsis*, *Hyella* and *Myxosarcina* (see Rai et al. [2000] and Rikkinen [2002a]). The range of cyanobionts in cyanolichens where the mycobiont is a basidiomycete is rather limited. Only two cyanobacteria (*Chroococcus* and *Scytonema*) are reported as cyanobionts in basidiolichens (see Schenk, 1992).

In recent years, analyses of $tRNA^{Leu}$ (UAA) introns and 16S rDNA sequences have been used as genetic markers to study the diversity of *Nostoc* cyanobionts (Paulsrud and Lindblad, 1998a; Paulsrud et al., 1998b; Paulsrud et al., 2000; Paulsrud et al., 2001; Lohtander et al., 2002; Rikkinen et al., 2002b). These studies have shown that genetic variation among lichen-forming *Nostoc* strains is considerable. Within symbiotic *Nostoc* strains, there seem to be several subgroups. For example, one subgroup of *Nostoc* strains seems to occur only in epiphytic cyanolichens, whereas another includes strains that occur as cyanobionts in terricolous cyanolichens and other symbiotic systems (Rikkinen, 2002a; Rikkinen et al., 2002b).

The Lichen Thallus Lichen thalli have a stable and organized structure quite distinct from any of their symbionts. The thalli appear to be crustose (small lobes and scales; e.g., *Collema*), foliose (flat and dorsiventral lobes; e.g., *Peltigera*), or fruiticose (round or flat thalli, upright or hanging down from the substratum; e.g., *Stereocaulon*). In foliose or fruticose thalli, the fungal hyphae form an outer pseudoparenchymatous zone (the cortex) that covers or encloses a more loosely interwoven medulla. Within the thallus, the partners remain extracellular to each other and can be isolated and grown in culture, but the symbiosis is fairly stable in nature because of the balanced and synchronized growth and development of the symbionts. Thinner cell walls (less sheath material) and specialized hyphae and haustoria, showing transfer cell ultrastructure, enable close contact between the mycobiont and the cyanobiont. Since the bulk of the thallus consists of the heterotrophic mycobiont, the thallus interior is microaerobic (see Rai et al., 2000).

In bipartite lichens, cyanobionts either are dispersed throughout the thallus (e.g., *Collema*) or occupy a distinct layer below the upper cortex (e.g., *P. canina*). In tripartite lichens, the cyanobiont is located in cephalodia, which occur at the upper surface of the thallus (external cephalodia; e.g., in *P. aphthosa*) or inside the medulla (internal cephalodia; e.g., in *Nephroma arcticum*). In some cases internal cephalodia are found close to the lower surface of the thallus (e.g., in *P. venosa*). In tripartite lichens, direct contact between the cyanobiont and the phycobiont (green algal partner) is never direct.

Lichen symbioses perpetuate by direct transmission of the cyanobiont from one generation to the next, and as a result of the acquisition, by the mycobiont of fresh cyanobiont from the environment. For example, a lichen thallus can develop from propagules (phyllidia, isidia, soredia and hormocystangia) of a pre-existing thallus (direct transmission) or from fresh synthesis (fresh acquisition of cyanobiont from the environment). The former mode of transmission allows prolonged continuity of the partners. Similar modes of cyanobiont acquisition also apply to the development of cephalodia (see Rai et al., 2000).

Cyanobionts are essential for the formation of thalli or cephalodia in cyanolichens. They may stimulate thallus morphogenesis but do not determine the kind of thallus formed. The mycobiont determines the structure and chemistry of a cyanolichen. Different lichen fungi form different lichen thalli even if associating with the same cyanobiont (see Rai [1990], Rai et al. [2000], and Rai et al. [2002b]).

Because they are slow growing, the initiation and development of lichens is difficult to study in nature. Development of a lichen thallus afresh involves germination of the mycobiont spore, development of the hyphal mat, contact, recognition and acquisition of the cyanobiont, and structural-functional integration of the symbionts. While a thallus may result within months when starting from propagules, it takes years when starting from isolated partners. During laboratory synthesis of lichens, the partners initially form undifferentiated aggregates that later differentiate into thalli (see Rai et al., 2000). Fresh synthesis in nature may also start from myco-

biont hyphae that become detached and acquire fresh cyanobiont (Smith and Douglas, 1987).

Development of each cephalodium is a new event. External cephalodia develop on the main thallus by entrapment of a cyanobiont by hairs on the thallus surface, followed by involvement of medullary hyphae immediately below. Internal cephalodia may develop in a similar fashion starting with cyanobiont entrapment by cortical hyphae or rhizines. The cyanobiont, enmeshed by a thick layer of mycobiont, is pressed into the thallus where the cephalodium eventually develops. New cephalodia may develop from hormogonia released by earlier cephalodia (Stocker-Wörgötter, 1995), ensuring cyanobiont homogeneity among cephalodia of a thallus. In laboratory synthesis however, cephalodia developed by attachment of hyphae from primordia (containing cyanobiont and mycobiont) to the green thallus (Stocker-Wörgötter and Turk, 1994). The latter mode of cephalodia development, if prevalent in nature, should cause considerable heterogeneity among symbiont populations within a single thallus, but this is not the case. Occasional reports of different cyanobionts (see Rai, 1990) or different strains of a cyanobiont (Paulsrud et al., 2000) among cephalodia of a single thallus may, however, indicate instances of cephalodia development by capture of a fresh cyanobiont in some lichens. Entry of the cyanobiont for development of internal cephalodia is from the lower surface of the thallus, but occasionally, when the cyanobiont enters from above, the phycobiont layer is pressed deep into the medulla.

Recognition and Signal Exchange Between Partners For the right symbionts to enter into a lichen symbiosis, signal exchange must occur between the partners. Transformation of *Nostoc* colonies into the symbiotic state occurred without the necessity for direct contact with the mycobiont during resynthesis of *Peltigera praetextata* (Yoshimura and Yamamoto, 1991). This suggests that the substance responsible for *Nostoc* transformation may be a diffusible soluble substance from the mycobiont. The exact identity of such a substance is not known, but lichen-forming fungi do produce a large number of unique secondary metabolites and compounds, and their possible roles in signal exchange need to be investigated.

Lectins (glycoproteins) of mycobiont origin have been implicated in the recognition of the cyanobiont by a mycobiont (Rai, 1990; Kardish et al., 1991; Lehr et al., 1995; Lehr et al., 2000). Cyanobiont cell surfaces possess specific sugars, fimbrae (pili), and in some cases, lectins, which may have a role in recognition and adherence (Stewart et al., 1983; Kardish et al., 1991; see Rai et al., 2000).

Direct observations, lectin binding experiments and $tRNA^{Leu}$ intron analysis all indicate a broader cyanobiont-mycobiont specificity in lichens than that in other cyanobacterial symbioses. Different lichen species can have the same cyanobiont, and different cyanobionts have been reported among cephalodia of a single lichen thallus. Different *Nostoc* strains have been found in different lichen species from the same site, while different lichen species from distant places had the same *Nostoc* strain. In chimeroid thalli, both bipartite and tripartite morphotypes are reported to have the same cyanobiont strain (Paulsrud et al., 1998b; Paulsrud et al., 2000; Paulsrud et al., 2001). Overall, there is a great deal of cyanobiont diversity among the lichens, and much of it might be contributed by the mode of cyanobiont acquisition during the development of the lichen thallus and cephalodia (Rai et al., 2000).

Many cyanolichens share similar environmental requirements and may depend on a common pool of cyanobionts. Many cyanolichen species having identical cyanobiont strains co-occur in a particular habitat, forming characteristic communities or "guilds" (Rikkinen et al., 2002b). Within a guild, the cyanobionts of all lichens are closely related, but the mycobionts are not. While some guilds include different mycobiont genera or even families, some closely related mycobionts belong to different guilds (associate with different types of cyanobionts).

Structural-Functional Changes Cyanobionts undergo structural-functional changes in the symbiosis that permit a close interaction and development of nutrient exchange between the partners. These changes include increased cell size, altered cell shape, lack of polyphosphate reserves, fewer carboxysomes, less sheath material, and slower growth and cell division (Rai et al., 2000).

The cyanobionts are photosynthetically active and fix CO_2 via the C_3 pathway. In addition, there is a significant level of dark CO_2 fixation (15–20% of that in the light) via the C_4 pathway (Rai et al., 2000; Palmqvist, 2002). However, CO_2 fixation by cyanobionts in internal cephalodia, particularly those on the lower surface (e.g., *Peltigera venosa*), may be minimal due to low light and Rubisco. In the tripartite *Nephroma arcticum*, the *Nostoc* cyanobiont has 70% fewer carboxysomes compared with that in the bipartite *P. canina* (Bergman and Rai, 1989).

In free-living cyanobacteria, heterocysts are regularly spaced and represent about 5–10% of the cell population. There is a change in the spacing pattern of heterocysts and an increase in their frequency in the cyanobionts in tripartite lichens

(heterocyst frequency 15–35%) but not in bipartite lichens. Heterocyst frequency correlates with the status of fixed carbon in the cyanobiont; in bipartite lichens the cyanobiont bears the burden of providing both fixed nitrogen and fixed carbon to the mycobiont, whereas in tripartite lichens it provides fixed nitrogen only. Indeed heterocyst frequency increases when *Nostoc* isolates are grown in the dark with sugars. In many cyanobacterial-plant symbioses, where the cyanobiont receives fixed carbon from the plant host, heterocyst frequencies of up to 80% can occur (see Rai et al., 2002b).

In free-living cyanobacteria, glutamine synthetase (GS) is the primary ammonia assimilating enzyme and GS levels in heterocysts are twofold higher than those in vegetative cells (Bergman et al., 1985). In cyanobionts, the GS activity and protein levels decrease by over 90% and the remaining GS is uniformly distributed among heterocysts and vegetative cells (Bergman and Rai, 1989; Rai, 2002a). GS activity is undetectable in the mycobiont, but mycobiont hyphae in contact with cyanobiont cells show high levels of nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent glutamate dehydrogenase (GDH) activity.

Nitrogen fixation occurs in all lichens containing heterocystous cyanobionts. The rates are higher in tripartite lichens owing to the higher heterocyst frequency of the cyanobiont (see Rai et al. [2000] and Rai [2002a]). In contrast to free-living forms, cyanobionts in bipartite lichens and in excised cephalodia continue to fix N_2 even in the presence of nitrate or ammonia (Stewart and Rowell, 1977b; Rai et al., 1980). However, nitrogen fixation by the cyanobiont in cephalodia attached to the main thallus of the tripartite lichen *P. aphthosa* was repressed by nitrate and ammonia. The effect was obviously mediated via the phycobiont. Significant levels of N_2 fixation have also been reported in darkness and under these conditions ammonia has an inhibitory effect (Rai et al., 1981a; Rai et al., 1983b). As in the free-living forms, nitrogenase is located only in the heterocysts, despite the microaerobic conditions in lichen thalli (Bergman et al., 1986).

The extent of the changes described above varies from young to older, more mature parts of the thallus. While growth rate gradually declines, cell division and GS levels, the levels of N_2 fixation, CO_2 fixation, and heterocyst frequency of the cyanobiont increase. There is a parallel increase in the GDH activity in the mycobiont (Rowell et al., 1985; Hill, 1989; Rai, 2002a). Still undetermined is whether these changes are caused by the mycobiont or by endogenous regulation due to special environmental conditions offered by the host in the symbiosis.

NUTRIENT EXCHANGE Most studies on nutrient exchange relate to carbon and nitrogen transfer from the cyanobiont to the mycobiont in foliose lichens, particularly *Peltigera* species. Such nutrient transfer is biotrophic in nature and varies along the lichen thallus. From young to mature parts of the lichen thallus, the cyanobiont increases fixation and release of nitrogen and carbon. Specialized mycobiont hyphae and haustoria showing transfer cell ultrastructure (TCU) may play an important role in the nutrient exchange (see Rai et al., 2000).

In bipartite cyanolichens, 70–80% of the CO_2 fixed is released by the cyanobiont to the mycobiont. The transfer of fixed carbon occurs mostly in the light and in the form of glucose. Cyanobionts in tripartite lichens transfer little (<5% of CO_2 fixed) or no fixed-C to the mycobiont. Their primary role seems to be the provision of fixed nitrogen. It would be interesting to know whether the cyanobionts in internal cephalodia occurring deep in the medulla or on the undersurface of a lichen thallus actually receive any fixed carbon from the phycobiont (either directly or via the mycobiont). The glucose transferred to the mycobiont is converted to mannitol, which serves as both a C source and a physiological buffer. Mannitol production by lichenized fungi could be an effective way of sequestering the fixed carbon since other partners cannot use it. The mechanism underlying glucose transfer is not fully understood, but the glucose is thought to originate from a glucan pool rather than directly from CO_2 fixation. Altered cell wall synthesis may lead to a diversion of sugars from cell wall synthesis to simple release. Release of glucose declines sharply and stops soon after the isolation of the cyanobiont, indicating the influence of mycobiont and symbiotic conditions in the thallus on this process (Smith and Douglas, 1987; Meindl and Loos, 1990; Rai, 1990; Palmqvist, 2002).

^{15}N tracer studies in *P. aphthosa* (tripartite) and *P. canina* (bipartite) have concluded that fixed-N is transferred from cyanobiont to the mycobiont as ammonia (Rai et al., 1981b; Rai et al., 1983a). Over 90% of the N_2 fixed in *P. aphthosa* (and about 50% in *P. canina*) is released by the cyanobiont because GS in heterocysts is repressed. The partitioning of fixed-N among the partners is proportionate to their contribution to the thallus composition. In *P. aphthosa*, the ammonium released by the cyanobiont is primarily assimilated by the mycobiont in cephalodia, and the phycobiont receives fixed-N via the mycobiont. The mechanism of ammonia release by the cyanobiont and its uptake by the mycobiont at the cyanobiont-mycobiont interface has not been investigated. However, diffusion of NH_3 from heterocysts can occur in the absence

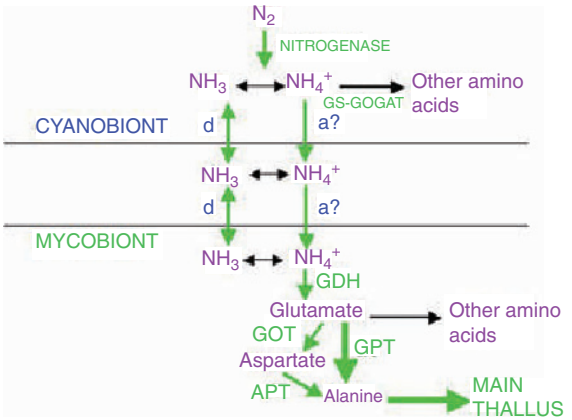

Fig. 12. Pathways of N-metabolism in *P. aphthosa*. a, active transport; and d, diffusion. GS, glutamine synthetase; GOGAT, glutamate synthase; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; GDH, glutamate dehydrogenase; and APT, aspartate-pyruvate transaminase.

of ammonia assimilation by GS. Ammonia assimilation in the mycobiont occurs via GDH followed by aminotransferases. In pulse-chase experiments, much of the ^{15}N label accumulated as alanine in the mycobiont of *P. aphthosa* cephalodia. Alanine could be the principal compound transferred to the rest of thallus (Fig. 12).

Ecological Significance Lichens are ubiquitous, occurring in terrestrial as well as aquatic habitats from the equator to the highest latitudes, at sea level to 9000 m altitude, and in the wettest to driest habitats. They are excellent colonizers of nutrient poor habitats (sand dunes, rocks, forest floors, and the surfaces of other vegetation), form dominant vegetation in tundra and arctic-alpine regions, and contribute significantly to the N-economy of these ecosystems. Lichens are good bioindicators of air pollution.

Cyanobionts endow mycobionts with N and C autotrophy and thereby widen their potential habitats. In a lichen thallus, cyanobionts gain a safe habitat and protection from uncertainty of fluctuating nutrient availability and climatic conditions in nature.

Outlook Many interesting aspects of the lichen symbioses remain to be elucidated. These include release and uptake of nutrients at the cyanobiont-mycobiont interface, cyanobiont acquisition, and regulatory mechanisms enforcing synchronized growth and development of the partners. Furthermore, whether the structural-functional changes in symbionts are a result of endogenous regulation due to the symbiotic environment (e.g., microaerobiosis, restricted growth, and cell division) or whether they are directly caused by the mycobiont will need to be resolved.

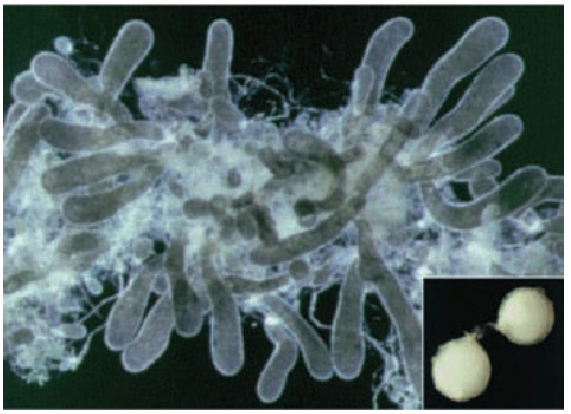

Fig. 13. The *Geosiphon* symbiosis, isolated from a laboratory culture on natural substrate, cleaned, and incubated in liquid medium. The large bladders are about 1.5 mm in length. The insert shows *Geosiphon* spores, which have a diameter of about 250 µm.

SECTION V: The Geosiphon Pyriformis—Nostoc Endocyanosis and its Relationship to the Arbuscular Mycorrhiza (AM)

Introduction

Geosiphon pyriformis (Kütz.) v. Wettstein (v. Von Wettstein, 1915) is presently the only known fungal endocyanosis (endocytobiotic association with a cyanobacterium). The coenocytic *Geosiphon* fungus forms unicellular, multinucleated cells ("bladders") of about 1–2 mm in size (Fig. 13), harboring endosymbiotic filamentous cyanobacteria of the genus *Nostoc*. Only five reports have shown that this symbiosis exists in nature at a range of sites from eastern Germany to Austria. Probably the symbiosis is geographically widespread but, owing to its small size, rarely reported. Presently, locations around the small village of Bieber in the Spessart Mountains (Germany) are the only known natural habitats worldwide (Mollenhauer, 1992; Schüßler and Kluge, 2001).

The species name "*Geosiphon pyriforme*" was used in the past for the fungus as well as for the symbiosis, since this association was often regarded as a "phycomycetous lichen." Nowadays endosymbiotic associations are often excluded from lichen definitions (Hawksworth and Honegger, 1994), and therefore the species name should be used for the fungus only (Schüßler, 2002). Moreover, phylogenetically the *Geosiphon* fungus belongs to the arbuscular mycorrhizal (AM) and related fungi (Fig. 14)

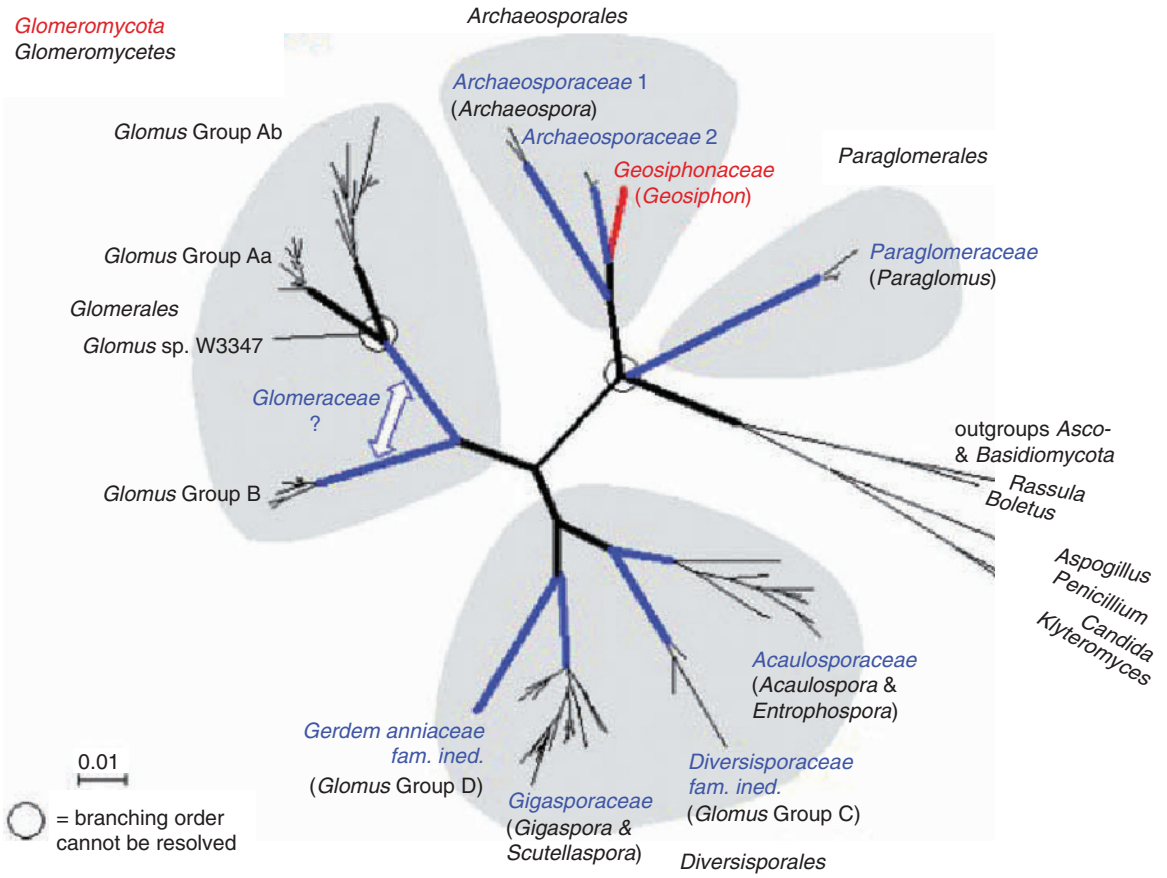

Fig. 14. Phylogenetic (maximum likelihood) tree of AM fungi (Glomeromycota), including *Geosiphon* (see Schüßler et al. [2001], Schwarzott et al. [2001], and Schüßler [2002]). For sequences used, updates, and details, see also (http://www.geosiphon.de) and (http://www.amf-phylogeny.com).

The association between the fungus and cyanobacteria here is referred to as the "*Geosiphon* symbiosis" and the species name of the fungus recently was corrected into its orthographically correct form, *Geosiphon pyriformis* (Schüßler, 2002).

The Symbionts: *Geosiphon pyriformis* and *Nostoc punctiforme*

Analysis of small-subunit ribosomal RNA (SSU rRNA) genes (Schüßler et al., 2001; Schwarzott et al., 2001) and also the morphology and formation of the fungal spores (Schüßler et al., 1994) have shown that the fungal partner of the *Geosiphon* symbiosis belongs to an ancestral branch within the *Glomeromycota*, a new fungal phylum in which the "arbuscular mycorrhizal (AM) and related fungi" (usually referred to as "AM fungi") are placed (Schüßler et al., 2001). In Fig. 14, the molecular phylogeny of the *Glomeromycota*, including *Geosiphon* (Geosiphonaceae and Archaeosporales), is shown. Therefore, the *Geosiphon* symbiosis is also attracting interest from the field of AM research. The AM symbiosis is formed by more than 80% of vascular plants, and moreover, also by lower plants (Read et al., 2000; Schüßler, 2000), where it is also called "AM," though these plants do not possess roots. This huge number of plants forming AM indicates that the AM is one of the most important factors in land ecosystems (Smith and Read, 1997).

The endosymbiont of *Geosiphon* is *Nostoc punctiforme*. Various strains of *Nostoc punctiforme* from other symbiotic systems (e.g., *Anthoceros*, *Blasia* and *Gunnera*) are capable of forming this symbiosis (Mollenhauer, 1992). In the field, *Geosiphon* was always found together with *Anthoceros* and *Blasia*, and its cyanobionts can infect the bryophytes and vice versa. In laboratory cultures, a *Nostoc punctiforme* strain is used that was originally isolated from the *Geosiphon* symbiosis by Mollenhauer.

Geosiphon also harbors another prokaryotic endosymbiont, the so-called "BLOs" ("bacteria-like organisms;" Fig. 15), which are not surrounded by a host membrane (Schüßler at al. 1996; Schüßler and Kluge, 2001). These endosymbiotic bacteria show the typical ultrastructure of those living in most AM fungi. Since these

bacteria are found in diverse branches of the *Glomeromycota*, they have to be considered as ancient symbionts, which are horizontally transferred and have probably lived as symbionts within these fungi for many hundreds of million years. Not much is known about these bacteria. The BLOs of fungi belonging to one glomeromycotan family, the Gigasporaceae, are better investigated. However, these BLOs are enclosed by a host membrane, seem to be a distinct type of bacteria, and may be unique for this branch within the *Glomeromycota*.

The *Geosiphon* symbiosis is facultative for one of the partners—*Nostoc* can be cultivated without the fungus—and obligate for the other one—*Geosiphon*. However, the fungus is, conceivably, not necessarily restricted to the cyanobacteria as symbiotic partner. Likely, *Geosiphon* forms symbiotic associations not only with *Nostoc* but also with plants to form AM (Kluge et al., 2002; Fig. 16). However, this assumption is still speculative.

In any case, because it belongs to the Glomeromycota and has functional as well as structural similarities to the AM, the *Geosiphon* symbiosis probably can play a role in the future as a model system for the hard-to-investigate but extremely important AM symbiosis (Schüßler and Kluge, 2001; Schüßler, 2002).

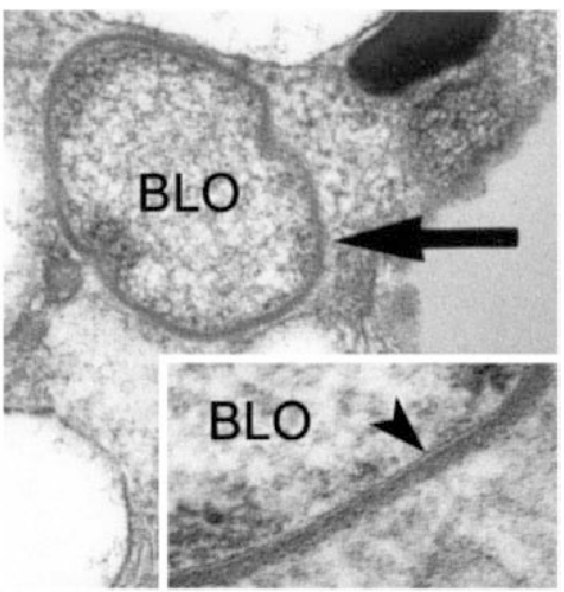

Fig. 15. Electron micrograph of a bacteria-like endosymbiont (BLO) in *Geosiphon*. The BLOs have a diameter of about 0.5 µm and are not enclosed by a host membrane (arrow). The insert shows the plasma membrane of the BLO (arrowhead), as well as its cell wall. The ultrastructure indicates that the BLOs are Gram-positive.

Infection Process, Development and Structure of the Symbiosis

THE INFECTION PROCESS Both symbiosis partners live in the upper layer and on the surface of humid soil, where they can contact each other. The specificity of the interaction between the partners is illustrated by two observations: 1) only certain *Nostoc punctiforme* strains are capable of entering into this symbiosis, and 2) before a successful interaction with the fungus can occur, *Nostoc* has to be at a specific stage of its lifecycle. This is represented by an early immobile stage of the cyanobacterial developmental cycle called a "primordium" (Mollenhauer et al., 1996). The motile filaments (hormogonia) of *Nostoc* and later developmental stages are not recognized by the fungus. When contacting *Nostoc*, the tip of the fungal hypha bulges out and surrounds a part of a cyanobacterial filament, thus incorporating the *Nostoc* cells (Fig. 17). Usually about 5–15 *Nostoc* cells are taken up by this process; exceptions are the heterocysts, which are cut off by the fungus when enclosing the filament. These events are also documented in a scientific film available in German and English (Mollenhauer and Mollenhauer, 1997).

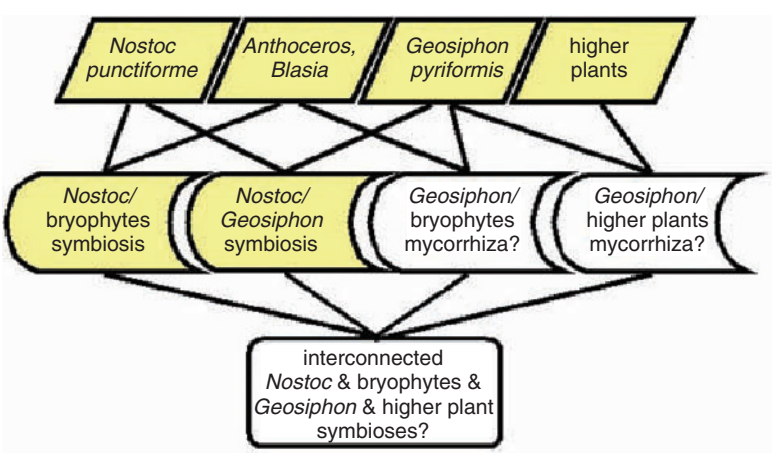

Fig. 16. A possible ecological "symbiosis network" among cyanobacteria, fungi, and plants. Associations which are not highlighted are hypothetical! Further endosymbionts like the BLOs in glomeromycotan fungi may also play an unknown but important role in this network, e.g., for N_2 fixation.

Studies using different lectins revealed that the envelope of the symbiosis-compatible *Nostoc* stage is labeled by a mannose specific lectin (ConA), whereas later stages (heterocysts and hormogonia) showed distinct lectin labeling patterns (Schüßler et al., 1997). Thus the alterations of extracellular glycoconjugates could be involved in partner recognition. Nowadays the symbiosis-compatible *Nostoc* stage is well characterized and a lectin mediated process is indicated by further studies (A. Schüßler and E. Wolf, unpublished observation).

Development of the Symbiosis Each individual *Geosiphon* bladder results from *Nostoc* incorporation by a hypha, as already recognized by Knapp (1933), and each bladder represents a polyenergid cell, coenocytic with the fungal mycelium. After incorporation by the fungus, the *Nostoc* cells first suffer severe stress, as indicated by bleaching of their photosynthetic pigments (Mollenhauer et al., 1996; Fig. 17). They are deformed during the early uptake stages, and some cells may die during this process. The outgrowings of the hyphal tip form an irregularly shaped structure (Fig. 17), which then swells, and after one week the young bladder shows a diameter of up to 100 µm (Fig. 18). Within the first three days, the enclosed *Nostoc* cells recover and then begin to multiply and regenerate heterocysts in about the same frequency as in the free-living filaments (Fig. 19). The endosymbiotic *Nostoc* cells then have an about sixfold larger volume compared with cells outside the bladder (Schüßler et al., 1996) and moreover, under phosphate limitation, divide much faster compared with the free-living ones within the first week (E. Wolf and A. Schüßler, unpublished observation). Individual *Geosiphon* bladders can reach more then 2 mm in length (Figs. 13 and 18) and show a turgor pressure of about 0.6 megapascals (MPa; Schüßler et al., 1995) and live for up to 6 months in laboratory cultures.

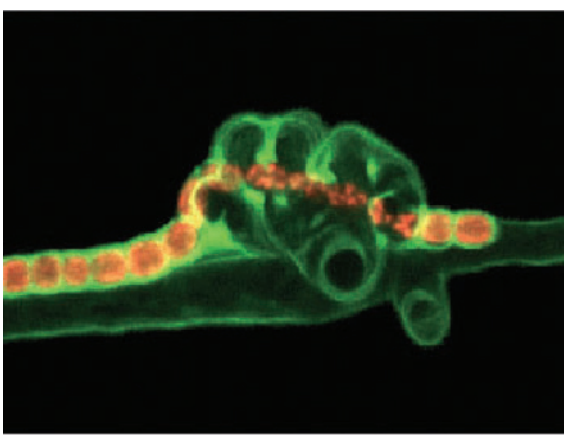

Fig. 17. Confocal laser scanning microscopic projection of a short hypha branching from a main hypha and enclosing a *Nostoc* filament. The extracellular polysaccharides of *Nostoc* and the outer layer of the fungal cell wall are labeled by the fluorescence-coupled lectin ConA (green). The *Nostoc* cells (red, about 4 × 3 µm) that are taken up by the fungal structure show deformations and lower and irregular pigment fluorescence.

Structure and Compartmentation of the Geosiphon Bladder Inside the bladder, the *Nostoc* cells are located within a single, cup-shaped, peripheral compartment, the so-called "symbiosome" (Fig. 20). Lectin labeling experiments combined with electron and fluorescence microscopy showed that the symbiosome space between the *Nostoc* cell-wall and the host symbiosome-membrane contains chitin (Schüßler et al., 1996). This means that the symbiosome-membrane synthesizes chitin and retains this feature from the plasma membrane, from which it was derived by invagination. Interestingly, the symbiotic interface between photobiont and fungus is very similar to that found in AM (Fig. 21). *Geosiphon* bladders are highly vacuolated and contain many lipid droplets in the basal part, which is in contact with the soil substrate. The latter causes a milky white

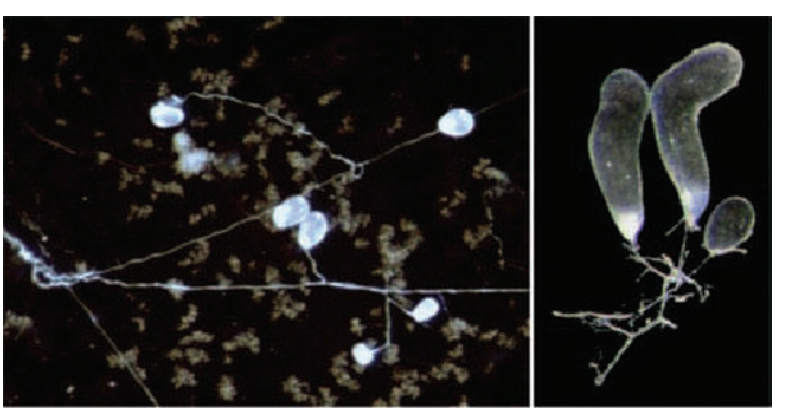

Fig. 18. Young *Geosiphon* bladders, 100–150 µm in size, formed at the fungal mycelium 7–10 days after initial uptake of the cyanobacteria (left). The irregular structures in the background are vegetatively growing *Nostoc* colonies. Two mature bladders of about 1 mm length, together with a young bladder, are shown at the right.

appearance of the bladder base. The dark appearing apical three quarters of the bladder contains the symbiosome and is located above ground. Although the endosymbiotic *Nostoc* cells have a much larger volume compared with free-living ones, they show a normal ultrastructure (Schüßler and Kluge, 2001). Interestingly, an early electron-microscope study of the *Geosiphon* symbiosis led to the "rules of the compartmentation of the eukaryotic cell," also strongly supporting the endosymbiosis theory of the origin of chloroplasts and mitochondria (Schnepf, 1964).

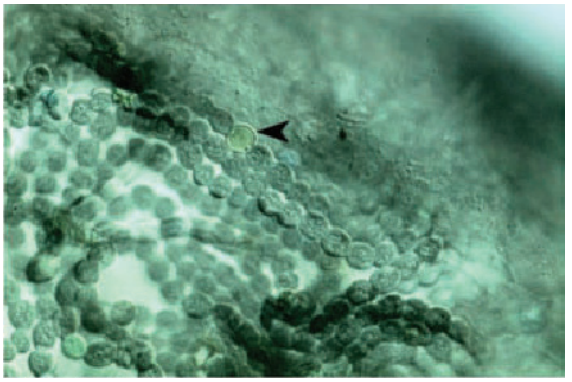

Fig. 19. Endosymbiotic *Nostoc*, about 7 × 6 µm in size, within a *Geosiphon* bladder. One heterocyst is in focus (arrowhead).

Signal Exchange Between Host and Cyanobacterium

Still unknown is what triggers the recognition and the morphological changes that occur during the establishment of the symbiosis. Microscopical studies give no hints for any chemotactic or otherwise directed growth towards the respective symbiosis partner. The growing fungal hyphae seem to contact compatible *Nostoc* stages by chance. However, these observations are mainly based on studies in liquid nutrient solution or on agar plates and might differ in nature. If a suitable stage is contacted by the growing hyphal tip, there have to be highly specific signal perceptions of unknown nature. This is clearly indicated by two facts: 1) the fungus recognizes a *Nostoc* stage (the primordium) only present for some hours in the *Nostoc* life cycle, and 2) the heterocysts are not taken up by the fungus but are cut off when the fungus encloses the vegetative part of the *Nostoc* filament (Fig. 17). The cyanobacteria considerably change their surface carbohydrate composition during the life cycle, and some indications point to a lectin-mediated partner recognition (Schüßler et al., 1997; A. Schüßler and E. Wolf, unpublished observation).

Host-Cyanobiont Interactions Post Infection

Morphological Modifications of the Symbiosis Partners The most obvious morphological

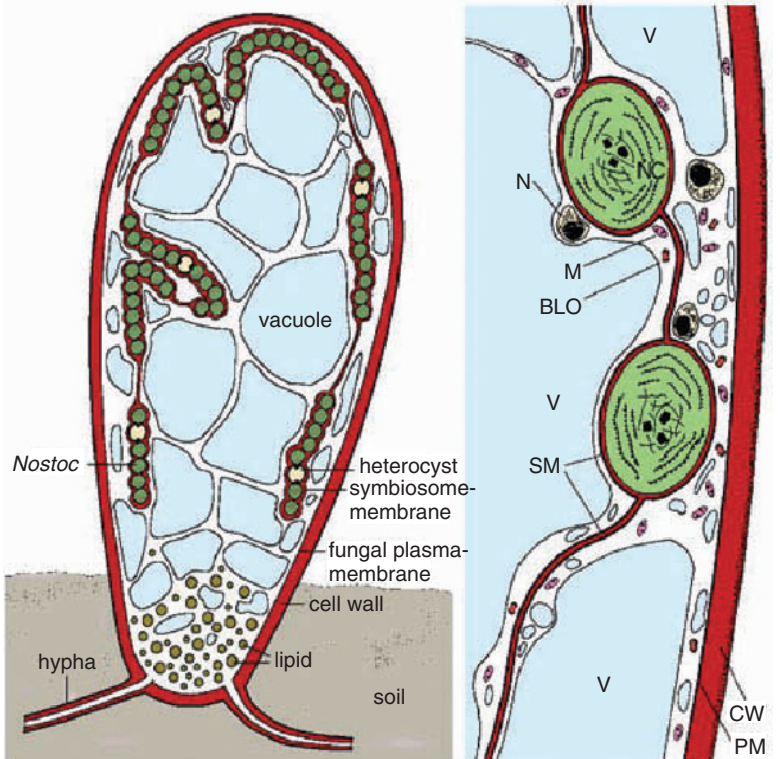

Fig. 20. Schemes of the compartmentation of the *Geosiphon* symbiosis. At the right a magnification of the peripheral part of the bladder is shown. Drawings are based on electron microscope observations. BLO, bacteria-like organism; CW, cell wall; M, mitochondrion; N, nucleus; NC, *Nostoc* cell; PM, plasma membrane; SM, symbiosome membrane; and V, vacuole.

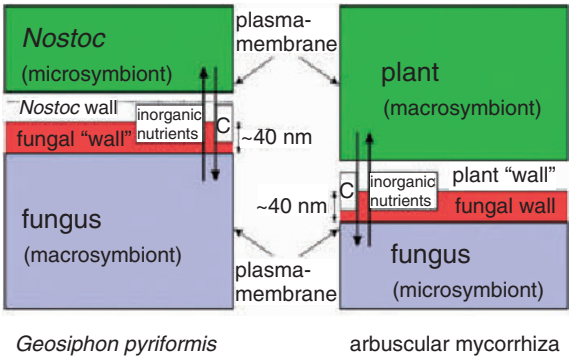

Fig. 21. Structure of the symbiotic interface and bidirectional nutrient flows in the *Geosiphon* symbiosis, in comparison with those in the arbuscular mycorrhiza (AM).

change taking place after partner recognition is the formation of the *Geosiphon* bladder (Figs. 13 and 18). Mature bladders represent big cells, which are coenocytic with the mycelium. They contain large vacuoles in the center and show a clear polarity, with a lipid-storing region in the basal part and the photosynthetically active symbiotic compartment (symbiosome) located in the apical part of the bladder (Fig. 20).

The symbiosome is derived from the invaginated plasma membrane of the fungus and contains the cyanobacteria, which also show an obvious morphological modification, namely an increase in size. Their cells show a six- to eightfold increase in volume compared with free-living vegetative cells. However, the ultrastructure of *Nostoc* in the symbiosis looks relatively normal. One alteration is that the outer membrane is hardly recognizable by electron microscopy. Heterocysts are formed with the same frequency as in free-living colonies, but their cell wall is thinner in the symbiosis, which may indicate a lower O_2 concentration in the surrounding milieu.

N_2 and CO_2 Fixation and Transfer ^{14}C-tracer studies have shown that the *Geosiphon* bladders fix CO_2 both in light and in darkness, whereas the rate of CO_2 fixation in light is much higher (Kluge et al., 1991). In light, largely phosphate esters, polyglucans, free sugars (including trehalose and raffinose), some amino acids and organic acids trap ^{14}C. In darkness, only malic and fumaric acids, together with some amino acids, appear as labeled products. High photosynthetic activity of the endosymbiotic *Nostoc* cells was also shown by photosystem II chlorophyll-fluorescence kinetics (Bilger et al., 1994). The symbiotic *Nostoc* cells achieve much higher steady-state quantum yields and electron transport rates when compared with free-living *Nostoc*.

The capacity for nitrogen fixation is indicated by the occurrence of heterocysts, and considerable nitrogenase activity is associated with the bladders (Kluge et al., 1992). In contrast to symbioses of *Nostoc* with plants (where usually a considerable increase in heterocyst frequency, compared with the free-living cyanobacteria, indicates N_2 fixation as the major role of the cyanobacteria), in *Geosiphon* the relative heterocyst number does not change. Here, the major role of the endosymbiotic *Nostoc* seems to be photosynthesis. However, matter exchange between the partners is still poorly investigated, and it is even possible that the second bacterial endosymbiont (BLO; Figs. 15 and 20), which is typical for many glomeromycotan fungi, contributes to N_2 fixation.

Ecological Importance

Why Is the Symbiosis Mutualistic? The fungal partner of the *Geosiphon* symbiosis belongs to the *Glomeromycota* (AM fungi, Fig. 14) and is, like these, an obligate symbiont. Still unknown is why glomeromycotan fungi are incapable of nonsymbiotic life. Possibly in the future special culture methods will be developed for *in vitro* growth of AM fungi, including *Geosiphon*. Generally in nature glomeromycotan fungi seem not to be capable of saprotrophic life but are dependent on their symbiosis partners for C-delivery. For *Geosiphon* bladders, only molecules with a radius <0.45 nm can pass through the cell wall (Schüßler et al., 1995). Such a pore size is too small for the uptake of sugar molecules from the outside but allows permeation of inorganic ions like phosphate. This might reflect the situation in nature. However, the fine hyphae growing into the substrate may show higher permeability. In any case, by incorporating *Nostoc*, the fungus obtains the needed source of organic compounds.

Nostoc also gains an advantage from the cooperation with the fungal host, which probably improves the supply of the endosymbiont with water, phosphate, and CO_2. Remarkably, all inorganic nutrients, except nitrogen, have to be delivered by the fungus, since the cyanobacteria live intracellularly (Figs. 19–21). Notably, as is usually also the case for the AM, the establishment of the *Geosiphon* symbiosis is strongly promoted by phosphate limitation, which is a severe stress for the photobiont. Preliminary studies moreover show that the intracellular cyanobacteria are protected against heavy metals. Therefore the endosymbionts seem to be protected against abiotic stress factors within the symbiosis.

Evolutionary Implications with Ecological Meaning Symbioses formed by AM fungi are very ancient. It has been proposed that the for-

mation of AM-like symbioses was the initial step allowing plants to colonize land (Pirozynsky and Malloch, 1975). What does this mean? If this hypothesis holds true, the land plants co-evolved with glomeromycotan fungi since their origin. This would have important consequences when interpreting plant ecosystems, since consequently plants probably show nutrient uptake (and other) mechanisms which evolved in dependence with the fungi, and vice versa.

Nowadays, several hints support this ecologically important assumption. AM symbiosis was already formed by the first land plants around 450–500 million years ago (Remy et al., 1994; Redecker et al., 2000). Since AM fungi probably are much older than land plants (Heckman et al., 2001), possibly the glomeromycotan fungi became dependent on the symbiotic carbon supply of photobionts even before land plants evolved (Schüßler, 2002). Therefore, glomeromycotan fungi may have adapted to symbiotic life more then 500 Myr ago, and the *Geosiphon* symbiosis might reflect such an early stage of land-colonizing AM fungal symbioses. Without fossil support this is speculative, but *Geosiphon* clearly shows the ability of glomeromycotan fungi to form symbioses with prokaryotic photoautotrophic organisms. Therefore, cyanobacterial symbioses formed by glomeromycotan fungi could have been an ecologically important step for the colonization of the land habitat. Since AM fungi form symbioses with >80% of the land plants, many ecologically and economically important questions have yet to be answered by future work; some studies may use the *Geosiphon* symbiosis as a model system for the AM (Schüßler, 2002). When performing ecophysiological studies, one should always bear in mind that in nature the mycorrhizas, and not the plant roots themselves, are the chief organs of nutrient uptake.

A Network among Fungi, Cyanobacteria, and Plants? Still unknown is whether *Geosiphon* also forms AM symbioses with plants. Since *Geosiphon* belongs to the monophyletic clade comprising AM fungi, it is tempting to speculate about this. Unpublished results of D. Schwarzott and A. Schüßler show that *Geosiphon* rRNA genes can be PCR amplified from plant roots and *Anthoceros* growing in the natural habitat of *Geosiphon*. However, this is not necessarily evidence for a location within the root and AM formation. The problem is that cleaning the root surface completely from attached fungal hyphae seems impossible, although this is ignored in some published studies. Therefore the sensitive nested PCR approach may be detecting some tiny amounts of externally attached hyphae. Future studies with in situ techniques will have to show whether *Geosiphon* is forming an AM. If this indeed would be the case, one may imagine a complex network of ecological importance (Fig. 16).

Within such a network, symbiotic *Nostoc* could be exchanged between *Geosiphon* and bryophytes, and *Geosiphon* could simultaneously form endosymbiosis with *Nostoc* and AM with plants, thus, for example, delivering N_2 fixed by the cyanobacteria to the plants.

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Literature Cited

Adams, D. G., and P. Duggan. 1999. Heterocyst and akinete differentiation in cyanobacteria: Tansley Review No. 107. New Phytol. 144:3–33.

Adams, D. G. 2000. Symbiotic interactions. *In:* B. Whitton and M. Potts (Eds.) Ecology of Cyanobacteria: Their Diversity in Time and Space. Kluwer Academic Publishers. Dordrecht, The Netherlands. 523–561.

Adams, D. G. 2002. Cyanobacteria in symbiosis with hornworts and liverworts. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 117–135.

Ahmadjian, V. 1993. The Lichen Symbiosis. John Wiley. New York, NY.

Anbudurai, P. R., T. S. Mor, I. Ohad, S. V. Shestakov, and H. B. Pakrasi. 1994. The ctpA gene encodes the C-terminal processing protease for the D1 protein of the photosystem II reaction center complex. Proc. Natl. Acad. Sci. USA 91:8082–8086.

Aptroot, A. 1998. Aspects of the integration of the taxonomy of lichenized and non-lichenized pyrenocarpoous ascomycetes. Lichenologist 30:501–514.

Aulfinger, H., E. B. Braun-Howland, S. Kannaiyan, and S. A. Nierzwicki-Bauer. 1991. Ultrastructural changes of the endosymbionts of Azolla microphylla during megaspore germination and early plantlet development. Can. J. Bot. 69:2489–2496.

Babic, S. 1996. Hormogonia Formation and the Establishment of Symbiotic Associations between Cyanobacteria and the Bryophytes Blasia and Phaeoceros [PhD thesis]. University of Leeds. Leeds, UK.

Baker, J., B. Entsch, and D. B. McKay. 2003. The cyanobiont in an Azolla fern is neither Anabaena nor Nostoc. FEMS Microbiol. Lett. 229:43–47.

Basile, D. V. 1990. Morphological role of hydroxyproline containing proteins in liverworts. *In:* R. N. Chopra and S. C. Bhatia (Eds.) Bryophyte Development: Physiology and Biochemistry. CRC Press. Boca Raton, FL. 225–243.

Bergman, B., P. Lindblad, A. Pettersson, E. Renström, and E. Tiberg. 1985. Immuno-gold localization of glutamine synthetase in a nitrogen-fixing cyanobacterium (Anabaena cylindrica). Planta 166:329–334.

Bergman, B., P. Lindblad, and A. N. Rai. 1986. Nitrogenase in free-living and symbiotic cyanobacteria: Immunoelectron microscopic localization. FEMS Microbiol. Lett. 35:75–78.

Bergman, B., and A. N. Rai. 1989. The Nostoc-Nephroma symbiosis: Localization, distribution pattern and levels of key proteins involved in nitrogen and carbon metabolism of the cyanobiont. Physiol. Plant. 77:216–224.

Bergman, B., A. N. Rai, C. Johansson, and E. Söderbäck. 1992a. Cyanobacterial-plant symbioses. Symbiosis 14:61–81.

Bergman, B., C. Johansson, and E. Söderbäck. 1992b. The Nostoc-Gunnera symbiosis. New Phytol. 122:379–400.

Bergman, B., A. Matveyev, and U. Rasmussen. 1996. Chemical signalling in cyanobacterial-plant symbioses. Trends Plant Sci. 1:191–197.

Bergman, B. 2002a. The Nostoc-Gunnera symbiosis. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 207–232.

Bergman, B., and B. Osborne. 2002b. The Gunnera-Nostoc symbiosis. *In:* B. Osborne (Ed.) Commentaries on Cyanobacterial Symbioses. Royal Irish Academy. Dublin, Ireland. 35–39.

Bergman, B., U. Rasmussen, and A. N. Rai. 2003. Cyanobacterial associations. *In:* C. Elmerich and W. E. Newton (Eds.) Associative Nitrogen-fixing Bacteria and Cyanobacterial Associations. Kluwer Academic Publishers. Dordrecht, The Netherlands.

Bilger, W., B. Büdel, R. Mollenhauer, and D. Mollenhauer. 1994. Photosynthetic activity of two developmental stages of a Nostoc strain (Cyanobacteria) isolated from Geosiphon pyriforme (Mycota). J. Phycol. 30:225–230.

Black, K. G., R. Parsons, and B. A. Osborne. 2002. Uptake and metabolism of glucose in the Nostoc-Gunnera symbiosis. New Phytol. 153:297–305.

Bonnett, H. T., and W. B. Silvester. 1981. Specificity in the Nostoc-Gunnera endosymbiosis. New Phytol. 89:121–128.

Brasell, H. M., S. K. Davies, and J. P. Mattay. 1986. Nitrogen fixation associated with bryophytes colonizing burnt sites in Southern Tasmania, Australia. J. Bryol. 14:139–149.

Braun-Howland, E. B., and S. A. Nierzwicki-Bauer. 1990. Azolla-Anabaena symbiosis: Biochemistry, physiology, ultrastructure and molecular biology. *In:* A. N. Rai (Ed.) CRC Handbook of Symbiotic Cyanobacteria. CRC Press. Boca Raton, FL. 65–117.

Bushnell, T. 1998. Molecular Identification of Endosymbionts Present in the Leaf Cavities of Azolla caroliniana and Azolla mexicana [PhD thesis]. Rensselaer Polytechnic Institute. Troy, NY. 1–222.

Calvert, H. E., and G. A. Peters. 1981. The Azolla-Anabaena relationship. IX: Morphological analysis of leaf cavity hair populations. New Phytol. 89:327–335.

Campbell, E. L., and J. C. Meeks. 1989. Characteristics of hormogonia formation by symbiotic Nostoc spp. in response to the presence of Anthoceros punctatus or its extracellular products. Appl. Environ. Microbiol. 55:125–131.

Campbell, E. L., and J. C. Meeks. 1992. Evidence for plant-mediated regulation of nitrogenase expression in the Anthoceros-Nostoc symbiotic association. J. Gen. Microbiol. 138:473–480.

Campbell, E. L., K. D. Hagen, M. F. Cohen, M. L. Summers, and J. C. Meeks. 1996. The devR gene product is characteristic of receivers of two-component regulatory systems and is essential for heterocyst development in the filamentous cyanobacterium Nostoc sp. strain ATCC 29133. J. Bacteriol. 178:2037–2043.

Campbell, E. L., B. Brahamsha, and J. C. Meeks. 1998. Mutation of an alternative sigma factor in the cyanobacterium Nostoc punctiforme results in increased infection of its symbiotic plant partner, Anthoceros punctatus. J. Bacteriol. 180:4938–4941.

Campbell, E. L., F. C. Y. Wong, and J. C. Meeks. 2003. DNA binding properties of the HrmR protein of Nostoc punctiforme responsible for transcriptional regulation of genes involved in the differentiation of hormogonia. Molec. Microbiol. 47:573–582.

Canini, A., M. G. Caiola, and M. Mascini. 1990. Ammonium content, nitrogenase activity and heterocyst frequency within the leaf cavities of Azolla filiculoides Lam. FEMS Microbiol. Lett. 71:205–210.

Carrapico, F., and R. Tavares. 1989. New data on the Azolla-Anabaena symbiosis II: Cytochemical and immunocytochemical aspects. *In:* F. A. Skinner, R. M. Boddey, and I. Fendrik (Eds.) Nitrogen Fixation with Non-legumes. Kluwer Academic Press. Dordrecht, The Netherlands. 95–100.

Carrapico, F. 1991. Are bacteria the 3rd partner of the Azolla-Anabaena symbiosis? Plant Soil 137:157–160.

Cohen, M. F., J. G. Wallis, E. L. Campbell, and J. C. Meeks. 1994. Transposon mutagenesis of Nostoc sp. strain ATCC 29133, a filamentous cyanobacterium with multiple cellular differentiation alternatives. Microbiology 140:3233–3240.

Cohen, M. F., and J. C. Meeks. 1997. A hormogonium regulating locus, hrmUA, of the cyanobacterium Nostoc punctiforme strain ATCC 29133 and its response to an extract of a symbiotic plant partner Anthoceros punctatus. Molec. Plant-Microbe Interact. 10:280–289.

Cohen, M. F., J. C. Meeks, Y. Cai, and C. P. Wolk. 1998. Transposon mutagenesis of heterocyst-forming filamentous cyanobacteria. Meth. Enzymol. 297:3–17.

Cohen, M. F, and H. Yamasaki. 2000. Flavonoid-induced expression of a symbiosis-related gene in the cyanobacterium Nostoc punctiforme. J. Bacteriol. 182:4644–4646.

Cohen, M. F., Y. Sakihama, Y. C. Takagi, T. Ichiba, and H. Yamasaki. 2002. Synergistic effect of doexyanthocyanins from symbiotic fern Azolla spp. on hrmA gene induction in the cyanobacterium Nostoc punctiforme. Molec. Plant-Microbe Interact. 15:875–882.

Costa, J.-L., P. Paulsrud, J. Rikkinen, and P. Lindblad. 2001. Genetic diversity of Nostoc symbionts endophytically associated with two bryophyte species. Appl. Environ. Microbiol. 67:4393–4396.

Costa, J.-L., and P. Lindblad. 2002. Cyanobacteria in symbiosis with cycads. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 195–205.

Dalton, D. A., and J. M. Chatfield. 1985. A new nitrogen-fixing cyanophyte-hepatic association: Nostoc and Porella. Am. J. Bot. 72:781–784.

de Roissart, P., C. Jucqued, L. Watetkeyn, P. Berghmans, and C. Van Hove. 1994. First evidence for the cutinic nature of the envelope at the interface of Azolla and its endophytes. *In:* N. A. Hegazi (Ed.) Nitrogen Fixation with Non-legumes: The Sixth International Symposium. The American University in Cairo Press. Cairo, Egypt. 133–138.

Do, V. C., I. Watanabe, W. J. Zimmerman, T. A. Lumpkin, and T. de Waha Gaillonville. 1989. Sexual hybridization among Azolla species. Can. J. Bot. 67:3482–3485.

Duckett, J. G., A. K. S. K. Prasad, D. A. Davies, and S. Walker. 1977. A cytological analysis of the Nostoc-bryophyte relationship. New Phytol. 79:349–362.

Enderlin, C. S., and J. C. Meeks. 1983. Pure culture and reconstitution of the Anthoceros-Nostoc symbiotic association. Planta 158:157–165.

Fiedler, G., A. Muro-Pastor, E. Flores, and I. Maldener. 2001. NtcA-dependent expression of the devBCA operon, encoding a heterocyst-specific ATP-binding cassette transporter in Anabaena spp. J. Bacteriol. 183:3795–3799.

Fisher, R. F., and S. R. Long. 1992. Rhizobium-plant signal exchange. Nature 357:655–660.

Forni, C., M. Grilli Caiola, and S. Gentili. 1989. Bacteria in the Azolla-Anabaena symbiosis. *In:* F. A. Skinner, R. M. Boddey, and I. Fendrik (Eds.) Nitrogen Fixation with Non-legumes. Kluwer Academic Press. Dordrecht, The Netherlands. 83–88.

Forni, C., S. Gentili, C. Van Hove, and M. Grilli Caiola. 1990. Isolation and characterization of the bacteria living in the sporocarps of Azolla filiculoides Lam. Ann. Microbiol. 40:235–243.

Galun, M. 1988. Handbook of Lichenology. CRC Press. Boca Raton, FL.

Gantar, M., N. W. Kerby, and P. Rowell. 1993. Colonization of wheat (Triticum vulgare L.) by N_2-fixing cyanobacteria. III: The role of a hormogonia-promoting factor. New Phytol. 124:505–513.

Gates, J. E., R. W. Fisher, and R. A. Candler. 1980. The occurrence of corynoform bacteria in the leaf cavity of Azolla. Arch. Microbiol. 127:163–165.

Gebhardt, J. S., and S. A. Nierzwicki-Bauer. 1991. Identification of a common cyanobacterial symbiont associated with Azolla spp. through molecular and morphological characterization of free-living and symbiotic cyanobacteria. Appl. Environ. Microbiol. 57:2141–2146.

Gorelova, O. A., O. I. Baulina, A. G. Shchelmanova, T. G. Korzhenevskaya, and M. V. Gusev. 1996. Heteromorphism of the cyanobacterium Nostoc sp., a microsymbiont of the Blasia pusilla moss. Microbiology 65:719–726.

Gratwicke, B., and B. E. Marshall. 2001. The impact of Azolla filiculoides Lam. on animal biodiversity in streams in Zimbabwe. Afr. J. Ecol. 39:216–218.

Hawksworth, D., and R. Honegger. 1994. The lichen thallus: A symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. *In:* M. A. J. Williams (Ed.) Plant Galls: Organisms, Interactions, Populations. Clarendon Press. Oxford, UK. The Systematics Association, Special Volume No. 49:77–98.

Heckman, D. S., D. M. Geiser, B. R. Eidell, R. L. Stauffer, N. L. Kardos, and S. B. Hedges. 2001. Molecular evidence for the early colonization of land by fungi and plants. Science 293:1129–1133.

Herdman, M., and R. Rippka. 1988. Cellular differentiation: Hormogonia and baeocytes. Meth. Enzymol. 167:232–242.

Herrero, A., A. M. Muro-Pastor, and E. Flores. 2001. Nitrogen control in cyanobacteria. J. Bacteriol. 183:411–425.

Hill, D. J. 1975. The pattern of development of Anabaena in the Azolla-Anabaena symbiosis. Planta 122:178–184.

Hill, D. J. 1977. The role of Anabaena in the Azolla-Anabaena symbiosis. New Phytol. 78:611–616.

Hill, D. J. 1989. The control of cell cycle in microbial symbionts. New Phytol. 112:175–184.

Hill, M. P., and I. G. Oberholzer. 2002. Laboratory host range testing of the flea beetle, Pseudolampsis guttata (Leconte) (Coleoptera:Chrysomelidae), a potential natural enemy for red water fern, Azolla filiculoides Lamarck (Pteridophyta:Azollaceae) in South Africa. Coleopts. Bull. 56:79–83.

Janes, R. 1998a. Growth and survival of Azolla filiculoides in Britain. I: Vegetative reproduction. New Phytol. 138:367–375.

Janes, R. 1998b. Growth and survival of Azolla filiculoides in Britain. II: Sexual reproduction. New Phytol. 138:377–384.

Johansson, C., and B. Bergman. 1992. Early events during the establishment of Gunnera/Nostoc symbiosis. Planta 188:403–413.

Johansson, C., and B. Bergman. 1994. Reconstruction of the symbiosis of Gunnera manicata Linden: Cyanobacterial specificity. New Phytol. 126:643–652.

Joseph, C. M., and J. C. Meeks. 1987. Regulation of expression of glutamine synthetase in a symbiotic Nostoc strain associated with Anthoceros punctatus. J. Bacteriol. 169:2471–2475.

Kaplan, D., and G. A. Peters. 1988. Interaction of carbon metabolism in the Azolla-Anabaena symbiosis. Symbiosis 6:53–68.

Kardish, N., L. Silberstein, G. Fleminger, and M. Galun. 1991. Lectins from the lichen Nephroma laevigatum Ach.: Localization and function. Symbiosis 11:47–62.

Kimura, J., and T. Nakano. 1990. Reconstitution of a Blasia-Nostoc symbiotic association under axenic conditions. Nova Hedwigia 50:191–200.

Kluge, M., D. Mollenhauer, and R. Mollenhauer. 1991. Photosynthetic carbon assimilation in Geosiphon pyriforme (Kützing) F.v. Wettstein, an endosymbiotic association of fungus and cyanobacterium. Planta 185:311–315.

Kluge, M., D. Mollenhauer, R. Mollenhauer, and R. Kape. 1992. Geosiphon pyriforme, an endosymbiotic consortium of a fungus and a cyanobacterium (Nostoc), fixes nitrogen. Bot. Acta 105:343–344.

Kluge, M., D. Mollenhauer, E. Wolf, and A. Schüßler. 2002. The Nostoc-Geosiphon endocytobiosis. *In:* A. N. Rai, B. Bergman, U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 19–30.

Knapp, E. 1933. Über Geosiphon pyriformis Fr.v. Wettst., eine intrazelluläre Pilz-Algen-Symbiose. Ber. Dtsch. Bot. Ges. 51:210–217.

Knight, C. D., and D. G. Adams. 1996. A method for studying chemotaxis in nitrogen-fixing cyanobacterium-plant symbioses. Physiol. Molec. Plant Pathol. 49:73–77.

Kobiler, D., A. Cohen-Sharon, and E. Tel-Or. 1981. Recognition between the N_2-fixing Anabaena and the water fern Azolla. FEBS Lett. 133:157–160.

Kobiler, D., A. Cohen-Sharon, and E. Tel-Or. 1982. Lectins are involved in the recognition between Anabaena and Azolla. Israel J. Bot. 31:324–328.

Komarek, J., and K. Anagnostidis. 1989. Trichormus azollae (Strasb.): Modern approaches to the classification system of cyanophytes 4-Nostocales. Arch. Hydrobiol. Algol. Stud. 56:303–345.

Lamb, J. R., S. Tugendreich, and P. Hieter. 1995. Tetratrico peptide repeat interactions: To TPR or not to TPR? Trends Biochem. Sci. 20:257–259.

Large, M. F., and J. E. Braggins. 1993. Spore morphology of New Zealand Azolla filiculoides Lam. (Salviniaceae). New Zealand J. Bot. 31:419–423.

Lechno-Yossef, S. 2002a. Identification and characterization of bacteria associated with the water fern Azolla sp. [PhD thesis]. Rensselaer Polytechnic Institute. Troy, NY. 1–132.

Lechno-Yossef, S., and S. A. Nierzwicki-Bauer. 2002b. Azolla-Anabaena symbiosis. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 153–178.

Lee, K. Y., C. M. Joseph, and J. C. Meeks. 1988. Glutamine synthetase specific activity and protein concentration in symbiotic Anabaena associated with Azolla caroliniana. Ant. v. Leeuwenhoek 54:345–355.

Lehr, H., G. Fleminger, and M. Galun. 1995. Lectin from the lichen Peltigera membranacea (Ach.) Nyl.: Characterization and function. Symbiosis 18:1–13.

Lehr, H., M. Galun, S. Ott, H. M. Jahns, and G. Fleminger. 2000. Cephalodia of the lichen Peltigera aphthosa (L.) Willd.: Specific recognition of the compatible photobiont. Symbiosis 29:357–365.

Lejeune, A., A. Cagauan, and C. Van Hove. 1999. Azolla research and development: Recent trends and priorities. Symbiosis 27:333–351.

Liaimer, A., A. Matveyev, and B. Bergman. 2001. Isolation of host plant induced cDNAs from Nostoc sp. PCC 9229 forming symbiosis with the angiosperm Gunnera spp. Symbiosis 31:293–307.

Liaimer, A., and B. Bergman. 2003. Phytohormones in cyanobacteria: Occurrence and perspectives. *In:* Proceedings from Plant-Microbe Interaction Conference, St Petersburg.

Lin, C., Z. Z. Liu, D. Y. Zheng, L. F. Tang, and I. Watanabe. 1989. Re-establishment of symbiosis to Anabaena free Azolla. Sci. China Ser. B-Chem. 32:551–559.

Lindblad, P., B. Bergman, and S. A. Nierzwicki-Bauer. 1991. Immunocytochemical localization of nitrogenase in bacteria symbiotically associated with Azolla spp. Appl. Environ. Microbiol. 57:3637–3640.

Lohtander, K., I. Oksanen, and J. Rikkinen. 2002. A phylogenetic study of Nephroma (lichen-forming Ascomycota). Mycol. Res. 106:777–787.

Martin, W., T. Rujan, E. Richly, A. Hansen, S. Cornelsen, T. Lins, D. Leister, B. Stoebe, M. Hasegawa, and D. Penny. 2002. Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proc. Natl. Acad. Sci. USA 99:12246–12251.

McConnachie, A. J., M. P. Hill, and M. J. Byrne. 2004. Field assessment of a frond-feeding weevil, a successful biological control agent of red waterfern, Azolla filiculoides, in southern Africa. Biol. Control 29:326–331.

Meeks, J. C., C. S. Enderlin, K. L. Wycoff, J. S. Chapman, and C. M. Joseph. 1983. Assimilation of $^{13}NH_4^+$ by Anthoceros grown with and without symbiotic Nostoc. Planta 158:384–391.

Meeks, J. C., C. S. Enderlin, C. M. Joesph, J. S. Chapman, and M. W. L. Lollar. 1985a. Fixation of [^{13}N] N_2 and transfer of fixed nitrogen in the Anthoceros-Nostoc symbiotic association. Planta 164:406–414.

Meeks, J. C., N. Steinberg, C. M. Joseph, C. S. Enderlin, P. A. Jorgensen, and G. A. Peters. 1985b. Assimilation of exogenous and dinitrogen-derived $^{13}NH_4^+$ by Anabaena azollae separated from Azolla caroliniana Willd. Arch. Microbiol. 142:229–233.

Meeks, J. C. 1988. Symbiotic associations. Meth. Enzymol. 167:113–121.

Meeks, J. C. 1990. Cyanobacterial-bryophyte associations. *In:* A. N. Rai (Ed.) CRC Handbook of Symbiotic Cyanobacteria. CRC Press. Boca Raton, FL. 43–63.

Meeks, J. C. 1998. Symbiosis between nitrogen-fixing cyanobacteria and plants. Bioscience 48:266–276.

Meeks, J. C., E. Campbell, K. Hagen, T. Hanson, N. Hitzeman, and F. Wong. 1999. Developmental alternatives of symbiotic Nostoc punctiforme in response to its symbiotic partner Anthoceros punctatus. *In:* G. A. Peschek, W. Löffelhardt, and G. Schmetterer (Eds.) The Phototrophic Prokaryotes. Kluwer Academic/Plenum Publishers. New York, NY. 665–678.

Meeks, J. C., and J. Elhai. 2002. Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol. Molec. Biol. Rev. 66:94–121.

Meindl, D., and E. Loos. 1990. Release of glucose by Nostoc species isolated from the lichen Peltigera horizontalis. Plant Sci. 72:283–288.

Mellor, R. B., G. M. Gadd, P. Rowell, and W. D. P. Stewart. 1981. A phytohaemagglutinin from the Azolla-Anabaena symbiosis. Biochem. Biophys. Res. Commun. 99:1348–1353.

Milano, V. 2003. Identification of microbionts associated with the water fern Azolla [masters thesis]. Rensselaer Polytechnic Institute. Troy, NY. 1–49.

Mollenhauer, D. 1992. Geosiphon pyriformis. *In:* W. Reisser (Ed.) Algae and Symbiosis: Plants, Animals, Fungi, Viruses, Interactions Explored. Biopress. Bristol, UK. 339–351.

Mollenhauer, D., R. Mollenhauer, and M. Kluge. 1996. Studies on initiation and development of the partner association in Geosiphon pyriforme (Kütz.) v. Wettstein, a unique endocytobiotic system of a fungus (Glomales) and the cyanobacterium Nostoc punctiforme (Kütz.) Hariot. Protoplasma 193:3–9.

Mollenhauer, D., and R. Mollenhauer. 1997. Endosymbiosis between Nostoc and Geosiphon pyriforme. Institut für den Wissenschaftlichen Film (IWF). Göttingen, Germany. Film-no. C1955.

Nash, T. H. 1996. Lichen Biology. Cambridge University Press. Cambridge, UK.

Newton, J. W., and A. I. Herman. 1979. Isolation of cyanobacteria from the aquatic fern, Azolla. Arch. Microbiol. 120:161–165.

Nierzwicki-Bauer, S. A., H. Aulfinger, and E. B. Braun-Howland. 1989. Ultrastructural characterization of an inner envelope that confines Azolla endosymbionts to the leaf cavity periphery. Can. J. Bot. 67:2711–2719.

Nierzwicki-Bauer, S. A., and H. Aulfinger. 1991. Occurrence and ultrastructural characterization of bacteria in association with and isolated from Azolla caroliniana. Appl. Environ. Microbiol. 57:3629–3636.

Nilsson, M., B. Bergman, and U. Rasmussen. 2000. Cyanobacterial diversity in geographically related and distant host plants of the genus Gunnera. Arch. Microbiol. 173:97–102.

Orr, J., and R. Haselkorn. 1982. Regulation of glutamine synthetase activity and synthesis in free-living and symbiotic Anabaena spp. J. Bacteriol. 152:626–635.

Osborne, B., F. Doris, A. Cullen, R. McDonald, G. Campbell, and M. Steer. 1991. Gunnera tinctoria: An unusual invader. Bioscience 41:224–234.

Osborne, B. A., A. Cullen, P. W. Jones, and G. J. Campbell. 1992. Use of nitrogen by the Nostoc-Gunnera tinctoria (Molina) Mirbel symbiosis. New Phytol. 120:481–487.

Osborne, B. 2002a. Commentaries on cyanobacterial symbioses. Proc. Roy. Irish Acad. 102B(1): 35–39.

Osborne, B. A., and J. I. Sprent. 2002b. Ecology of Nostoc-Gunnera symbiosis. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 207–232.

Palmqvist, K. 2002. Cyanolichens: Carbon metabolism. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 73–96.

Paulsrud, P., and P. Lindblad. 1998a. Sequence variation of the $tRNA^{Leu}$ intron as a marker for genetic diversity and specificity of symbiotic cyanobacteria in some lichens. Appl. Environ. Microbiol. 64:310–315.

Paulsrud, P., J. Rikkinen, and P. Lindblad. 1998b. Cyanobiont specificity in some Nostoc-containing lichens and in a Peltigera aphthosa photosymbiodeme. New Phytol. 139:517–528.

Paulsrud, P., J. Rikkinen, and P. Lindblad. 2000. Spatial patterns of photobiont diversity in some Nostoc-containing lichens. New Phytol. 146:291–299.

Paulsrud, P., J. Rikkinen, and P. Lindblad. 2001. Field experiments on cyanobacterial specificity in Peltigera aphthosa. New Phytol. 152:117–123.

Pennell, R. I. 1992. Cell surface arabinogalactan proteins, arabinogalactans and plant development. *In:* J. A. Collow and J. R. Green (Eds.) Perspectives in Plant Cell Recognition. Cambridge University Press. Cambridge, UK. 105–121.

Perkins, S. K., and G. A. Peters. 1993. The Azolla-Anabaena symbiosis: Endophyte continuity in the Azolla life-cycle is facilitated by epidermal trichomes. I: Partitioning of the endophytic Anabaena into developing sporocarps. New Phytol. 123:53–64.

Peters, G. A., and H. E. Calvert. 1983. The Azolla-Anabaena azollae symbiosis. *In:* L. J. Goff (Ed.) Algal Symbiosis. A Continuum of Interaction Strategies. Cambridge University Press. Cambridge, UK. 109–145.

Peters, G. A., D. Kaplan, J. C. Meeks, K. M. Buzby, B. H. Marsh, and J. L. Corbin. 1985. Aspects of nitrogen and carbon interchange in the Azolla-Anabaena symbiosis. *In:* P. W. Ludden and J. E. Burris (Eds.) Nitrogen Fixation and CO_2 Metabolism. Elsevier Science. New York, NY. 213–222.

Peters, G. A., and S. K. Perkins. 1993. The Azolla-Anabaena symbiosis: Endophyte continuity in the Azolla life-cycle is facilitated by epidermal trichomes. II: Reestablishment of the symbiosis following gametogenesis and embryogenesis. New Phytol. 123:65–75.

Pirozynski, K. A., and D. W. Malloch. 1975. The origin of land plants: A matter of mycotrophism. BioSystems 6:153–164.

Plazinski, J. 1990a. The Azolla-Anabaena symbiosis. *In:* P. M. Gresshoff (Ed.) Molecular Biology of Symbiotic Nitrogen Fixation. CRC Press. Boca Raton, FL. 51–75.

Plazinski, J., R. Taylor, W. Shaw, L. Croft, B. G. Rolfe, and B. E. S. Gunning. 1990b. Isolation of Agrobacterium sp. strain from the Azolla leaf cavity. FEMS Microbiol. Lett. 70:55–59.

Plazinski, J., Q. Zheng, R. Taylor, L. Croft, B. G. Rolfe, and B. E. S. Gunning. 1990c. DNA probes show genetic variation in cyanobacterial symbionts of the Azolla fern and a closer relationship to free-living Nostoc strains than to free-living Anabaena strains. Appl. Environ. Microbiol. 56:1263–1270.

Plazinski, J., L. Croft, R. Taylor, Q. Zheng, B. G. Rolfe, and B. E. S. Gunning. 1991. Indigenous plasmids in Anabaena azollae: Their taxonomic distribution and existence of regions of homology with symbiotic genes of rhizobium. Can. J. Microbiol. 37:171–181.

Rai, A. N., P. Rowell, and W. D. P. Stewart. 1980. NH_4^+ assimilation and nitrogenase regulation in the lichen Peltigera aphthosa Willd. New Phytol. 85:545–555.

Rai, A. N., P. Rowell, and W. D. P. Stewart. 1981a. Nitrogenase activity and dark CO_2 fixation in the lichen Peltigera aphthosa Willd. Planta 151:256–264.

Rai, A. N., P. Rowell, and W. D. P. Stewart. 1981b. $^{15}N_2$ incorporation and metabolism in the lichen Peltigera aphthosa Willd. Planta 152:544–552.

Rai, A. N., P. Rowell, and W. D. P. Stewart. 1983a. Interactions between cyanobacterium and fungus during $^{15}N_2$-incorporation and metabolism in the lichen Peltigera aphthosa Willd. Arch. Microbiol. 134:136–142.

Rai, A. N., P. Rowell, and W. D. P. Stewart. 1983b. Mycobiont-cyanobiont interactions during dark nitrogen fixation by the lichen Peltigera aphthosa. Physiol. Plant. 57:285–290.

Rai, A. N., M. Borthakur, S. Singh, and B. Bergman. 1989. Anthoceros-Nostoc symbiosis: Immunoelectronmicroscopic localization of nitrogenase, glutamine synthetase, phycoerythrin and ribulose-1,5-bisphosphate carboxylase/oxygenase in the cyanobiont and the cultured (free-living) isolate Nostoc 7801. J. Gen. Microbiol. 135:385–395.

Rai, A. N. 1990a. Cyanobacteria in Symbiosis. *In:* A. N. Rai (Ed.) CRC Handbook of Symbiotic Cyanobacteria. CRC Press. Boca Raton, FL. 1–7.

Rai, A. N. 1990b. Cyanobacterial-fungal symbioses: The cyanolichens. *In:* A. N. Rai (Ed.) Handbook of Symbiotic Cyanobacteria. CRC Press. Boca Raton, FL. 9–41.

Rai, A. N., E. Söderbäck, and B. Bergman. 2000. Tansley Review No. 116, Cyanobacterium-plant symbioses. New Phytol. 147:449–481.

Rai, A. N. 2002a. Cyanolichens: Nitrogen metabolism. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 97–115.

Rai, A. N., B. Bergman, and U. Rasmussen (Eds.). 2002b. Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands.

Rasmussen, U., C. Johansson, and B. Bergman. 1994. Early communication in the Gunnera-Nostoc symbiosis: Plant-induced cell differentiation and protein synthesis in the cyanobacterium. Molec. Plant-Microbe Interact. 7:696–702.

Rasmussen, U., C. Johansson, A. Renglin, C. Petersson, and B. Bergman. 1996. A molecular characterization of the Gunnera-Nostoc symbiosis: Comparison with Rhizobium- and Agrobacterium-plant interactions. New Phytol. 133:391–398.

Rasmussen, U., and M. Svenning. 2001. Characterization by genotypic methods of symbiotic Nostoc strains isolated from five species of Gunnera. Arch. Microbiol. 176:204–210.

Rasmussen, U., and M. Nilsson. 2002. Cyanobacterial diversity and specificity in plant symbioses. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 313–328.

Read, D. J., J. G. Duckett, R. Francis, R. Ligrone, and A. Russel. 2000. Symbiotic fungal associations in "lower" land plants. Phil. Trans. R. Soc. Lond. B 355:815–831.

Redecker, D., R. Kodner, and L. E. Graham. 2000. Glomalean fungi from the Ordovician. Science 289:1920–1921.

Reinke, J. 1873. Untersuchungen über die Morphologie der Vegetationsorgane von Gunnera. *In:* J. Reinke (Ed.) Morphologische Abhandlungen. Verlag W. Engelman. Leipzig, Germany. 45–123.

Remy, W., T. N. Taylor, H. Hass, and H. Kerp. 1994. Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc. Natl. Acad. Sci. USA 91:11841–11843.

Renzaglia, K. S. 1982. A comparative developmental investigation of the gametophyte generation in the Metzgeriales (Hepatophyta). Bryophytorum Bibliotheca 24:1–238.

Rikkinen, J. 2002a. Cyanolichens: An evolutionary overview. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis. Kluwer Academic Publishers. Dordrecht, The Netherlands. 31–72.

Rikkinen, J., I. Oksanen, and K. Lohtander. 2002b. Lichen guilds share related cyanobacterial symbionts. Science 297:357.

Rodgers, G. A., and W. D. P. Stewart. 1974. Physiological interrelations of the blue-green alga Nostoc with the liverworts Anthoceros and Blasia. Br. Phycol. J. 9:223.

Rodgers, G. A., and W. D. P. Stewart. 1977. The cyanophyte-hepatic symbiosis. I: Morphology and physiology. New Phytol. 78:441–458.

Rowell, P., A. N. Rai, and W. D. P. Stewart. 1985. Studies on the nitrogen metabolism of the lichen Peltigera aphthosa and Peltigera canina. *In:* D. H. Brown (Ed.) Lichen Physiology and Cell Biology. Plenum Press. New York, NY. 145–160.

Saunders, R. M. K., and K. Fowler. 1993. The supraspecific taxonomy and evolution of the fern genus Azolla (Azollaceae). Plant Syst. Evol. 184:175–193.

Schaede, R. 1951. Über die Blaualgensymbiose von Gunnera. Planta 39:154–170.

Schenk, H. E. A. 1992. Cyanobacterial symbioses. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. Berlin, Germany. 4:3819–3854.

Schnepf, E. 1964. Zur Feinstruktur von Geosiphon pyriforme. Arch. Mikrobiol. 49:112–131.

Schüßler, A., D. Mollenhauer, E. Schnepf, and M. Kluge. 1994. Geosiphon pyriforme, an endosymbiotic association of fungus and cyanobacteria: The spore structure resembles that of arbuscular mycorrhizal (AM) fungi. Bot. Acta 107:36–45.

Schüßler, A., E. Schnepf, D. Mollenhauer, and M. Kluge. 1995. The fungal bladders of the endocyanosis Geosiphon pyriforme, a Glomus-related fungus: Cell wall permeability indicates a limiting pore radius of only 0.5 nm. Protoplasma 185:131–139.

Schüßler, A., P. Bonfante, E. Schnepf, D. Mollenhauer, and M. Kluge. 1996. Characterization of the Geosiphon pyriforme symbiosome by affinity techniques: Confocal laser scanning microscopy (CLSM) and electron microscopy. Protoplasma 190:53–67.

Schüßler, A. 2000. Glomus claroideum forms an arbuscular mycorrhiza-like symbiosis with the hornwort Anthoceros punctatus. Mycorrhiza 10:15–21.

Schüßler, A., and M. Kluge. 2001. Geosiphon pyriformis, an endocytosymbiosis between fungus and cyanobacteria, and its meaning as a model system for arbuscular mycorrhizal research. *In:* B. Hock (Ed.) The Mycota. Springer-Verlag. New York, NY. 9:151–161.

Schüßler, A., T. Meyer, H. Gehrig, and M. Kluge. 1997. Variations of lectin binding sites in extracellular glycoconjugates during the life cycle of Nostoc punctiforme, a potentially endosymbiotic cyanobacterium. Eur. J. Phycol. 32:233–239.

Schüßler, A., D. Schwarzott, and C. Walker. 2001. A new fungal phylum, the Glomeromycota: Phylogeny and evolution. Mycol. Res. 105:1413–1421.

Schüßler, A. 2002. Molecular phylogeny, taxonomy, and evolution of Geosiphon pyriformis and arbuscular mycorrhizal fungi. Plant Soil 244:75–83.

Schwarzott, D., C. Walker, and A. Schüßler. 2001. Glomus, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is non-monophyletic. Molec. Phylogenet. Evol. 21:190–197.

Sergeeva, E., A. Liaimer, and B. Bergman. 2002. Evidence for biosynthesis and release of the phytohormone indole-3-acetic acid by cyanobacteria. Planta 215:229–238.

Serrano, R., F. Carrapico, and R. Vidal. 1999. The presence of lectins in bacteria associated with the Azolla-Anabaena symbiosis. Symbiosis 27:169–178.

Shannon, B. T., J. E. Gates, and S. M. McCowen. 1993. DNA base composition of eubacteria isolated from 4 species of Azolla. Symbiosis 15:165–175.

Silvester, W. B., and D. R. Smith. 1969. Nitrogen fixation by Gunnera-Nostoc symbiosis. Nature 224:1231.

Silvester, W. B. 1976a. Endophyte adaptation in Gunnera-Nostoc symbiosis. *In:* P. S. Nutman (Ed.) Symbiotic Nitrogen Fixation in Plants. Cambridge University Press. Cambridge, UK. 521–541.

Silvester, W. B., and P. J. McNamara. 1976b. The infection process and ultrastructure of the Gunnera-Nostoc symbiosis. New Phytol. 77:135–141.

Silvester, W. B., R. Parsons, and P. W. Watt. 1996. Direct measurement of release and assimilation of ammonia in the Gunnera-Nostoc symbiosis. New Phytol. 132:617–625.

Singh, P., and D. Singh. 1997. Azolla-Anabaena symbiosis. *In:* K. Ddarwal (Ed.) Biotechnological Approaches in Soil Microorganisms for Sustainable Crop Production. Scientific Press. Jodhpur, India. 93–107.

Smith, D. C., and A. E. Douglas. 1987. The Biology of Symbiosis. Edward Arnold. London, UK.

Smith, S. E., and D. J. Read. 1997. Mycorrhizal Symbiosis, 2nd ed. Academic Press. London, UK.

Söderbäck, E., P. Lindblad, and B. Bergman. 1990. Developmental patterns related to nitrogen fixation in the Nostoc-Gunnera magellanica Lam. symbiosis. Planta 182:355–362.

Söderbäck, E. 1992a. Developmental Patterns in the Nostoc-Gunnera Symbiosis [doctoral thesis]. Stockholm University. Stockholm, Sweden.

Söderbäck, E., and B. Bergman. 1992b. The Nostoc-Gunnera magellanica symbiosis: Phycobiliproteins, carboxysomes and Rubisco in the microsymbiont. Physiol. Plant. 8:425–432.

Söderbäck, E., and B. Bergman. 1993. The Nostoc-Gunnera symbiosis: Carbon fixation and translocation. Physiol. Plant. 89:125–132.

Solheim, B., and M. Zielke. 2002. Associations between cyanobacteria and mosses. *In:* A. N. Rai, B. Bergman, and U. Rasmussen (Eds.) Cyanobacteria in Symbiosis.

Kluwer Academic Publishers. Dordrecht, The Netherlands. 137–152.

Steinberg, N. A., and J. C. Meeks. 1989. Photosynthetic CO_2 fixation and ribulose bisphosphate carboxylase/oxygenase activity of Nostoc sp. strain UCD 7801 in symbiotic association with Anthoceros punctatus. J. Bacteriol. 171:6227–6233.

Steinberg, N. A., and J. C. Meeks. 1991. Physiological sources of reductant for nitrogen fixation activity in Nostoc sp. strain UCD 7801 in symbiotic association with Anthoceros punctatus. J. Bacteriol. 173:7324–7329.

Stewart, W. D. P., and G. A. Rogers. 1977a. The cyanophyte-hepatic symbiosis. II: Nitrogen fixation and the interchange of nitrogen and carbon. New Phytol. 78:459–471.

Stewart, W. D. P., and P. Rowell. 1977b. Modifications of nitrogen-fixing algae in lichen symbiosis. Nature (Lond.) 265:371–372.

Stewart, W. D. P., P. Rowell, and A. N. Rai. 1983. Cyanobacteria-eukaryotic plant symbioses. Ann. Microbiol. Inst. Pasteur 134B:205–228.

Stock, P. A., and W. B. Silvester. 1994. Phloem transport of recently fixed nitrogen in the Gunnera-Nostoc symbiosis. New Phytol. 126:259–266.

Stocker-Wörgötter, E., and R. Turk. 1994. Artificial resynthesis of the photosymbiodeme Peltigera leucophlebia under laboratory conditions. Cryptogr. Bot. 4:300–308.

Stocker-Wörgötter, E. 1995. Experimental cultivation of lichens and lichen symbionts. Can. J. Bot. 73S:579–589.

Tabita, F. R. 1994. The biochemistry and molecular regulation of carbon dioxide metabolism in cyanobacteria. *In:* D. A. Bryant (Ed.) The Molecular Biology of Cyanobacteria. Kluwer Academic Publishers. Dordrecht, The Netherlands. 437–467.

Tandeau de Marsac, N. 1994. Differentiation of hormogonia and relationships with other biological processes. *In:* D. A. Bryant (Ed.) The Molecular Biology of Cyanobacteria. Kluwer Academic Publishers. Amsterdam, The Netherlands. 825–842.

Tehler, A., J. S. Farris, D. L. Lipscomb, and M. Kallersjo. 2000. Phylogenetic analysis of the fungi based on large rDNA data sets. Mycologia 92:459–474.

Tel-Or, E., T. Sandovsky, D. Kobiler, C. Arad, and R. Weinberg. 1983. The unique symbiotic properties of Anabaena in the water fern Azolla. *In:* G. C. Papageorgiou and L. Packer (Eds.) Prokaryotes: Cell Differentiation and Function. Elsevier Science. New York, NY. 303–314.

Towata, E. M. 1985. Morphometric and cytochemical ultrastructural analyses of the Gunnera kaalensis/Nostoc symbiosis. Bot. Gaz. 146:293–301.

Uheda, E., and S. Kitoh. 1991. Electron microscopic observations of the envelopes of isolated algal packets of Azolla. Can. J. Bot. 69:1418–1419.

Uheda, E., and W. B. Silvester. 2001. The role of papillae during the infection process in the Gunnera-Nostoc symbiosis. Plant Cell Physiol. 42:780–783.

Van Coppenolle, B., I. Watanabe, C. Van-Hove, G. Second, N. Huang, and S. R. McCouch. 1993. Genetic diversity and phylogeny analysis of Azolla based on DNA amplification by arbitrary primers. Genome 36:686–693.

Van Coppenolle, B., S. R. McCouch, I. Watanabe, N. Huang, and C. Van Hove. 1995. Genetic diversity and phylogeny analysis of Anabaena azollae based on RFLP detected in Azolla-Anabaena azollae DNA complexes using nif gene probes. Theor. Appl. Genet. 91:589–597.

Van Hove, C., and A. Lejeune. 1996. Does Azolla have any future in agriculture? *In:* M. Rahman (Ed.) Biological nitrogen fixation associated with rice production. Kluwer Academic Press. Dordrecht, The Netherlands. 83–94.

Veys, P., L. Waterkeyn, A. Lejeune, and C. Van Hove. 1999. The pore of the leaf cavity of Azolla: Morphology, cytochemistry and possible functions. Symbiosis 27:33–57.

Veys, P., A. Lejeune, and C. Van Hove. 2000. The pore of the leaf cavity of Azolla: Interspecific morphological differences and continuity between the cavity envelopes. Symbiosis 29:33–47.

Veys, P., A. Lejeune, and C. Van Hove. 2002. The pore of the leaf cavity of Azolla: Teat-cell differentiation and cell wall projections. Protoplasma 219:31–42.

Von Wettstein, F. 1915. Geosiphon Fr. v. Wettst., eine neue, interessante Siphonee. sterr. Bot. Z. 65:145–156.

Wallace, W. H., and J. E. Gates. 1986. Identification of eubacteria isolated from leaf cavities of 4 species of the N-fixing Azolla fern as Arthrobacter Conn and Dimmick. Appl. Environ. Microbiol. 52:425–429.

Wang, C.-M., M. Ekman, and B. Bergman. 2004. Expression of cyanobacterial genes involved in heterocyst differentiation and dinitrogen fixation along a plant symbiosis development profile. Molec. Plant-Microbe Interact. 17(4):436–443.

Wanntorp, L., H.-E. Wanntorp, B. Oxelman, and M. Källersjö. 2001. Phylogeny of Gunnera. Plant Syst. Evol 226:85–107.

Watanabe, I., M. T. Lapis-Tenorio, T. S. Ventura, and B. C. Padre. 1993. Sexual hybrids of Azolla filiculoides with A. microphylla. Soil Sci. Plant Nutr. 39:669–676.

Watanabe, I. 1994. Genetic enhancement and Azolla collection-problems in applying Azolla-Anabaena symbiosis. *In:* N. A. Hegazi, M. Fayez, and M. Monib (Eds.) Nitrogen Fixation with Non-legumes. The American University in Cairo Press. Cairo, Egypt. 437–450.

Watanabe, I., and C. Van Hove. 1996. Phylogenetic, molecular, and breeding aspects of Azolla-Anabaena symbiosis. *In:* J. M. Camus, M. Gibby, and R. J. Jones (Eds.) Pteridology in Perspective. Royal Botanic Gardens. Kew, UK. 611–619.

Watts, S. D., C. D. Knight, and D. G. Adams. 1999. Characterisation of plant exudates inducing chemotaxis in nitrogen-fixing cyanobacteria. *In:* G. A. Peschek, W. Löffelhardt, and G. Schmetterer (Eds.) The Phototrophic Prokaryotes. Kluwer Academic/Plenum Publishers. New York, NY. 679–684.

Watts, S. 2001. Signalling in the Nostoc-plant Symbiosis [PhD thesis]. University of Leeds. Leeds, UK.

Wei, W. X., G. Y. Jin, N. Zhang, and J. Chen. 1988. Studies of hybridization in Azolla. *In:* K. H. Singh, and K. U. Kramer (Eds.) Proceedings of the International Symposium on Systematic Pteriodology. China Sciientific and Technical Press. Beijing, China. 135–139.

West, N., and D. G. Adams. 1997. Phenotypic and genotypic comparison of symbiotic and free-living cyanobacteria from a single field site. Appl. Environ. Microbiol. 63:4479–4484.

West, N. J., D. G. Adams, P. R. Sisson, R. Freeman, and P. M. Hawkey. 1999. Pyrolysis mass spectrometry analysis of free-living and symbiotic cyanobacteria. Ant. v. Leeuwenhoek 75:201–206.

Wolk, C. P. 2000. Heterocyst formation in Anabaena. *In:* Y. V. Brun and L. J. Shimkets (Eds.) Prokaryotic Development. ASM Press. Washington, DC. 83–104.

Wong, F. C. Y., and J. C. Meeks. 2001. The hetF gene product is essential to heterocyst differentiation and affects HetR function in the cyanobacterium Nostoc punctiforme. J. Bacteriol. 183:2654–2661.

Wong, F. C. Y., and J. C. Meeks. 2002. Establishment of a functional symbiosis between the cyanobacterium Nostoc punctiforme and the bryophyte Anthoceros punctatus requires genes involved in nitrogen control and initiation of heterocyst differentiation. Microbiology 148:315–323.

Wouters, J., S. Janson, and B. Bergman. 2000. The effect of exogenous carbohydrates on nitrogen fixation and hetR expression in Nostoc PCC 9229 forming symbiosis with Gunnera. Symbiosis 28:63–76.

Yoshimura, I., and Y. Yamamoto. 1991. Development of Peltigera praetextata lichen thalli in culture. Symbiosis 11:109–117.

Zheng, W. W., M. Nilsson, B. Bergman, and U. Rasmussen. 1999. Genetic diversity and classification of cyanobacteria in different Azolla species by the use of PCR fingerprinting. Theor. Appl. Genet. 99:1187–1193.

Zhou, R., and C. P. Wolk. 2002. Identification of an akinete marker protein in Anabaena variabilis. J. Bacteriol. 184:2529–2532.

Zimmerman, W. J., I. Watanabe, and T. A. Lumpkin. 1991. The Anabaena-Azolla symbiosis: Diversity and relatedness of neotropical host taxa. Plant Soil 137:167–170.

Prokaryotes (2006) 1:364–402
DOI: 10.1007/0-387-30741-9_15

CHAPTER 2.2

Symbiotic Associations Between Ciliates and Prokaryotes

HANS-DIETER GÖRTZ

Introduction

Ciliates are frequently colonized by bacteria. This is partly due to the cytology and biology of these highly evolved heterotrophic protozoa (Hausmann and Bradbury, 1996). As phagotrophic predators on microorganisms, they bear a high risk of microbial, namely bacterial, infections: ingested bacteria may resist digestion, escape from the phagosomes, and persist in the cells as endocytobionts. Traditionally, intracellular bacteria in ciliates have been termed "symbionts" (Preer et al., 1974). Phagocytosis appears to be the usual way infectious bacteria enter their host cells. In addition to intracellular bacteria, ectosymbionts may be intimately associated with ciliates. The most intriguing example is certainly the epixenosomes of *Euplotidium*, which even defend their host against predators (Petroni et al., 2000; see also "Terms in Symbiosis Research").

The bacterial symbionts of ciliates comprise a variety of bacteria in many unrelated genera of different subgroups of proteobacteria. They are alike in that their habitat is the cytoplasm or the nucleus of a ciliate cell. Many of these symbionts appear to be well adapted to their environment; they are no longer free living and have genomes that are reduced in size, indicating a lengthy period of symbiont-host association. Most of the symbionts are not infectious, but a few are *Holospora*: Infectious Bacteria of the Nuclei. These infectious symbionts have developed specific features that guarantee uptake and transport to the intracellular sites where they can multiply. In most cases, it is not clear whether the symbionts provide their hosts with a selective advantage; under laboratory conditions, most of the symbionts have proved to be dispensable. On the other hand, it is significant that the majority of cells of *Paramecium biaurelia* freshly collected from nature contain symbionts (Beale et al., 1969), and work by Landis (Landis, 1981; Landis, 1987) and Kusch (2001) gives evidence that paramecia that bear bacteria of the genus *Caedibacter* (formerly called "kappa particles") have a selective advantage over those that are symbiont-free, see "The Killer Trait in *Paramecium*". Moreover, *Polynucleobacter necessarius* (formerly called "omikron particles") and the closely related omikron-like symbionts occurring in several freshwater *Euplotes* species have been shown to be necessary for survival of their hosts (Heckmann, 1975; Heckmann et al., 1983b; see also "Prokaryotic Symbionts of *Euplotes*"). Since in these cases the hosts depend on their symbionts and those in turn depend upon their hosts, they are no longer free living—the distinction between "symbiont" on the one hand and "organelle" on the other hand becomes blurred.

Ciliates can usually be handled easily, and some of them—particularly species of the *Paramecium aurelia* complex—have been investigated so thoroughly that they can be manipulated in a variety of ways. This has led to a wealth of information about their symbionts that has been reviewed extensively by Preer et al. (1974), Soldo (1974a), Quackenbush (1988), and Pond et al. (1989). It is not possible to list all the types of symbionts of ciliates encountered. Their number is very large, and certainly many more will be added to this list. Few of them, however, have been deposited in stock cultures and have been investigated in such a way that they can easily be identified when found again. An even smaller number have been described with validated binomial names, in keeping with the international rules of nomenclature. Especially these last ones will be discussed in detail in this chapter. A lag phase in the description of new symbionts in ciliates for several years may be partly due to the fact that intracellular bacteria could not be investigated with classical microbiological methods, since they cannot be grown outside their host cells. With the new powerful techniques for detection and phylogenetic classification such as polymerase chain reaction (PCR) and fluorescence in situ-hybridization (FISH) now available, this is changing.

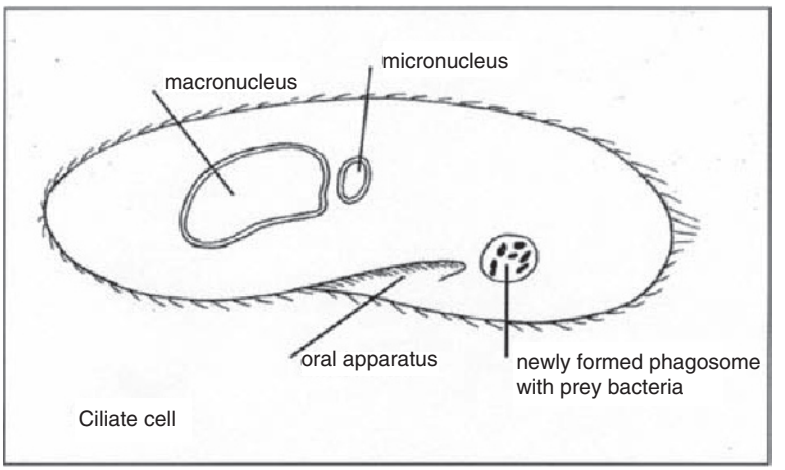


Fig. 1. The ciliate cell. Both macronucleus and micronucleus are encircled by the nuclear envelope with nuclear pores (not shown). Note that the nuclear envelopes have a perinuclear space that may be colonized by certain bacteria. The oral apparatus/cytostome is the only site where phagocytosis takes place.

The Ciliate Cell as a Microcosm

Ciliates—phylum Ciliophora—are large protozoa that offer a variety of suitable habitats within their cells with plenty of space for bacteria (Fig. 1). The ciliate cell is characterized by a highly organized cortex consisting of the plasma membrane, flat vesicles termed "alveoles" underneath the plasma membrane, and a network of microtubules and other cytoskeletal elements. Cilia, the motile organelles of ciliates, are anchored to the cytoskeleton.

Ciliates are heterotrophic. Because of their solid cortex of three membranes and multiple cytoskeletal systems, phagocytosis occurs only at a preformed structure termed the "oral apparatus." This is a complex metaorganelle consisting of a funnel-like pouch with batteries of cilia and a highly ordered cytoskeletal basket at the bottom of which new phagosomes are formed. Newly formed phagosomes are acidified and fuse with primary lysosomes (Fok and Allen, 1988). As known from other professional phagocytes such as macrophages, ingested bacteria are attacked by acidification, oxidative burst, and lysosomal enzymes; see The Nuclei of Ciliates. In spite of these attacks, certain bacteria with appropriate features may survive and even escape from the phagosomes. In the cytoplasm of ciliates, bacteria may prevail in symbiontophorous vesicles or naked (i.e., not encircled by host membranes; Sitte, 1993). Symbionts may also live in the large, somatic macronuclei as well as in the much smaller, generative micronuclei. Of those bacteria that are, however, nuclear-specific, certain species are only found in micronuclei, and others only in macronuclei, of their specific hosts. Bacteria have even been found in the perinuclear space and in the endoplasmic reticulum (Fokin and Karpov, 1995). The ciliate cell thus may be regarded as a microcosm that might contain even different symbionts at any given time.

The Nuclei of Ciliates

Characteristically ciliates have two types of nuclei: small generative micronuclei and large somatic macronuclei (Paulin, 1996). Ciliates multiply by binary division and sexually propagate by conjugation. The latter includes meiosis of the micronucleus, resorption of the old macronucleus, and formation of a new macronucleus from the synkaryon that will also give rise to the new micronucleus. Whereas cytoplasmic symbionts may be simply distributed to daughter cells during binary division, endonuclear symbionts may even make use of the nuclear division machinery. Both micronuclear mitosis and macronuclear division are closed: the nuclear envelopes are maintained, keeping nucleoplasm and cytoplasm separated throughout the cell cycle. Endonuclear symbionts (see "Terms in Symbiosis Research") are therefore caught in the nuclei unless they have developed means of passing through the nuclear envelopes. During conjugation of the host cell, resorption of the old macronucleus may be deleterious to endonuclear symbionts that are not adapted to this process; bacteria are then digested in the process of resorption. New macronuclei developing after conjugation are free of bacteria unless they are infected anew.

In spite of the problems arising for bacteria colonizing nuclei, endonuclear symbionts are frequently found in ciliates because in the nuclei, symbionts should: 1) have access to the most complete supply of metabolites, (2) be more assured of distribution/segregation to the daughter cells at cell division, and (3) be pro-

tected from cellular defense mechanisms. The last reason is more obvious when the fact that symbionts in nuclei are naked—not encircled by host membranes—and less vulnerable to attacks by lytic enzymes, which would be deleterious to nuclear structures such as chromatin, is considered.

The History of Symbiont Research in Ciliates

Prokaryotes living in ciliates were first noticed over a century ago by J. Müller (1856). Rod-shaped structures were observed in the macronuclei and micronuclei of a number of ciliates, and less commonly, in their cytoplasm. In the beginning, it was not clear whether they were parasites or spermatozoa, because the micronucleus was considered to be a testis and the macronucleus an ovary, while chromosome filaments and endonuclear symbionts were mistaken for spermatozoa. This view was corrected by Bütschli (1876), who also wrote the first review on parasites in ciliates (Bütschli, 1889). Accounts of early observations of bacteria in protozoa that followed this initial period were reviewed by Kirby (1941), Wichterman (1953), and Ball (1969).

Interest in prokaryotic symbionts of ciliates arose again in the 1950s when it was discovered that a killer phenotype is frequently associated with them. The possibility that certain ciliate strains kill other strains by liberating a toxic agent into the medium was first expressed by Sonneborn (1938), who noted this phenomen in paramecia during experiments on mating types that involved mixing different strains (see "The Killer Trait in *Paramecium*"). He found that under certain conditions, conjugation could be brought about between killers and sensitive paramecia so that genetic analysis of these traits became technically feasible. Sonneborn (1943) demonstrated that the killer phenotype was an inherited trait transmitted via cytoplasmic particles, which he named "kappa." His findings aroused great interest among geneticists and other biologists because it furnished one of the first clear examples of a cytoplasmic genetic factor. That kappa was an endosymbiont was not known at that time. From data obtained in studies using X-rays, Preer (1948b) determined that kappa was similar in size to bacteria. He subsequently demonstrated the presence of kappa in the cytoplasm of killer paramecia as Feulgen-staining bodies (Preer, 1950). In the following years, cytological, biochemical, and physiological studies by a number of workers established that kappa was actually a Gram-negative bacterium. In 1974, it was given a binomial designation—*Caedobacter taeniospiralis*, which has since been changed to *Caedibacter taeniospiralis* (for a detailed review, see Preer et al., 1974, and Preer and Preer, 1984).

After the initial discovery of the first killer paramecia, other types were found. Siegel (1953) described "mate killers," whose toxins act only after cell-to-cell contact is made during conjugation, and Schneller (1958) described "rapid-lysis" killers, which may injure sensitives in 10 min and kill them in 30 min, a process that is much more rapid than when kappa mediates killing. Over decades, bacterial symbionts had been studied most extensively in the *Paramecium aurelia* species complex (for reviews, see Beale et al., 1969; Preer et al., 1972, 1974; Preer and Preer, 1984; Gibson, 1974; and Soldo, 1974a). They have also been found and studied in other ciliates, particularly in *Paramecium caudatum* (for reviews, see Ossipov, 1981; Görtz, 1983, 2001) and in *Euplotes* species (reviewed by Heckmann, 1983a; see "Prokaryotic Symbionts of *Euplotes*"). Many cases of simultaneous infections of ciliate cells by two or more different bacteria have been observed (Görtz, 1987, 1992a; Görtz unpublished). In each of these killer paramecia, particles were found that later proved to be prokaryotic symbionts. One only needed to wash the paramecia free of bacteria, crush the ciliates, and observe the resulting preparations in a phase contrast microscope to ascertain the presence of the symbionts, see "Identification of Symbionts in *Paramecium*". This procedure also revealed, however, that some strains carry endosymbionts without showing any kind of killing ability. The symbionts of nonkiller paramecia were named "nu" (Sonneborn et al., 1959b).

The presence of endo- and episymbionts of marine and freshwater ciliates living in anaerobic habitats has been generating increasing interest among ecologists and microbiologists. In these habitats, many of the symbionts were identified as methanogenic bacteria (Stumm and Vogels, 1989; Fenchel and Finlay, 1991a; Finlay and Fenchel, 1992; see "Prokaryotic Symbionts of Ciliates from Anaerobic Environments").

We may expect to find many more prokaryotic symbionts of ciliates, once further host species are studied. The description of episymbionts that have a defensive function for their host *Euplotidium* by Rosati, Verni, and colleagues (Rosati, 1999a) is a fantastic example, see "Prokaryotic Ectosymbionts of *Euplotidium*". Only recently these episymbionts have been identified as belonging to the *Verrucomicrobia* (Petroni et al., 2000).

Terms in Symbiosis Research

The following terms are used in this chapter or in the references cited:

Symbiosis—the living together of dissimilarly named organisms. Sometimes the term is used in a restricted sense in the sense of mutualism
Mutualism—associations which involve mutual benefit (symbiosis in its restricted sense)
Parasitism—associations in which there is overt exploitation of one associate
Endosymbiosis—the symbiont lives within its host, intracellular symbiosis
Episymbiosis—the symbiont lives attached to the surface of its host cell
Episymbiont—microorganism living attached to the surface of a host cell
Epibiont—synonymous with episymbiont
Ectosymbiont—synonymous with episymbiont
Xenosome—intracellular symbiont in a broad sense
Endocytobiont—intracellular symbiont in a broad sense of symbiosis
Epixenosome—episymbiont in a broad sense

For definitions of the terms, see Henry (1966), Read (1970), Corliss (1985), Margulis and Fester (1991), Sitte (1993), and Rosati (1999a).

Prokaryotic Symbionts of *Paramecium*

With respect to endosymbionts, *Paramecium* is by far the best-studied ciliate genus. In the *Paramecium aurelia* species complex, consisting of 14 sibling species described by Sonneborn (1975) and named *P. primaurelia* to *P. quadecaurelia*, many different types of endosymbionts have been discovered. They have been thoroughly reviewed by Preer et al. (1974) and descriptions appeared in earlier editions of this book (Preer, 1981; Heckmann and Görtz, 1991). Valuable information on isolation and identification of symbionts is taken from these earlier editions. In the present edition, descriptions of further endosymbionts were added and the information about many symbionts has increased owing to the availability of new techniques. Among the symbionts where a wealth of data has been presented are the *Holospora* bacteria. They differ from most of the *Paramecium* symbionts in being infectious and in their ability to invade nuclei for reproduction. The holosporas are being investigated to elucidate the mechanisms that allow a prokaryote to invade a eukaryote. Being relatively large, the holospora can be seen even at relatively low magnification and are therefore used to monitor their route of infection and the changes they undergo after entering a cell.

Habitat and Biology of the Bacterial Symbionts of *Paramecium*

Paramecia are aerobic ciliates from fresh water and brackish water habitats. Many of the paramecia brought in from nature are found to contain symbionts. Usually, there are hundreds and sometimes even thousands of symbionts per paramecium (see Figs. 2 and 3). Endosymbionts may be found in the micronucleus, macronucleus, perinuclear space and cytoplasm of paramecia. Different types of symbiont invade different parts of the ciliate cell and they are, moreover, often adapted to one *Paramecium* species only. The cell compartment in which a symbiont multiplies and the *Paramecium* species in which multiplication occurs are therefore important taxonomic characters. Though it had been observed that bacterial endosymbionts may prevent secondary infections by another bacterium in laboratory cultures (Ossipov et al., 1975; Görtz, 1983), double and even triple infections are found in cells of natural populations (Görtz, 1992a; Kusch et al., 2000).

Several symbionts have been shown to require the presence of specific *Paramecium* genes for

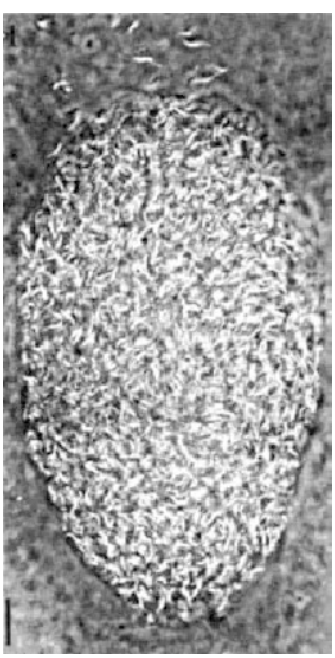


Fig. 2. Vegetative macronucleus of *Paramecium biaurelia* stock 562. The spiral endosymbionts filling the macronucleus are cells of *Holospora caryophila*. A few symbionts are also visible in the cytoplasm. Osmium-lacto-orcein preparation. Whole mount, bright phase contrast. Bar = 10 μm. From Preer (1969).

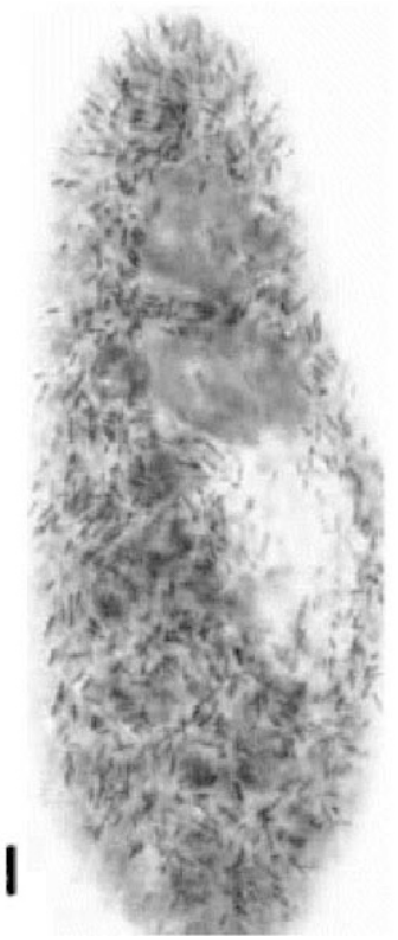


Fig. 3. *Paramecium tetraurelia* stock 239 bearing endosymbiont *Lyticum flagellatum*, seen as dark-staining rods in the cytoplasm. Osmium-lacto-orcein preparation, whole mount, dark phase contrast. Bar = 10 µm. From Preer et al. (1974).

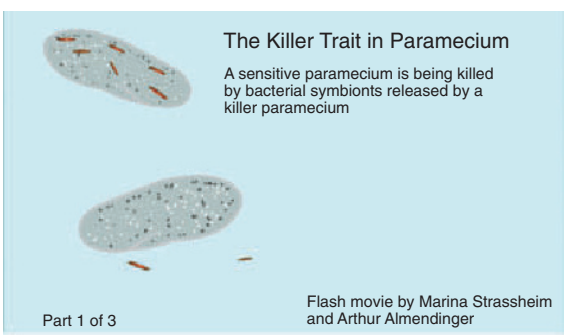
their maintenance (Sonneborn, 1943; Siegel, 1953; Schneller et al., 1959; Gibson and Beale, 1961; Fujishima and Fujita, 1985). lt is not known whether the genes that assure maintenance of the symbionts are active—e.g., providing the symbionts with some essential metabolite—or whether they are merely inactive alleles, the active ones preventing growth of an "invader" (Preer et al., 1974). In this connection, it should be mentioned that the species 3, 7, 9, 10, 11, 12, 13 and 14 have never been found to contain symbionts (Preer and Preer, 1984); however, not all species of the *P. aurelia* complex have been studied with the same thoroughness. lt has been argued that symbionts profit from living inside a paramecium by being better protected from digestion, as compared with free-living species of bacteria, and that symbionts are provided with a convenient and abundant supply of nutrients (Beale et al., 1969). Which metabolites of the host are used, however, is not known.

Although many of the symbionts have a smaller genome size than free-living bacteria (Soldo and Godoy, 1973a) and some of the associations were suggested to be very ancient (Preer, 1977), no indications for a transfer of genes from symbiont to host nucleus have been discovered as has been found in the case of mitochondria (Gellissen and Michaelis, 1987). Schmidt (1984), studying the association of *Caedibacter varicaedens* with *P. biaurelia*, was unable to obtain evidence for a sharing of the translational systems of host and symbiont. His observations indicate that all major proteins found in *Caedibacter* are synthesized in the symbiont itself.

In only a few cases, it has been demonstrated that host cells may profit from bearing symbionts; Soldo (1963) and Soldo and Godoy (1973b) found that it was not necessary to provide a *Paramecium* stock bearing *Lyticum flagellatum* (formerly called "lambda particles") with folic acid, while the same stock freed of this symbiont required the vitamin. Holtzman (1959) observed that *P. pentaurelia* bearing *Pseudocaedibacter falsus* was more resistant to killer paramecia bearing *Lyticum flagellatum* than *P. pentaurelia* strains that were free of symbionts, and Landis (Landis, 1981; Landis, 1987) showed that under natural conditions, paramecia with killer properties have a selective advantage over nonkillers, see "The Killer Trait in *Paramecium*". On the other hand, in the laboratory, the symbionts are all dispensable and many of them are lost when paramecia are cultured for some time. The reason for such loss is in most cases a rapid multiplication of the paramecia, resulting in a dilution of the symbionts and then in their loss. Although there is little information about what occurs in nature, it appears unlikely that reinfection of paramecia that have lost their intracellular bacteria plays a major role for most of the symbionts (Kusch et al., 2001).

Most symbionts of ciliates are not infectious and are propagated in the host population with the divisions of host cells only. An exception to this rule is provided by symbionts of the Holosporaceae. In addition to being propagated with host cell division, they develop forms specialized for infection (infectious forms) that are released and infect new cells upon being taken up with food. Unlike most other endosymbionts, they tend to harm their hosts and can therefore be regarded as parasites, although it cannot be excluded that under certain conditions, the host cells may have advantages from the presence of these symbionts (Görtz, 1983). Interactions between *Holospora* and its host *Paramecium* are described in more detail in the section entitled "Biology of the Holosporaceae."

THE KILLER TRAIT IN *PARAMECIUM* Many of the symbionts of *Paramecium* confer on their hosts the ability to produce toxins capable of killing sensitive *Paramecium* strains of the same species and even of other species, if the toxins are liberated into the medium. The toxin producers are called "killers" and their victims "sensitives." If the toxins act only during the period of cell-to-cell contact at conjugation, the toxin producers are called "mate killers." Different killer stocks of *Paramecium* induce different prelethal symptoms in sensitives mixed with killers. These symptoms include spinning, vacuolization, paralysis, formation of aboral humps, and rapid lysis. The symptoms were taken as crucial characters for identification of a symbiont. The value of such symptoms for identification and determination of the symbionts must, however, be doubted, see "Identification of Symbionts in *Paramecium*". In addition to making their hosts capable of producing toxins, the symbionts also confer upon the hosts specific resistance to the toxins produced. When a symbiont is lost from a killer strain, the paramecia lose both toxin production and toxin resistance (Sonneborn, 1959a).



Video sequence showing killer trait. For the video, see the online version of *The Prokaryotes.*

Killer strains have been reported not only for species of the *P. aurelia* complex but also for *P. caudatum* (Schmidt et al., 1987b; Schmidt et al., 1988), *P. bursaria* (Chen, 1955; Dorner, 1957), and *P. polykaryum* (Takayanagi and Hayashi, 1964). In the latter two species, however, symbionts were not observed, although it is likely that they were present and were responsible for the killing properties of the paramecia.

A feature unique to the killer symbionts of the genus *Caedibacter* is the ability to produce R bodies. These are proteinaceous ribbons, 20–30 μm long, coiled inside the bacterial cell to form a hollow cylindrical structure (Figs. 4–6). This structure has a diameter of about 0.4 μm in all species and is about 0.4 μm long, except in *C. caryophilus* where the R bodies are approximately 0.8 μm in width and length. The R bodies unroll when ingested into a phagosome and also under certain in vitro conditions, e.g., when placed at low pH (Preer et al., 1966).

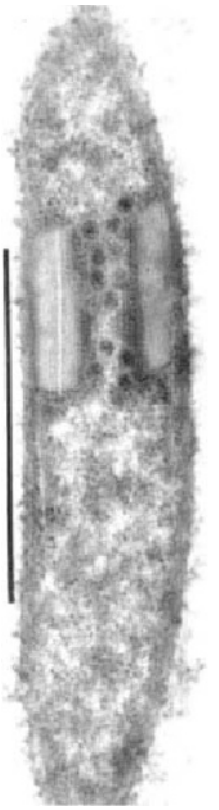


Fig. 4. Electron micrograph of longitudinal section of *Caedibacter varicaedens*, endosymbiont of *Paramecium biaurelia* stock 7 with an R body. Note the numerous dark-staining phages inside the coiled body. Bar = 1 μm. From Preer and Jurand (1968).

It has been suggested that the R bodies play an important role in the killing mediated by *Caedibacter*. Mueller (1963) and Smith-Sonneborn and Van Wagtendonk (1964) demonstrated that only *Caedibacter* particles that contained R bodies were toxic to sensitive paramecia. In addition, R bodies purified from certain strains of *C. varicaedens* have been shown to be toxic to sensitive paramecia (Preer and Preer, 1964; Preer et al., 1972). However, neither the toxin itself nor its mode of action have been identified, nor is it known how paramecia are protected from the toxic action of their own symbionts.

Early observations suggested that the genetic determinants of R bodies are plasmids or bacteriophages that have lost the ability to lyse their host cells—the symbiotic bacteria—upon maturation of the virions (Preer and Preer, 1967; Preer et al., 1974). Dilts (1976) isolated plasmid DNA from *C. taeniospiralis* 51 and suggested

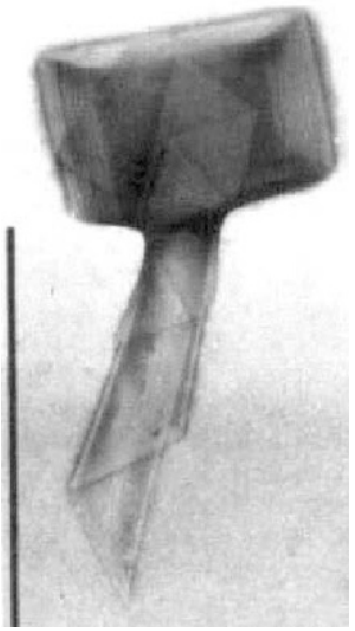


Fig. 5. Electron micrograph of an R body isolated from *Caedibacter taeniospiralis* of *Paramecium tetraurelia* stock 51. The R body begins to unroll from the inside. Negative staining with phosphotungstic acid. Bar = 1 μm. From Preer et al. (1972).

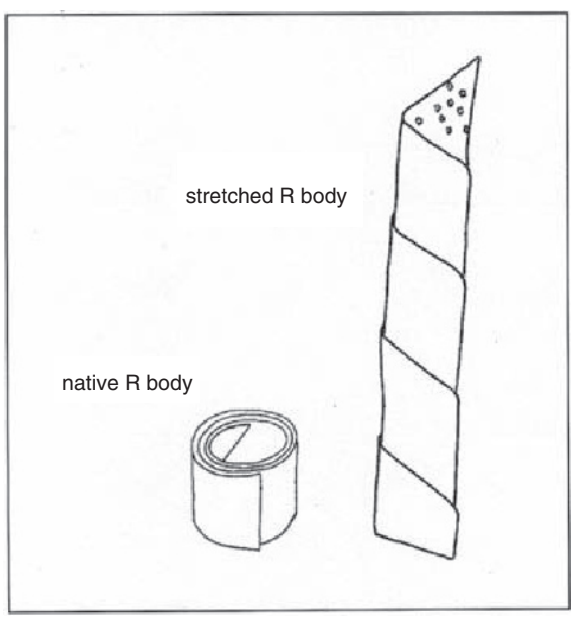


Fig. 6. R bodies of *Caedibacter*. R bodies (refractile bodies) are coiled proteinaceous ribbons. By appropriated triggers, such as acid pH, they are induced to unroll.

that the extrachromosomal DNA might be the determinant of the R bodies. Further investigation revealed that plasmids are present in all strains of *C. taeniospiralis* and that they are highly homologous as determined by restriction mapping (Quackenbush, 1983a). Further evidence that the genetic determinant for R-body synthesis resides on the plasmid was presented by Quackenbush and Burbach (1983b), who cloned portions of a plasmid and obtained expression of the R-body-encoding sequences in *Escherichia coli*. Analysis of various subclones allowed them to determine the approximate location of the R body-encoding sequence. The DNA required for type 51 R body synthesis is about 1.8 kbp in size and has been completely sequenced (see Pond et al., 1989; D. P. Heruth, 1987). However, none of the R body-producing *E. coli* clones was found to be toxic to sensitive paramecia. The DNA sequence required for toxin production has not yet been located. It is believed to reside also on the plasmid (Pond et al., 1989). The R bodies may unroll and destroy phagosomal membranes when ingested, and apparently they play a role in killing mediated by *Caedibacter* (Preer et al., 1974), but their presence is not required for the host cell to resist being killed.

R bodies of some *Caedibacter* species are associated with phage capsids (Fig. 4). The proteins of these R bodies and perhaps also the toxin are encoded on bacteriophage genomes (Quackenbush, 1988; Schmidt et al., 1988; Pond et al., 1989; Fokin and Görtz, 1993). Only *Caedibacter* bearing phages or plasmids may confer the killer trait to their hosts. Heruth et al. (1994) have characterized and sequenced three genes (*rebA*, *rebB* and *rebC*) for synthesis and assembly of R body synthesis in *C. taeniospiralis*. These genes are independent transcriptional units on a plasmid. Two polymerization events were found to be involved in R body assembly: one event requires RebB and RebC; the other requires all three proteins. The RebC protein is apparently involved in posttranslational modifications of RebA and RebB, both of them showing peptide species with different molecular weights. The role of a fourth protein whose gene has not been sequenced remains unknown (Kanabrocki et al., 1986; Heruth et al., 1994).

Dilts and Quackenbush (1986b) have provided evidence that R bodies are required for the killing trait to be expressed but not for resistance of the ciliate host to killing mediated by *C. taeniospiralis*. They described a mutant strain of *C. taeniospiralis* 169 that simultanously lost the ability to produce R bodies and to kill sensitive paramecia, but still rendered its host resistant to killing. Investigations of the R body-encoding plasmid isolated from the mutant revealed that a transposon-like element had been inserted within the R body-encoding region, thereby eliminating R body production. Two separate mutational events occurred in the same cell,

one inactivating the R body-encoding sequence and the other inactivating the toxin-encoding sequence. This shows that R bodies are crucial to expression of the killer trait. Their exact role remains, however, unknown. Evidence indicates that their action probably involves delivery of the toxin to the sensitive paramecium and to its target site by unrolling and penetrating the food vacuole membrane (Dilts, 1986a). Evidence that R bodies are required for killing but not for resistance of the host has also been obtained for *C. caryophilus*. Paramecia bearing mutant *C. caryophilus* with blocked R body synthesis no longer showed a killer trait but were resistant to the toxin (Schmidt et al., 1988).

The R bodies of other species of the genus were shown to be associated with icosahedral viral capsids (Preer and Jurand, 1968; Grimes and Preer, 1971). The capsids were in most cases found to contain DNA (Preer et al., 1971). The relationships between genomes of different phages of kappa and between R body-encoding plasmids and kappaphage genomes have been studied by restriction endonuclease analysis and by DNA-DNA hybridization (Quackenbush, 1978; Quackenbush et al., 1986). These studies demonstrate that the R body-encoding plasmids show little or no homology with kappaphages, and that there is also considerable diversity among the kappaphages.

R bodies have also been reported in the free-living bacteria *Pseudomonas taeniospiralis* (Lalucat et al., 1979) and *P. avenae* (Wells and Horne, 1983). However, with respect to antigenicity, genetic determinants, and other features, these R bodies appear to be unrelated to the R bodies of *Caedibacter* species (Bedingfield et al., 1984; Gibson, 1984; Meenaghan et al., 1984; Kanabrocki et al., 1986; Lalucat et al., 1986). These R bodies have also been reported for *Rhodospirillum centenum*, a nonsulfur photosynthetic bacterium (Favinger et al., 1989). Since R bodies are often present at low frequencies, it appears likely that R body-producing bacteria are more common than previously suspected. With respect to the shape of the ends and the mode of unrolling, the different types of R body vary (for details, see Preer et al., 1974; Quackenbush, 1988, and Pond et al., 1989).

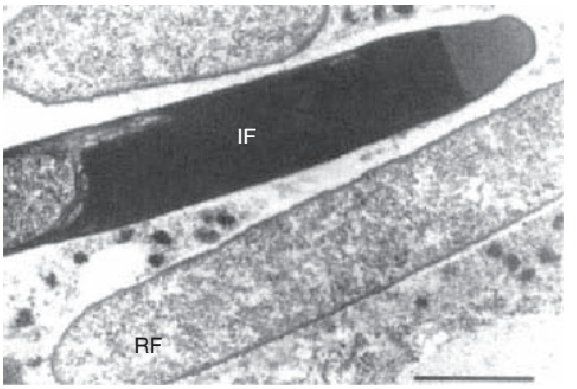


Fig. 7. *Holospora obtusa* in the macronucleus of *Paramecium caudatum*. IF, infectious form; and RF, reproductive form. Bar 1 μm. From Görtz et al. (1988).

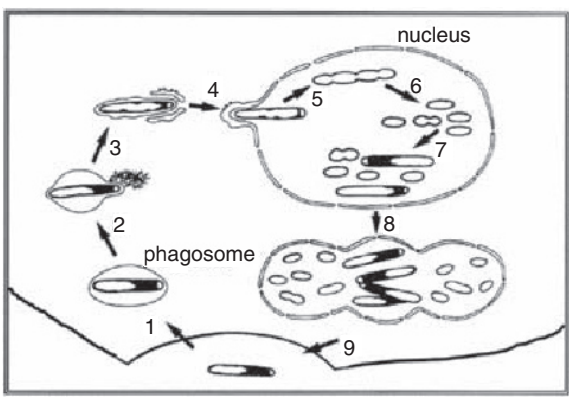
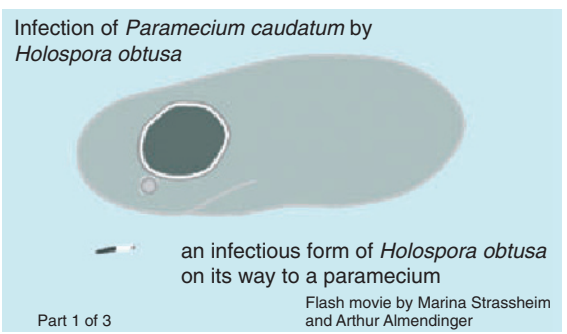


Fig. 8. Infection cycle of *Holospora obtusa*. 1) The infectious form of the bacterium (long rod) is ingested by a *Paramecium caudatum* cell into a phagosome (food vacuole). 2) Microfilaments are nucleated at the phagosomal membrane covering the bacterium protruding from the phagosome. 3) The bacterium is sluiced out of the phagosome. It remains encircled by a host membrane that forms a transport vesicle. Later on, the transport vesicle is surrounded by vesicles of endoplasmic reticulum. These vesicles finally form a secondary transport vesicle. 4) While the inner (phagosome-derived) membrane disintegrates, the membranes originating from endoplasmic reticulum fuse with the membranes of the nucleus, and the bacterium is incorporated into the macronucleus. 5) The nucleus of the bacterium constricts at several sites. 6) The bacterium divides into 4–10 short rods. By this multiple division, the reproductive form is established. 7) The reproductive form multiplies by binary fission. Some of the short rods grow longer and differentiate into infectious forms, see "Development of *Holospora*". 8) At the division of the host cell, mature infectious forms are collected in the connecting piece of the dividing nucleus and 9) are later released from the host cell (Ossipov, 1981; Görtz and Wiemann, 1989; Wiemann and Görtz, 1989).

Biology of Holosporaceae All *Holospora* species known up to the present are residents of the micro- or macronuclei of *Paramecium*; see The Ciliate Cell as a Microcosm. The bacteria grow and multiply exclusively in the host nuclei (Figs. 2 and 7) and have evolved mechanisms to invade nuclei as well as to escape from intact nuclei (Hafkine, 1890; Ossipov et al., 1975; Ossipov, 1981; Görtz, 1983; Fokin and Sabaneyeva, 1997; Fig. 8). *Holospora* species are host-specific, infecting only certain species of *Paramecium*, and nucleus-specific, infecting only the micronucleus or the macronucleus. Host cells remain vital with *Holospora* infections and it has been

shown that *Holospora*-bearing paramecia are able to grow, divide and even mate. However, conjugation is unsuccessful, as new macronuclei are apparently not functional (Görtz and Fujishima, 1983), and growth and physiological conditions of infected cells may be affected, depending on culture conditions. In starving *Paramecium* or after inhibition of host protein synthesis, most bacteria differentiate into the infectious form (Fujishima, 1993). Paramecia seem to have mechanisms to cure themselves from endonuclear symbionts. Namely, in "wrong" hosts, infectious forms are digested having been phagocytosed or, if they enter the nucleus, may later be lysed within the nucleus (Skoblo et al., 1990; Ossipov et al., 1993; Fokin and Skovorodkin, 1991; Fokin and Skovorodkin, 1997). This lysis may be due to an unknown mechanism of cellular defense. After lysis of bacteria in the nucleus, host cells remain vital nevertheless.



Video sequence showing infection of *Paramecium* by *Holospora*. For the video, see the online version of *The Prokaryotes.*

Not only the infection cycle but also the development of the Holosporaceae is unique. The bacteria show a developmental cycle with a long infectious form and a short reproductive form (Fig. 9). It is, however, not just the length that characterizes infectious forms, but also their specific organization. Even short forms are infectious (Kawai and Fujishima, 2000). The infectious form is unique among bacteria. It is polarly organized and has a voluminous periplasm (Figs. 7 and 10–12) that contains a number of stage-specific proteins, some of which appear to be released during the infection process (Görtz and Wiemann, 1989a; Dohra et al., 1994; Dohra et al., 1997; Dohra and Fujishima, 1999a; Fujishima et al., 1990a; Wiemann and Görtz, 1991). The cytoplasm is condensed and located toward one end of the symbiont, while a voluminous periplasmic area is located mainly at the other end. The periplasm consists of fine granular, strongly osmiophilic material. Some less osmiophilic material is located at the end, distal from the cytoplasm. Obviously, the infectious form is physiologically inactive. It is a resting stage that may remain vital and infectious outside host cells for days to weeks. Infectious forms were therefore regarded as spores ("holospores;" Hafkine, 1890; Ossipov, 1981). The mode of development and reactivation is, however, different from formation and hatching of endospores (Görtz and Wiemann, 1989a; Fig. 11).

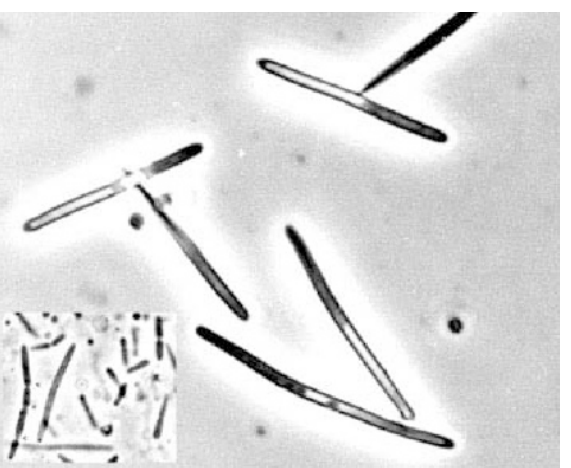


Fig. 9. Phase contrast micrograph of *Holospora obtusa* isolated from *Paramecium caudatum*. Note the peculiar appearance of the infectious forms reflecting their fine structure (Fig. 8). Inset: Developmental stages of *Holospora obtusa*. At the left a bacterium with three constrictions (arrowheads) undergoing multiple division is seen.

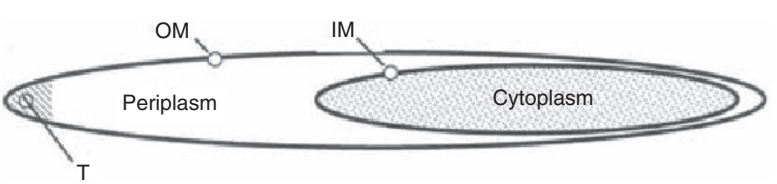


Fig. 10. Diagram of infectious form of *Holospora*. The cytoplasm is condensed and occupies only half the volume of the bacterial cell. Accordingly, the periplasm is extremely voluminous. IM, the inner membrane (cell membrane); OM, the outer membrane; and T, the less electron dense, apically oriented material in the periplasm (special tip).

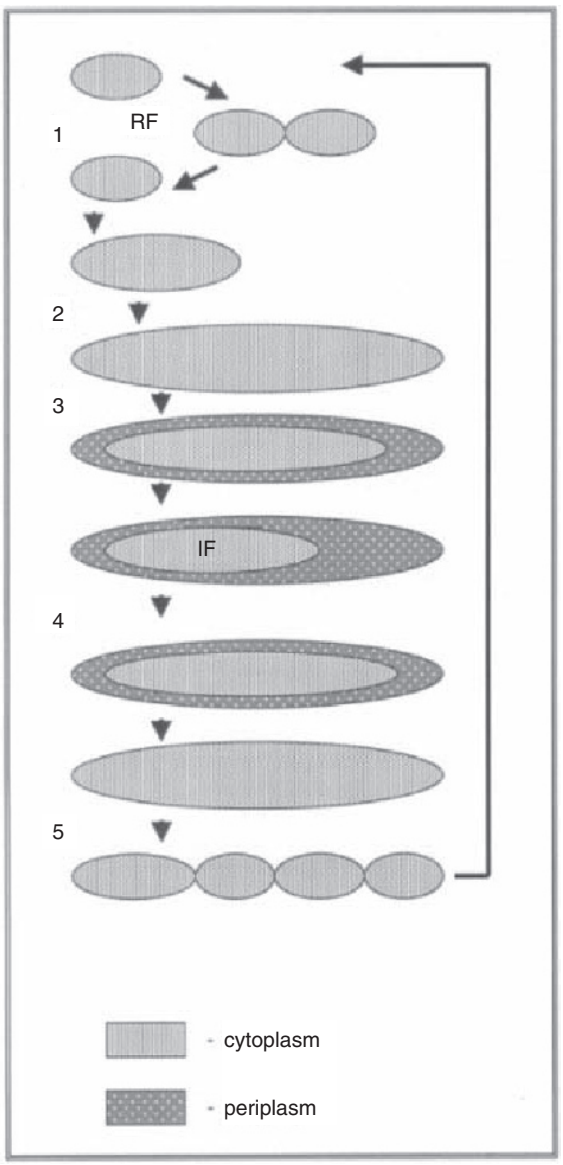


Fig. 11. Developmental cycle of *Holospora*. 1) Reproductive forms (RF) multiply by binary fission. 2) Some reproductive forms grow longer. 3) After having reached their final length, the cytoplasm condenses and proteins are deposited in the periplasm until the periplasm makes up more than half of the total cell volume. At the end of this differentiation, the infectious form (IF) is established. 4) When an infectious form is ingested into a phagosome of a suitable host, it starts to develop into the reproductive form. The cytoplasm expands, while periplasmic proteins are assumed to be secreted. 5) Once the periplasm has almost completely disappeared, the bacterium constricts at several points and undergoes a multiple division. By this division, the reproductive form is established anew.

When an infective form has been taken up by a paramecium and is on its way into the nucleus, the periplasmic material disappears while the cytoplasm expands (Fig. 11). It is assumed that the periplasm of the infectious form contains

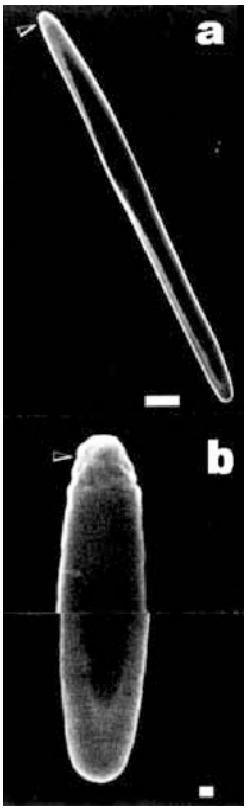


Fig. 12. Scanning electron micrograph (SEM) of the isolated infectious form of *Holospora obtusa*. a) One pole (arrows) of the infectious forms appears rough compared to the other pole. Bar = 1 μm. b) Ends of the bacterium at higher magnification. Bar = 0.1 μm. From Fujishima et al. (1990).

substances that interact with host membranes during the infection process (Görtz et al., 1988; Görtz and Wiemann, 1989a). The further development of the infectious form into the short, reproductive form is completed in the nucleus. Induction of the development of infectious forms appears to be activated by specific triggers of host cells. Acidification of the phagosome and possibly also the actions of oxidative radicals and lysosomal enzymes may be such triggers for the development of infectious forms of *Holospora* and may initiate a recognition mechanism depending upon protein-protein interactions between bacterium and host cell (Wiemann and Görtz, 1991; Fujishima et al., 1997). Apparently, there is a stepwise interaction between invading bacterium and host cell. This is indicated by the observation that the equipment of the invading infectious form with surface proteins changes dramatically (Görtz et al., 1992b).

Certain proteins were immunolocalized in the periplasm of the infectious form (Fig. 13). During the invasion process, some of these proteins were found on the surface of the bacteria after their ingestion into the phagosome, and others

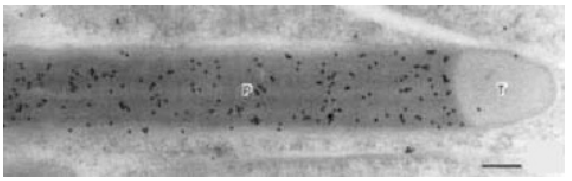


Fig. 13. Immunoelectron micrograph of Holospora obtusa in the macronucleus of *Paramecium caudatum*, treated with a monoclonal antibody specific for a 39-kDa periplasmic protein of the infectious form. Secondary label with gold-anti-mouse IgG. Bar = 0.2 μm. From Dohra et al. (1994).

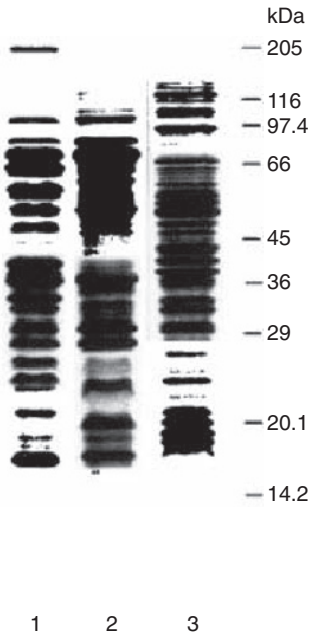


Fig. 14. SDS-polyacrylamide gel electrophoresis of total protein of *Holospora obtusa* and *Escherichia coli*. Lane 1, *H. obtusa*, infectious form (from 2×10^7 cells); lane 2, *H. obtusa*, reproductive form (from 2×10^8 cells); and lane 3, *E. coli* (from 5×10^7 cells). The position and size (kDa) of marker proteins are indicated on the right. From Görtz et al. (1988).

were associated with the phagosomal membrane (Görtz et al., 1990; Fujishima et al., 1990b; Dohra et al., 1994, 1997; Wiemann and Görtz, 1991; H.-D. Görtz, unpublished results). This is what would be expected if such proteins were used for communication with host membranes. Released periplasmic proteins could also protect the bacteria against lysosomal enzymes of the host or inactivate such enzymes. Dohra et al. (1997) have sequenced the gene of a small periplasmic protein of 5.4 kDa. The gene is highly expressed in the intermediate form, a transitional stage in the development from the reproductive into the infectious form of the bacterium. It has been suggested that the protein may function in the recognition process in the early phase of infection. Amino acid sequence similarities with other polypeptides have not been found. Binding experiments of biotinylated macronuclear proteins on blotted *Holospora* proteins had revealed binding to a 50-kDa polypeptide (Ehrsam and Görtz, 1999). The 50-kDa protein was immunolocalized in the periplasm and on the surface of *H. obtusa* on sections for light and electron microscopy and may function in the recognition process during infection. Dohra et al. (1998) have identified a GroEL-like protein in *H. obtusa* that is selectively expressed in the reproductive form.

The infectious form differs from the reproductive form not only in morphology, but also in the protein patterns produced on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Figs. 14 and 15). Some proteins specific for the infectious form of *H. obtusa* are located in the periplasm, as shown by means of immune electron microscopy (Fig. 15) using poly- and monoclonal antibodies as probes against these proteins (Wiemann and Görtz, 1991; Dohra et al., 1994).

The fine structure of the reproductive forms of species of *Holospora* is that of Gram-negative bacteria. Freeze-fracture studies of *H. obtusa* revealed that the outer membrane of the reproductive form has a density of intramembrane particles (IMPs) that is similar to that of the inner membrane. In contrast to this, the outer membrane of the infectious form was found to contain only very few IMPs (Görtz et al., 1989b), which corresponds to the observation that the infectious form shows only a limited number of surface proteins (Görtz et al., 1992b). These differences between the two forms correlate with a difference in the behavior of the two forms during the division of the host nuclei. The infectious forms of *H. obtusa* are concentrated in the connecting piece around the separation spindle of the dividing nucleus and are later released from the host cell. The reproductive forms are transported to the poles, and in this way, get into the daughter nuclei (Ossipov et al., 1975; Ossipov, 1981; Görtz and Dieckmann, 1980; Wiemann, 1989a; Wiemann and Görtz, 1989b). Apparently, *H. obtusa* makes use of the division apparatus of the host nucleus.

The physiology of *Holospora* and the triggers needed at different stages of its develoment are still enigmatic. It appears that during development of the infectious into the reproductive form, protein synthesis of the host cell is needed (Dohra and Fujishima, 1999b). It has also been shown that induction of the development of the reproductive form into the infectious form depends upon protein synthesis of the host cell

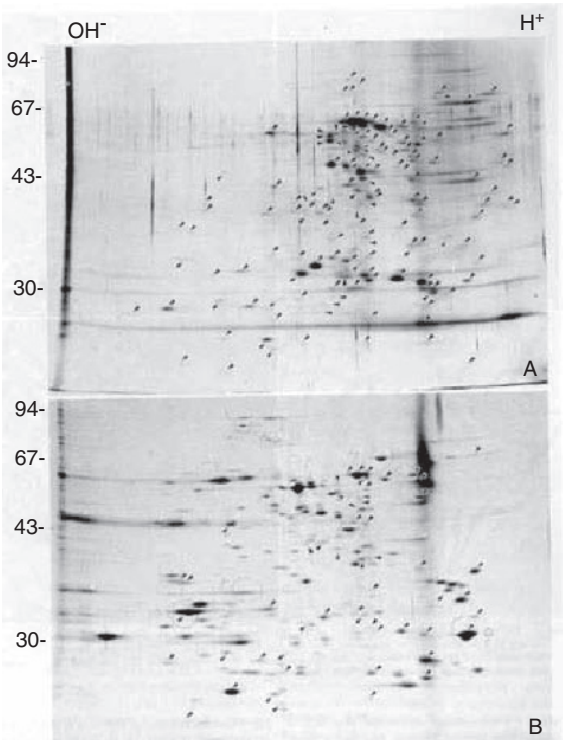
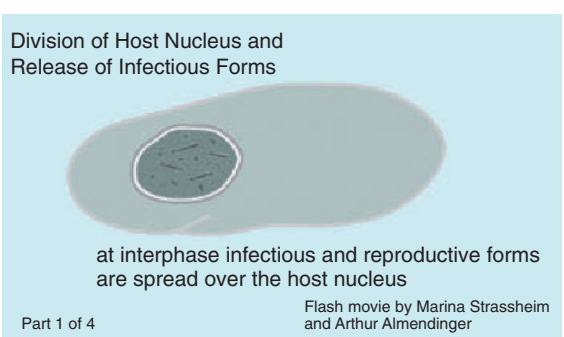


Fig. 15. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of the reproductive (A) and the infectious (B) forms of *Holospora obtusa*. Spots being unique for either of the forms were labeled with single arrowheads and those common to both of the forms were indicated with white-rimmed arrowheads. From Fujishima et al. (1990).

(Fujishima, 1993). Freiburg (1985) presented evidence that RNA polymerase of infected macronuclei of *Paramecium* had a 5-fold higher activity than that of uninfected nuclei (Freiburg, 1985).



Video sequence showing release of *Holospora*. For the video, see the online version of *The Prokaryotes*.

Isolation of Symbiont-Bearing *Paramecium*

Prokaryotic symbionts of *Paramecium* are not free living. They can only be found in their host cells. To investigate the symbionts, paramecia have to be isolated from natural habitats. The techniques used in collecting and cultivating paramecia have been described by Sonneborn (Sonneborn, 1950; Sonneborn, 1970). Paramecia are easily detected in samples of pond or lake water and even in rivers with the help of a dissection microscope. For most species of *Paramecium*, the highest abundance is found in the eulitoral or in the microaerobic areas just above the anaerobic layer of detritus on the water level, where the abundance of bacteria and small eukaryotes is high. In the pelagial—the free water body of a lake—the abundance of paramecia may be low. Few species of *Paramecium* are found in brackish water, and no marine species are known (Wichterman, 1953; Fokin and Chivilev, 1999). *Paramecia* do not form cysts and, unless they have been introduced with the water added, cannot be obtained from infusions of straw or hay. When brought in from nature, the paramecia must be grown slowly at low temperature (about 16°C) without adding any antibiotics, if the purpose is to look for symbionts. Their presence can be monitored as described in the section "Identification of Symbionts in *Paramecium*."

If the paramecia are grown at high fission rates, their symbionts often become diluted and may even be lost. Natural populations of *Paramecium* are infected with symbionts to varying degrees. Most strains carry only one kind of symbiont. A culture containing only one type of symbiont may be obtained, therefore, by isolating a single paramecium and growing it into a clone. If a strain of *Paramecium* does carry more than one type of symbiont, it is often possible to obtain pure cultures by growing the strain at a high fission rate until the symbionts are diluted down to no more than one symbiont per paramecium. Cells isolated at that time and cultured at a low fission rate will grow into populations containing one type of symbiont only. This technique has also been used to separate different types of symbionts (Preer Jr., 1948a).

Culturing Paramecia with Symbionts A method of culturing paramecia involves the use of bacterized cereal leaf medium (Sonneborn, 1970). For a stock solution, 75 g of dried cereal leaves (a mixture of wheat and barley leaves that were harvested before growth of ears) are boiled for 15 min in 1 liter of distilled water. The solution is then filtered, diluted with water (1 : 30), buffered with Na_2HPO_4, and autoclaved. A day before use, the autoclaved medium is inoculated with *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Micrococcus luteus* or another bacterium suitable as food for *Paramecium*. The medium should have a pH of about 7. The growth rate of *Paramecium* can be regulated by varying the temperature and the amount of bacterized medium that is added to a culture.

Other Culture Methods An even simpler method is to culture paramecia in autoclaved straw or hay infusions. However, this method is less reproducible than the one using cereal leaf medium. For preparing the straw or hay medium, 3–5 g of straw or hay per liter of distilled water is boiled, filtered and buffered with Na_2HPO_4 (pH 6.8–7.0), and then autoclaved. After cooling and a day before use, the medium is inoculated with bacteria.

Unfiltered straw medium, with the straw remaining in the medium, may be used for maintaining stock cultures. In these cultures, paramecia with or without symbionts can be kept for months at low temperatures (6–16°C) without special care. Another, even safer, method for maintaining stocks is to freeze the paramecia and to keep them in liquid nitrogen, from which they may be recovered with their symbionts on thawing (Simon and Schneller, 1973). A great number of *Paramecium* stocks bearing symbionts are maintained in the frozen state at the American Type Culture Collection, Rockville, MD, USA. It appears, however, that some symbionts may be lost after prolonged maintenance in liquid nitrogen.

Some strains of *Paramecium* can also be grown axenically. For sterile growth, the paramecia must first be washed free of bacteria. This is achieved by allowing the paramecia to swim through sterile medium for a time sufficient to permit the bacteria in the food vacuoles to be eliminated (Sonneborn, 1950; Van Wagtendonk and Soldo, 1970). A simple technique modified from Heatherington (1934) is to place several paramecia at one edge of a hole of a depression slide filled with wash fluid, allow them to swim to the other side, and then to transfer them to a new depression. The procedure should be repeated four times before the cells are left in the fifth wash for an hour. They should then be transferred to a new depression and this should be repeated hourly for another 4 hours. A helpful tool for this method and for the growth of small numbers of paramecia is the so-called "three-spot depression slide," also called the "Sonneborn slide." This is a thick slide with three depressions (Fig. 16). For sterile washing, the paramecia must be taken up each time in a new sterile micropipette with as little fluid as possible; slides and wash fluid have to be sterile. The use of antibiotics to obtain bacteria-free cultures is to be avoided because such substances may harm the symbionts.

A method for culturing paramecia without bacteria involves use of the photoautotrophic alga *Chlamydomonas reinhardii* as a food organism (Preer et al., 1974). The medium contains 1 g of yeast autolysate, 0.25 g sodium acetate, 0.625 g of cereal leaves, and 0. 125 g of Na_2HPO_4 in 1 liter of double-distilled water. The medium is dispensed into test tubes, autoclaved and stored. Before it is used, the medium is inoculated with a small number of *Chlamydomonas* and incubated under light for two days. A single bacterium-free paramecium, put into a tube half-filled with this *Chlamydomonas* medium, usually will multiply and ingest most of the algae within 3 to 4 days.

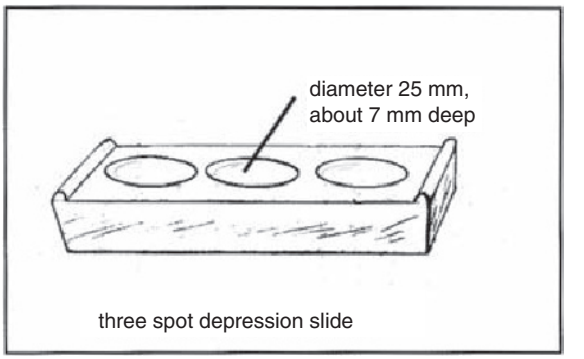


Fig. 16. Three-spot depression slide. Each depression has the volume of about 1.5 ml (Fig. 8). The depression slides may be labeled by pencil on the etched sides. Kept in a moist chamber, the slides may be covered by glass plates supported by the elevated edges of the slides.

Most of the axenic media used for growing paramecia are based on a recipe initially designed by Soldo et al. (1966). Recent modifications by Thiele et al. (1980) and Schönefeld et al. (1986) have proved particularly valuable for large-scale cultures of *P. tetraurelia*. A modification (Soldo, 1987a) that should be capable of supporting the axenic growth of paramecia and many other ciliates of freshwater and marine origin is shown below.

Medium for Axenic Growth of Marine and Freshwater Ciliates (Soldo, 1987a)

Proteose peptone	10.00 g
Trypticase	5.0 g
Yeast nucleic acid	1.0 g
Biopterin	0.5 mg
Folic acid	0.5 mg
Nicotinamide	2.5 mg
D-Pantothenate, Ca	7.5 mg
Pyridoxal hydrochloride	2.5 mg
Riboflavin	2.5 mg
Thiamine hydrochloride	0.01 mg
DL-Thioctic acid	0.01 mg
Phospholipid (oleate-containing)	250 mg
Stigmasterol	2 mg
Distilled water (or seawater)	1 liter

The medium is prepared in distilled water for freshwater ciliates and in seawater (density 1.015–1.026 g/ml) for marine forms. The final pH is 7.2. Stigmasterol is added from a stock solution (0.5 g of stigmasterol dissolved in 100 ml of absolute ethanol, stored at 4°C in a tightly capped plastic bottle) by injection into the culture medium from a syringe.

It is important to transfer paramecia gradually from a bacterized to an axenic medium; the ciliates need to be allowed to adapt slowly. Several procedures have been proposed for this transfer (see, e.g., Van Wagtendonk and Soldo, 1970, and Fok and Allen, 1979). An adapting medium (called "VS medium") based on that of Allen and Nerad (1978) is one containing all the vitamins of the axenic medium given above plus stigmasterol, but not the other components, the pH being adjusted to 7.0. In VS medium, bacteria (e.g., *Klebsiella pneumoniae* previously grown in a tryptone medium) are suspended and adjusted to $OD_{590} = 3.0$ by dilution. The bacteria are then dispensed in 1-ml portions in screw-cap tubes and placed in a deep freeze. The final medium is prepared, about a week's supply at a time, by adding 1 ml of the frozen bacterized VS medium to 9 ml of unbacterized VS medium. This medium is autoclaved and inoculated with paramecia. The protozoa grow in this medium at a rate of 1/2 to 2 fissions per day. Not all stocks of paramecium can be adapted to an axenic medium. Furthermore, some media were found to support growth of paramecia, but the latter were not able to maintain their symbionts under these conditions.

Purificaton of Symbionts from *Paramecium*

The purification of symbionts from paramecia (i.e., separation from host cell material) has been achieved in a number of ways: passage of cell homogenates through ion-exchange cellulose columns (Smith, 1961; Mueller, 1963), through filter paper columns (Preer et al., 1966), and centrifugation (Soldo et al., 1970) have all been used. For many symbionts, Percoll gradient centrifugation proved to be the method of choice (Görtz et al., 1988; Fujishima et al., 1990a). Depending on the type of symbionts, different methods of purification may be neccessary. For example, the R-body-containing *Caedibacter caryophila* can best be isolated with the help of a discontinuous 70% Percoll gradient, whereas isolation of *Caedibacter caryophila* containing no R bodies is better achieved by means of an ECTEOLA (anion exchanger) column (Schmidt et al., 1987c; Schmidt et al., 1988). For the isolation of holosporas, two different approaches have been followed. One method starts with homogenization of the host cells, and the other involves separation of the macronuclei from the cytoplasm and then isolation of the symbionts. The latter method involving prior isolation of nuclei (Freiburg, 1985) proved especially useful for preparing clean reproductive forms of the symbionts because it avoids contamination of the bacteria with food vacuoles. For isolation of nuclei, the paramecia are lysed in a buffer containing 10 mM Tris, pH 7.9, 0.25 M sucrose, 3 mM $CaCl_2$, 8 mM $MgCl_2$ plus 0.1 mM phenylmethylsulfonyl fluoride, 0.1% (w/v) spermidine, and 0.2% Nonidet P40 by gently stirring the cells in an ice-bath and subsequently passing the suspension 5–10 times through a 20-ml pipette. The nuclei are concentrated on a cushion of 1.6 M sucrose by spinning for 10 min at 700 *g*. The purified nuclei are then homogenized in a Mg-free buffer, and the bacteria are pelleted. Two different forms of the bacteria, the infectious and reproductive forms, can be separated by sedimentation. The infectious form sediments at 350 *g* within 10 min, while the reproductive form remains in the supernatant (Görtz et al., 1988).

Protocols for isolating holosporas directly from cell homogenates have been published by Fujishima and Nagahara (1984b), Schmidt et al. (1987a), and Görtz et al. (1988). Infected cells are homogenized in sodium phosphate buffer with a hand homogenizer. The Teflon pestle should fit tightly. The homogenate is then centrifuged at about 3000 *g* for 10 min. The pellet is resuspended in buffer and centrifuged in a preformed continuous gradient of 70% Percoll for 12 min at 40,000 *g*. To maintain the gradient, it is advisable to use a centrifuge with an acceleration rate control. For *H. obtusa*, *H. elegans* and *H. undulata*, the infectious forms then concentrate in a sharp band that is usually uncontaminated with food bacteria and cell debris (microscopical control). The reproductive forms do not form a band in a continuous Percoll gradient. These forms can be obtained with the help of a discontinuous gradient (at 10,000 *g*) where—depending on the species—they form a layer above ca. 60% Percoll. Most of the cell organelles of *P. caudatum* do not enter at the 50% Percoll step.

The isolation procedures described here have been helpful for studying ultrastructural details and protein composition of symbionts, their R bodies, and their DNA. Attempts to culture *Holospora* endosymbionts outside their hosts have been unsuccessful, as has been the case for other symbionts of aerobic ciliates.

Identification of the *Paramecium* Symbionts

Most of the endosymbionts are easy to observe. Paramecia are collected from ponds, lakes or rivers and brought into the laboratory. With the help of a micropipette, a few cells are placed on a microscope slide, covered with a cover glass, and then observed with a light microscope using phase contrast optics. Crushing of the paramecia is sometimes necessary (Preer and Stark, 1953). The classification of the endosymbionts of *Paramecium* is traditionally based on the following characteristics: morphology and killing activity, the species in which they live, DNA base ratios

calculated from the buoyant density (Bd) or from thermal denaturation profiles (T_m), and interstrain DNA/DNA hybridizations. Type strains, which have been deposited at the American Type Culture Collection (ATCC), are listed below by their ATCC numbers. Some type strains have also been deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia, and can be obtained from that source.

Since endosymbionts do not multiply outside their hosts, characterization based on metabolism and growth, as is customary for bacteria, is not possible. Instead, morphological and biological features and sometimes data on the G+C content of the symbiont's DNA must be used for identification. Nowadays, sequencing of ribosomal genes after PCR amplification and classification by fluorescence in situ hybridization (FISH) using oligonucleotide probes complementary to ribosomal sequences (Fig. 17) have become powerful tools for classification of uncultured intracellular bacteria (Amann et al., 1991; Amann et al., 1995). Nonetheless, detection and sometimes a preliminary identification are still possible with the help of a light microscope equipped with phase contrast, namely, after staining with orcein (Beale and Jurand, 1966; see "Orcein Staining of Intracellular Bacteria").

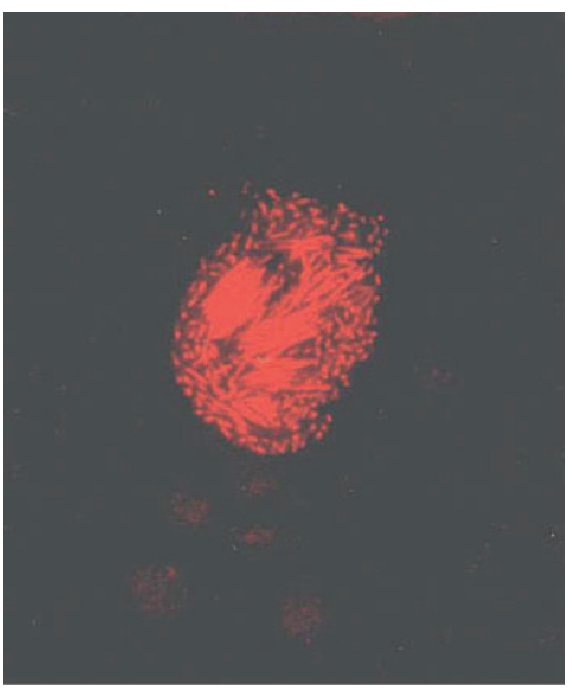
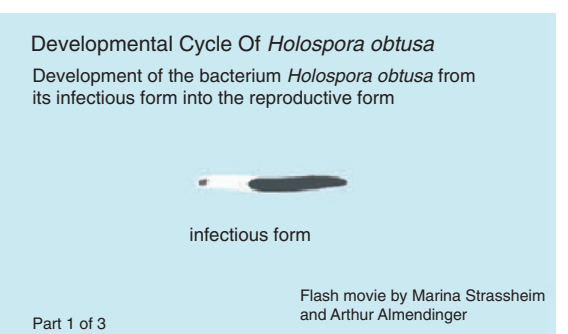


Fig. 17. Fluorescence in situ hybridization (FISH) using an oligonucleotide probe specific for *Holospora obtusa*. Short and long rods of holosporas in the host macronucleus are labeled. Faintly, phagosomes with prey bacteria are visible. From Fokin et al. (1996).

In addition to microscopic observation, killer tests should be performed when a stock is brought in from nature. Standard sensitives (e.g., cells of stock 152 of *P. triaurelia*) may be employed in such tests. Equal volumes of the culture to be tested and a culture of sensitive cells are mixed in a depression slide and observed with appropriate controls for prelethal effects. Mate killing can only be detected by mating sensitive cells with symbiont bearers. Any symbiont-free strain that will mate with an unknown strain is usually adequate. The mode of killing and the features of R bodies (if present), i.e., shape of the ends and the mode of unrolling of the different types of R body (Fig. 6), have been used for classification and determination of killer symbionts (for details, see Preer et al., 1974; Quackenbush, 1988, and Pond et al., 1989). It has now become evident that R bodies and their genetic basis, plasmids or phages, are not adequate for classification of their bacterial hosts (Beier et al., 2001).

In contrast to the killer trait, other biological features may still be of great value for the classification and determination of certain symbiotic bacteria. Such features may be the high infectivity, unique life cycle, and also unique morphology of the infectious forms of the species of the genus *Holospora*; see "Biology of the Holosporaceae". These bacteria are host-specific as well as nucleus-specific, i.e., they infect only one type of nucleus, either the micronuclei or the macronuclei (Ossipov, 1973; Ossipov et al., 1975; Fig. 1; see The Ciliate Cell as a Microcosm). *Holospora* bacteria establish short, reproductive forms that undergo binary fission as well as long, infective forms that leave the paramecium and infect others very efficiently (Ossipov and Podlipaev, 1977; Wiemann and Görtz, 1989b; Wiemann, 1989a; Fujishima et al., 1990b; Figs. 9–11). Namely, the infectious forms have a structure unique among bacteria and their structure may well be used for identification.



Video sequence showing the development of *Holospora*. For the video, see the online version of *The Prokaryotes*.

Orcein Staining of Intracellular Bacteria
A very useful staining technique that allows observations of intracellular bacteria in regular bright field and/or phase contrast is given by Beale and Jurand (1966): Paramecia are placed in a small drop on a slide, and as much of the fluid as possible is withdrawn by a micropipette or filter paper. The paramecia are lightly fixed by exposure to OsO_4 vapor for 6–10 s and immediately stained with a small drop of lacto-aceto-orcein (1 g of orcein dissolved in 25 ml of hot 45% acetic acid, mixed with 25 ml of lactic acid, diluted with water 1 : 1 and then filtered). A coverslip is placed over the drop of stained paramecia and is lightly pressed down, flattening but not disrupting the cells. The preparations can then be observed with a 100X oil immersion objective. To remove lipids that sometimes obscure observation of endosymbionts, the paramecia can be treated with a drop of acetone or a 3 : 1 mixture of ethanol and acetic acid before staining. Orcein-stained preparations can be made permanent by either applying vaseline around the edges of the cover slip or by the following procedure. The slide with the orcein preparation and cover slip is rapidly cooled in liquid nitrogen. The slide is immediately lifted off the cover slip with a razor blade. Cells will remain on the slide. The preparation is dehydrated by dipping it briefly into 50%, 70% and absolute ethanol and adding a small drop of Euparal or other ethanol-soluble resins. The resin layer between slide and cover slip should be extremely thin for optimal optical conditions.

The Taxa of Prokaryotic Symbionts of *Paramecium*

Genus *Caedibacter* (Preer and Preer, 1982)
Cells are straight rods or coccobacilli, 0.4–1.0 μm in diameter and 1.0–4.0 μm in length. Usually less than 10% but sometimes up to 50% of the symbionts contain refractile (R) bodies (Figs. 4–6). Cells containing R bodies are usually longer than those that do not. In addition to R bodies, many spherical phage-like structures or covalently closed circular DNA plasmids are present. The symbionts are Gram-negative and nonmotile. The G+C content of the DNA is 40–44 mol%. Earlier work of this extensively studied genus has been reviewed by Preer et al., 1974; Preer and Preer, 1984, and Pond et al., 1989.

An analysis of the 16S rDNA of *Caedibacter caryophilus*, a killer symbiont dwelling in the macronucleus of *Paramecium caudatum*, revealed an unusual insertion of 194 bp that was not present in mature 16S rRNA (Springer et al., 1993). It was shown that *C. caryophila* contained fragmented 16S rRNA. Insertion of more than 100 bp has been reported for bacterial 23S rDNAs (Roller et al., 1992). Comparable intervening sequences in 16S rRNAs have not been described in free-living or intracellular bacteria. Results obtained by Beier et al. (2001) give evidence that the genus *Caedibacter* is polyphyletic. Apparently, R bodies and their genetic basis, plasmids or phages, are not adequate for classification of their bacterial hosts. The following species have been described:

Caedibacter taeniospiralis *(Preer and Preer, 1982)* Cells are rods, 0.4–0.7 μm in diameter and 1.0–2.5 μm long. The G+C content is 41 mol%. They are found in the cytoplasm of *P. tetraurelia* only. Their R bodies (Fig. 5) unroll from the inside and contain plasmids. Ingestion of R-body-containing symbionts by sensitive paramecia results in the development of clear small blisters on their surface in 2 to 3 h. Between 4 and 5 h, a bulge develops first in the posterior part of the oral side and later moves to the posterior aboral side. The position of the bulge gave rise to the designation "hump killer." Death takes place in 1 to 2 days, the corpses remaining intact for some time. *Caedibacter taeniospiralis* of *P. tetraurelia* stock 51K has been affiliated to γ-subgroup of Proteobacteria according to its ribosomal gene-sequences (Beier et al., 2001). The type strain is found in ATCC strain 30632 (stock 51 of *P. tetraurelia*; see Preer et al., 1974).

Caedibacter varicaedens *(Quackenbush, 1982)* The cells are rods, 0.4–1.9 μm in diameter and 2.0–4.0 μm long. The G+C content of the DNA is 40–41 mol%. They are found in the cytoplasm of *P. biaurelia*. Different strains cause vacuolization, paralysis, or rapid reverse rotation while swimming (spin-killing) of sensitive cells. After 4–6 h of swimming, vigorous rotation to the right becomes nearly uninterrupted, and then becomes slower. R bodies (Fig. 4) unroll from the outside. The outer terminus of the unrolled R body is blunt. The R body is usually associated with bacteriophage capsids. The type strain is found in ATCC 30637 (stock 7 of *P. biaurelia*; see Preer et al., 1974).

Caedibacter pseudomutans *(Quackenbush, 1982)* These are cigar-shaped rods, approximately 0.5 μm in diameter and 1.5 μm long. The G+C content is 44 mol%. They are found in the cytoplasm of *P. tetraurelia* and cause rapid reverse rotations of sensitive paramecia while swimming (spin-killers). The R bodies are of the *C. varicaedens* type. The type strain is found in ATCC strain 30633 (strain 51 ml of *P. tetraurelia*; see Preer et al., 1974).

Caedibacter paraconjugatus *(Quackenbush, 1982)* Cells are small rods and contain phage-like structures. Less than 1% of the symbionts contain R bodies of the *C. varicaedens* type. They are found in the cytoplasm of *P. biaurelia*. Ingestion of symbionts by sensitive strains does not produce any observable toxic effect. Cell-to-cell contact between host and sensitive paramecia is required for toxic effects to be observed in the sensitive paramecia (mate-killers). The type strain is found in ATCC strain 30638 (stock 570 of *P. biaurelia*; see Preer et al., 1974).

Caedibacter caryophilus *(Schmidt et al., 1987b; Euzeby, 1997)* Cells are rods, 0.4–0.7 μm wide and up to 2.5 μm long. Those with R bodies are larger than those without R bodies. The G+C content is 34.6 mol%. They are found in the macronucleus of *P. caudatum*. The R bodies unroll from the inside (with blunt outer terminus and sharp inner terminus) and are associated with phages. Width of R bodies after isolation is 0.8 μm. Sensitive strains are killed by paralysis. Coinfections with *Holospora obtusa*, *H. undulata* and *H. elegans* may occur in natural populations (H.-D. Görtz, unpublished observations). According to its ribosomal gene sequences, *C. caryophilus* has been affiliated to the α-subgroup of Proteobacteria, the closest relative being *Holospora obtusa* (Springer et al., 1993). The type strain is found in ATCC strain 50168 (clone C221 of *P. caudatum*; see Schmidt et al., 1987b).

Genus *Pseudocaedibacter* (Quackenbush, 1982) Cells are rods, 0.25–0.7 μm in diameter and 0.5–4.0 μm long. They do not produce R-body-containing cells. The symbionts are Gram negative and nonmotile. The G+C content of the DNA is 35–39 mol%. The genus includes some species that confer a killer trait on their hosts, some that render them mate killers, and others that do not produce any killing ability. Four species have been described:

Pseudocaedibacter conjugatus *(Quackenbush, 1982)* Formerly called mu particles, these cells are rods, 0.3–0.5 μm in diameter and 1.0–4.0 μm long. The G+C content of the DNA is 35–37 mol%. They are found in the cytoplasm of *P. primaurelia* and *P. octaurelia* where a mate-killer phenotype of the hosts is produced. The type strain is found in ATCC strain 30796 (stock 540 of *P. primaurelia*; see Preer et al., 1974).

Pseudocaedibacter minutus *(Quackenbush, 1982)* Formerly known as "gamma particles," these cells are rods, often double but also single rods 0.25–0.35 μm in diameter and 0.5–1 μm long. The G+C content of the DNA is 38 mol%. They are found in the cytoplasm of *P. octaurelia*. In the host cell, the symbiont is surrounded by an extra set of membranes, apparently continuous with the endoplasmic reticulum of the host. The paramecia which bear these symbionts are very strong killers that cause sensitives to develop vacuoles, swell, finally become spherical and lyse. Death occurs after about 8 h. The type strain is found in ATCC strain 30699 (stock 214 of *P. octaurelia*; see Preer et al., 1974).

Pseudocaedibacter falsus *(Quackenbush, 1982)* Formerly called "nu" and "pi particles," these cells are rods, 0.4-0.7 μm in diameter and 1.0–1.5 μm long. The G+C content of the DNA is 36 mol%. They have no known toxic actions and are found in the cytoplasm of *P. biaurelia*, *P. tetraurelia* and *P. pentaurelia*. The type strain is found in ATCC strain 30640 (stock 1010 of *P. biaurelia*; see Preer et al., 1974).

Pseudocaedibacter glomeratus *(Fokin and Ossipov, 1986)* These are small rods, about 0.3 μm wide and up to 1.2 μm long. They are found in the cytoplasm of *P. pentaurelia* and have no known toxic actions. Symbionts are individually enclosed in vacuoles which are tightly associated with the endoplasmic reticulum. The type strain is strain Bp 171 of *P. pentaurelia* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia).

Caedobacter chlorellopellens *(Skoblo et al., 1985)* This organism, a symbiont living in the cytoplasm of *P. bursaria* and found to be antagonistic to symbiotic chlorellae, was named "*Caedobacter chlorellopellens*" by Skoblo et al. (1985), who did not know that the genus name *Caedobacter* had been changed to *Caedibacter*. *Caedibacter* was then restricted to symbionts that produce R bodies and confer killer traits upon their host paramecia (Preer and Preer, 1982). The symbiont in *P. bursaria* is egg-shaped, rarely rod-shaped, 0.35 μm wide and up to 1.4 μm long. Since no R bodies were observed and the cells have no known toxic actions on sensitives, placement of the species in the genus *Pseudocaedibacter* seems more plausible. A renomination of the species seems necessary but should be done only after ribosomal sequences have been obtained.

Genus *Lyticum* (Preer and Preer, 1982) Cells are large rods, 0.6–0.8 μm in diameter and 3.0–5.0 μm long. They resemble bacilli in general appearance and bear numerous peritrichous flagella (Figs. 3 and 18) but are not obviously motile. They are enclosed in vacuoles in the cyto-

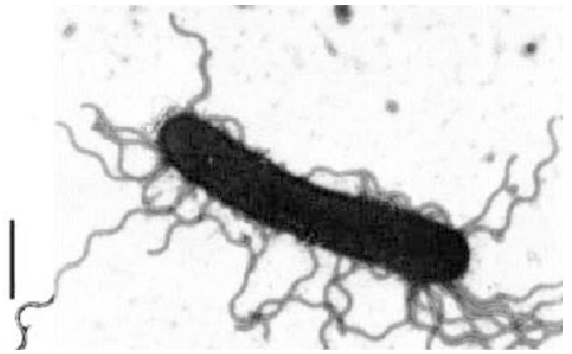


Fig. 18. Electron micrograph of *Lyticum flagellatum* of *Paramecium octaurelia* stock 327. Negative staining with phosphotungstic acid. Bar = 1 μm. From Preer et al. (1974).

plasm of their hosts. They do not contain R bodies. The G+C content is 27 mol% in one type and 45–49 mol% in another type. The symbionts are Gram negative. They produce toxins that kill sensitive paramecia by lysing them within 10–20 min (rapid-lysis killing). Two species have been described:

Lyticum flagellatum *(Preer and Preer, 1982)* Formerly known as "lambda particles," these cells are straight rods, 0.6–0.8 μm in diameter and 2.0–4.0 μm long (Figs. 3 and 18). The G+C content of the DNA is 27 mol% in one strain and 49 mol% in another. They are found in the cytoplasm of *P. tetraurelia* and *P. octaurelia*. The type strain is found in ATTC strain 30700 (stock 299 of *P. octaurelia*; see Preer et al., 1974).

Lyticum sinuosum *(Preer and Preer, 1982)* Formerly, these were called "sigma particles." They are curved or spiral rods, 0.7–0.9 μm in diameter and 2.0–10 μm long, sometimes forming chains of 2–3 cells. The G+C content is 45 mol%. They are found in the cytoplasm of *P. biaurelia*. The type strain is found in ATCC strain 30696 (stock 114 of *P. biaurelia*; see Preer et al., 1974).

Genus *Pseudolyticum* (Boss et al., 1987) These are large symbionts with numerous flagella. No motility has been observed. They may contain refractile bodies consisting of polyhydroxybutyric acid. No killer activity and no infectivity could be detected. Only one species is known:

Pseudolyticum multiflagellatum (Boss et al., 1987) Cells are straight rods, 1.0–2.0 μm in diameter and 3.5–14 μm long. They are found in the cytoplasm of *P. caudatum*. The symbionts are individually enclosed in vacuoles. The membrane of these vacuoles forms numerous projections that are continuous with the endoplasmic reticulum. No toxic actions are known.

Genus *Tectibacter* (Preer and Preer, 1982) The species of this genus are distinguished from other symbionts by a layer of electron-dense material surrounding the outer of the two membranes. The symbionts have sparse peritrichous flagella and show slight motility on occasion. They appear not to be toxic. For a more detailed description, see Preer et al. (1974). Only one species has been described:

Tectibacter vulgaris (Preer and Preer, 1982) Formerly known as delta particles, these cells are straight rods, 0.4–0.7 μm in diameter and 1.0–2.0 μm long. Gram-negative. Hosts: *P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, *P. sexaurelia* and *P. octaurelia*. The type strain is found in ATCC strain 30697 (stock 225 of *P. sexaurelia*; see Preer, 1974).

Genus *Nonospora* (Fokin et al., 1987a) These rod-like symbionts live in the macronucleus. Flagella have not been observed. No toxic actions are known. The symbionts are retained in macronuclear fragments of exconjugants and enter macronuclear anlagen by fusion of old fragments with the anlagen (Fokin et al., 1987a). Only one species has been described:

Nonospora macronucleata (Fokin, Ossipov, Skoblo, Rautian, and Sabaneyeva 1987; Demar-Gervais, 1976). These symbionts are rodlike, 0.2–0.3 μm in diameter and mostly about 1.0 μm long, sometimes forming chains up to 10 μm long. The surface of the symbionts appears irregularly wavy in the electron microscope. They are found in the macronucleus of *P. caudatum*, often clustered in the center of the nucleus.

Genus *Holospora* (Gromov and Ossipov, 1981; Hafkine, 1890) Symbionts of this genus live in the micronucleus or the macronucleus (see The Ciliate Cell as a Microcosm) of *Paramecium* and exist in two forms: a short rod, 1.0–3.0 μm long and 0.5–1.0 μm wide that can replicate; and a long form 5.0–20 μm long and 0.8–1 μm wide that cannot replicate (Figs. 9–11). The long form is released from the host and can infect other paramecia (Fig. 8). The infective form is differentiated into a refractile portion with a less electron dense, pale tip and a posterior part that contains typical bacterial cytoplasm, stains with DNA-specific dyes, and appears dark in phase contrast (Fig. 9). Nine species have been described (see Table 1): *Holospora undulata*, *H. obtusa*, *H. elegans*, *H. acuminata*, *H. caryophila*, *H. recta*, *H. curviuscula*, *H. bacillata*, and *H. curvata*.

The striking similarity of the biology and cytology of the different *Holospora* species led to their classification in one genus, although a com-

parison of the protein patterns of *H. obtusa* and *H. elegans* revealed great differences. Moreover, the hybridization of the total DNAs and a comparison of DNA banding patterns after digestion with restriction enzymes indicate that the two species may not be as closely related as originally assumed (Schmidt et al., 1987a). The differences at the molecular level may suggest that the genus *Holospora* has coevolved with ciliates for a long time.

Holospora were the first intracellular bacteria in *Paramecium* for which the phylogenetic position was determined (Amann et al., 1991). *Holospora obtusa*, *H. elegans* and *H. undulata* belong to the α-group of Proteobacteria. The closest relative among other symbionts in ciliates was found to be *Caedibacter caryophilus*, and the closest relatives among other bacteria found up to now are *Rickettsia* and *Ehrlichia* (Amann et al., 1991; Springer et al., 1993). It is tempting to regard the striking biology (developmental cycle, host specificity for *Paramecium*, etc.) and the unique morphology of the infectious form as homologous features proving the close relationship and monophyletic origin of these bacteria. New observations, however, cast doubt on this possibility (Fokin et al., 1996).

The behavior of the infectious forms of certain *Holospora* species to assemble in the connecting piece of the dividing host nucleus is certainly highly advanced and must be regarded as an apomorphic feature. This behavior ensures that the infectious forms are specifically collected and released by the host cell. *Holospora caryophila*, *H. bacillata* and *H. curvata* do not share this feature with the other holosporas (Fokin et al., 1996; Fokin and Sabaneyeva, 1997), and it is not clear how the bacteria of these species leave their host nuclei. They either are more primitive than the other holosporas, as a quantitative separation of infectious forms and reproductive forms is not observed, or are phylogenetically not closely related. Two species groups may presently be distinguished in the genus *Holospora* (Table 1). It has been hypothesized that the unique behavior of the infectious form to deposit enormous amounts of periplasmic materials polarly could be encoded on a plasmid or phage genome. However, no plasmid or phage genome has been found (M. S. Rautian et al., unpublished results). It is consistent with this observation that the "more advanced" species tested (of which the infectious forms are collected in the separation spindle) were labelled by in situ-hybridization using an oligonucleotide probe designed for *H. obtusa* (Amann et al., 1991; Fokin et al., 1996).

An unnamed symbiont of this *Holospora*-type has repeatedly been observed in *Stentor multiformis* and in *S. polymorphus* (Görtz and Wiemann, 1987); another one has been observed in the peritrich ciliate *Zoothamnium pelagicum* by Laval (1970). Similar morphology and life cycle were also observed in bacterial symbionts in the ciliates *Trithigmostoma cucullulus* (Görtz and Maier, 1991) and *Spirostomum* sp. (H.-D. Görtz, unpublished observation). These observations indicate that symbionts of the genus *Holospora* are not restricted to *Paramecium*. Some strains of *Paramecium* were found to be harmed after infection with holosporas, whereas other strains apparently remained unaffected.

Table 1. Descriptions of *Holospora* spp.

Holospora[a]	Morphology and size of infectious form	Host *Paramecium*	Nucleus	Species group[b]
"*H. acuminata*"	Straight, tapered ends, length 4–6μm	*P. bursaria*	Micronucleus	I
"*H. bacillata*"	Straight, ends rounded, length 5–17μm	*P. woodruffi* *P. calkinsi*	Macronucleus	II
H. caryophila	Spiral, tapered ends, length 5–6μm	*P. biaurelia* *P. novaurelia* *P. caudatum*	Macronucleus	II
"*H. curvata*"	Curved, length 12–20 μm	*P. calkinsi*	Macronucleus	II
"*H. curviuscula*"	Curved, tapered ends, length 6–10 μm	*P. bursaria*	Macronucleus	I
H. elegans	Straight, tapered ends, length 7–18 μm	*P. caudatum*	Micronucleus	I
H. obtusa	Straight, tapered ends, length 7–20 μm	*P. caudatum*	Macronucleus	I
"*H. recta*"	Straight, tapered ends, length 10–15μm	*P. caudatum*	Micronucleus	I
H. undulata	Spiral, tapered ends, length 7–15μm	*P. caudatum*	Micronucleus	I

[a]Names in quotation marks are not validated.

[b]Species of group I are characterized by an escape mechanism of the infectious form from the host nucleus that is regarded as higher evolved. The infectious forms are collected in the connecting piece of the dividing nucleus and later released by the host cell (Fig. 8). Bacteria of species of group I react with a "*Holospora*-specific" oligonucleotide probe in fluorescence in situ hybridization (Fokin et al., 1996). For species of group II, the exact mode by which infectious forms leave their host nuclei is not known. In this group, infectious forms are not collected in the connecting piece of a dividing nucleus (Fokin et al., 1996; Fokin and Sabaneyeva, 1997). *Holospora* species of group II do not react with a "*Holospora*-specific" oligonucleotide probe in fluorescence in situ hybridization.

Holospora undulata *(Hafkine, 1890; Gromov and Ossipov, 1981)* This organism lives in the micronucleus of *P. caudatum*. Two forms are seen: a short, spindle-shaped, reproductive form, about 0.8 μm in diameter and 1.5–2.0 μm long, and a long, spiral-shaped infectious form that has tapered ends and is 7.0–15 μm long. The type strain is found in clone MI-48 of *P. caudatum* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia).

Holospora obtusa *(Hafkine, 1890; Gromov and Ossipov, 1981)* This organism lives in the macronucleus (Fig. 7). Reproductive forms are short rods, 0.8 μm in diameter and 1.5–2.5 μm long. Infectious forms are long rods with rounded ends, 0.8–1.0 μm in diameter and 7.0–20 μm long. Reflecting the fine structural organization, the infectious form shows a refractile part and a dark part in phase contrast light microsopy (Fig. 9). The type strain is found in clone M-115 of *P. caudatum* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia).

Holospora elegans *(Hafkine, 1890; Preer and Preer, 1982)* This organism lives in the micronucleus. Reproductive forms are short rods, 0.8 μm in diameter and 1.5–2.0 μm long. Infectious forms are long rods with tapered ends, 0.6–0.8 μm in diameter and 7.0–18 μm long. The type strain is found in ATCC strain 50008 (stock C1O1 of *P. caudatum*; Görtz and Dieckmann, 1980).

Holospora acuminata *(Ossipov, 1980)* This organism lives in the micronucleus of *P. bursaria*. Reproductive forms are fusiform rods, 0.6 μm in diameter and 2.0–2.5 μm long. lnfectious forms are straight fusiform rods with tapered ends, 0.8–1.0 μm in diameter and 4.0–6.0 μm long. The type strain is found in stock AC61-10 of *P. bursaria* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia).

Holospora caryophila *(Preer and Preer, 1982)* These organisms were formerly known as "alpha particles" (Preer, 1969), live in the macronucleus of *P. biaurelia* (Fig. 2), and may also be found in *P. caudatum* (Görtz, 1987). Reproductive forms are thin, fusiform rods, 0.4 μm in diameter and 1.0–3.0 μm long. Infectious forms are spiral shaped with tapered ends, 0.5 μm in diameter and 5.0–6.0 μm long. The type strain is found in ATCC strain 30694 (stock 562 of *P. tetraurelia*; see Preer et al., 1974).

Holospora recta *(Fokin, 1991)* This organism lives in the micronucleus of *P. caudatum*. Two forms are seen: a short, spindle-shaped, reproductive form, about 0.8 μm in diameter and 1.5–2.0 μm long, and a long, straight infectious form (one end rounded, one end tapered) 10–15 μm long.

Whereas all other *Holospora* spp. listed here definitely appear to be good species, this status has been questioned for *H. recta* by Rautian and Ossipov (M. S. Rautian and D. V. Ossipov, personal communication), who found that a strain of *H. elegans* had a number of individuals with *H. recta*-like features. The question may finally be resolved after molecular data have been obtained from analyses of the different strains.

Holospora curviuscula *(Borchsenius et al., 1983)* Cells live in the macronucleus of *P. bursaria*. They infect only certain strains of three syngens, and in other strains, the development into the infectious form is not completed. Occasionally, infection of both macro- and micronuclei was observed (Skoblo and Lebedeva, 1986). Reproductive forms are fusiform rods, 0.8 μm in diameter and 1.5–2.0 μm long. Infectious forms are slightly curved rods with tapered ends, approximately 0.8 μm in diameter and 6.0–10 μm long.

Holospora bacillata *(Fokin and Sabaneyeva, 1993)* This organism lives in the macronucleus of *Paramecium calkinsi* (Fokin and Sabaneyeva, 1993) and *Paramecium woodruffi* (Fokin et al., 1996; Fokin and Sabaneyeva, 1997). Infectious forms are 5–17.0 μm long, 0.7–0.8 μm wide, straight, rounded at both ends.

Holospora curvata *(Fokin and Sabaneyeva, 1993)* Cells live in the macronucleus of *Paramecium calkinsi* (Fokin and Sabaneyeva, 1993). Infectious forms are 12–20 μm long, 0.7–0.9 μm wide, curved, and rounded at both ends.

Symbionts of *Paramecium* Without Binomial Names Jenkins (1970) described a Gram-negative bacterium living within bulbous distensions of the outer membrane of the nuclear envelopes of both the micro- and macronucleus of a strain of *P. multimicronucleatum*. This symbiont is a very short rod, sometimes appearing nearly coccoid, approximately 0.35 μm in diameter with longer forms reaching 0.7 μm in length. It was named "epsilon."

Another symbiont, reported to occur in the perinuclear space, has been described by Fokin

(1988). It was found inside the nuclear cisternae of the macronuclear envelope of *P. duboscqui*. The symbiont is 0.3 µm in diameter and 0.7–1.4 µm long, looks spindle-shaped and is Gram negative. No killing activity was observed when symbiont bearers were tested against nonsymbiont bearers.

A symbiont studied by Estève (1978) occurs in the macronucleus of *P. caudatum* and confers a killer trait on its host. When it was investigated cytologically, a greatly enlarged macronucleus was observed to contain numerous kappa-like bacteria, some of which contained R bodies. Electron micrographs of this bacterium showed spherical phages inside the R bodies. Schmidt and coauthors assumed that the symbiont is identical with *Caedibacter caryophilus* described by them (Schmidt et al., 1987b).

A so far unnamed symbiont found by Görtz and Freiburg (1984) living in the micronucleus of *P. bursaria* is a small rod, 0.5 µm in diameter and up to 2 µm long. Its ultrastructure suggests that it is a Gram-negative bacterium. No flagella were found and no killing capacity of its host was observed. Another symbiont of the nonkiller type was discovered in the cytoplasm of *P. woodruffi*, a ciliate living in brackish water. The symbiont is a Gram-negative rod (0.2–0.8 µm in diameter and 0.6–2.5 µm long) that lacks flagella and contains hexagonal viroid particles (Fokin et al., 1987b).

In *P. sexaurelia* isolated from an aquarium with tropical fish, a bacterial endosymbiont was observed by Görtz (1981) to invade the macronucleus but not the micronucleus. Once the symbiont has entered the macronucleus, it tends to disappear from the cytoplasm. The bacterium is slightly curved with a diameter of 0.5–0.8 µm and up to 25 µm long. When present in the cytoplasm, the symbiont tends to be closely associated with food vacuoles. Once the symbiont has entered the macronucleus, it multiplies there without causing nuclear hypertrophy. After autogamy, the symbiont is found only in the cytoplasm and not in new macronuclear anlagen. It remains there until a new infection of the macronucleus occurs. The bacterium was observed again in a *P. sexaurelia* isolated by Fokin (Przybosz and Fokin, 1997) from a pond in the Wilhelma (Zoological and Botanical Garden, Stuttgart).

Table 2 summarizes the taxa of prokaryotic symbionts of *Paramecium* and provides a determinative key to their identification.

Table 2. Key to the prokaryotic symbionts of *Paramecium*.

I. Host paramecia are killers or mate killers
  A. Between 2 and 50% of the symbiont population contains R bodies
    1. Host paramecia are killers
      a. Kill by producing aboral humps on sensitive paramecia; R bodies unroll from inside at low pH, reroll at high pH; found in *P. tetraurelia*
      Caedibacter taeniospiralis
      b. Kill in ways other than producing aboral humps
        (1) Found in *P. tetraurelia*; R bodies unroll from the outside irreversibly when exposed to high temperature or certain detergents
        Caedibacter pseudomutans
        (2) Found in *P. biaurelia*; R bodies unroll from outside irreversibly when exposed to high temperature or certain detergents
        Caedibacter varicaedens
        (3) Found in *P. caudatum*, found in the macronucleus only; R bodies unroll from inside
        Caedibacter caryophilus
    2. Host paramecia are mate killers
      Caedibacter paraconjugatus
  B. Symbiont population does not contain R bodies
    1. Host paramecia are killers
      a. Rapid lysis killer; symbionts are large flagellated cells
        (1) Straight rods found in *P. tetraurelia*, *P. octaurelia*
        Lyticum flagellatum
        (2) Sinuous rods found in *P. biaurelia*
        Lyticum sinuosum (Fig. 3)
      b. Kill by vacuolization, symbionts are very small cells, often doublets
        Pseudocaedibacter minutus
    2. Host paramecia are mate-killers
      Pseudocaedibacter conjugatus
II. Host paramecia are nonkillers
  A. Symbionts are present only in the cytoplasm
    1. Symbionts lack flagella
      a. Host: *P. bursaria*; egg-shaped, rarely rod-shaped, up to 1.4 µm long. Antagonistic to symbiotic algae (*Chlorella*) of the host
      Caedobacter chlorellopellens

Table 2. *Continued*

b. Hosts: *P. biaurelia*, *P. tetraurelia*, *P. pentaurelia*; rods, up to 1.2 μm long
Pseudocaedibacter falsus
c. Host: *P. pentaurelia*; rods, up to 1.2 μm long; the symbiont-containing vacuole is surrounded by endoplasmic reticulum
Pseudocaedibacter glomeratus

2. Symbionts with flagella
a. Host: *P. caudatum*; rods; 3.5 to 14 μm long; refractile inclusions (polyhydroxy butyric acid); with numerous flagella, but no motility observed
Pseudolyticum multiflagellatum
b. Host: *P. primaurelia*; rods, up to 2 μm long, Gram negative with a thick surface layer visible in EM; with sparse flagella, occasionally slight motility; coexists with other symbionts such as *Caedibacter* and *Pseudocaedibacter*
Tectibacter vulgaris

B. Symbionts are present almost exclusively in the nuclei
1. Symbionts exclusively in the micronucleus, show two morphologically distinct forms, and are highly infectious
a. Long form of symbiont (infectious form) spiral-shaped, both ends tapered, 7–15 μm long; host: *P. caudatum*
Holospora undulata
b. Long form of symbiont (infectious form) straight rod with tapered ends
(1) Host: *P. caudatum*; long form 7–18 μm long
Holospora elegans
(2) Host: *P. bursaria*; long form 4–6 μm long
Holospora acuminata
c. Long form of symbiont (infectious form) straight rod, one end tapered, one end rounded, host: *P. caudatum*
Holospora recta

2. Symbionts exclusively in the macronucleus
a. Symbionts are highly infectious, two morphologically distinct forms often observable
(1) Long form slightly spiral shaped, with tapered ends, 5–6 μm long; host: *P. biaurelia* (sometimes also observed in *P. caudatum*)
Holospora caryophila (Fig. 2)
(2) Long form straight rod, with rounded ends, 7–20 μm long; host: *P. caudatum*
Holospora obtusa (Fig. 9)
(3) Long form straight rod, with rounded ends, 5–17 μm long; host: *P. calkinsi* or *P. woodruffi*
Holospora bacillata
(4) Long form slightly curved, with tapered ends, 6–10 μm long; host: *P. bursaria*
Holospora curviuscula
(5) Long form curved, both ends rounded, 12–20 μm long, host: *P. calkinsi*
Holospora curvata
b. Symbionts are weakly infectious, only one morphological form observable. Host: *P. caudatum*; rods small, about 1 μm long, sometimes forming "chains" that are up to 10 μm long; mostly aggregated in the center of the macronucleus; may coexist with *H. obtusa* or *H. undulata*
Nonospora macronucleata

Based on the key proposed by Preer (1981) and on information from articles cited in this Chapter.

Prokaryotic Symbionts of *Euplotes*

Endosymbiotic bacteria are also very common in *Euplotes*, a ciliate genus that comprises both freshwater and marine species (Heckmann, 1983a; Petroni et al., 2001). So far, only one of the symbionts has been given a binomial name. The others are still referred to by Greek letters, as was formerly customary for cytoplasmic elements. Several *Euplotes* symbionts remained unnamed or were provisionally given Latin letters as names when they were encountered. However, most of these symbionts are not well characterized.

Habitat of the *Euplotes* Symbionts

All except one of the bacterial symbionts of *Euplotes* are confined to the cytoplasm; see The Ciliate Cell as a Microcosm. They can easily be observed with a phase contrast microscope. Fixation and staining of *Euplotes* with aceto-carmine or lacto-aceto-orcein can be helpful for the observation and identification of these endosymbionts; see Orcein Staining of Intracellular Bacteria. When stained, *Polynucleobacter necessarius* (formerly omikron) and the closely related "omikron-like" bacteria reveal many nucleoids (Heckmann, 1975; Heckmann et al., 1983b; Figs. 19 and 20). It appears that most of the *Euplotes* symbionts cannot grow outside their hosts, although this has been investigated thoroughly only for *P. necessarius*. Like *Caedibacter* and other symbionts of the *P. aurelia* species complex, the *Euplotes* symbionts appear not to be infectious, at least under laboratory conditions, and some are killer symbionts similar to the killer symbionts of *Paramecium*; see The Killer

Trait in *Paramecium*. The symbionts found to date have not been observed to be harmful to their hosts, which, however, in some cases are converted by infection into killers or mate killers. *Polynucleobacter necessarius* and the closely related omikron-like bacteria are essential for their hosts (Heckmann, 1983a).

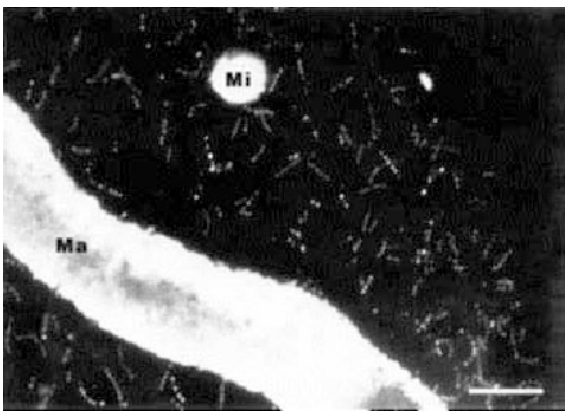


Fig. 19. Fluorescence micrograph of *Polynucleobacter necessarius* in the cytoplasm of a slightly crushed *Euplotes aediculatus* cell after staining with N,N'-diethylpseudoisocyanin chloride. The symbionts are revealed by the DNA-specific yellow fluorescence of their nucleoids. Ma, macronucleus; and Mi, micronucleus. Bar = 1 µm. From Heckmann (1975).

Isolation of the *Euplotes* Symbionts

Euplotes minuta, *E. crassus* and *E. vannus* are marine species. They can be isolated from water samples containing algae or detritus collected at the seashore. All these species can be easily grown in the laboratory in seawater with *Dunaliella salina* as the food organism (for details, see Heckmann, 1963). The other *Euplotes* species listed in Table 3 are freshwater

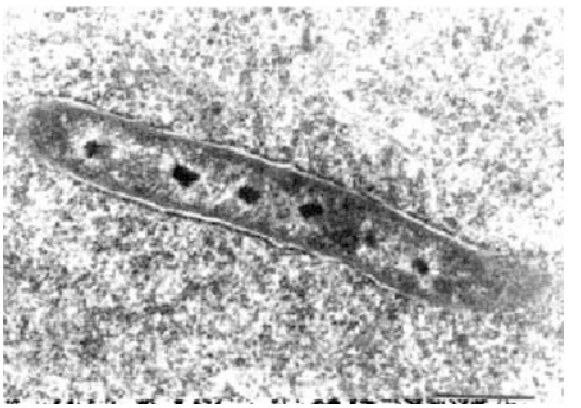


Fig. 20. Electron micrograph of *Polynucleobacter necessarius*. Longitudinal section. The ultrastructure of this symbiont resembles that of Gram-negative bacteria. Bar = 0.5 µm. From Heckmann (1975).

Table 3. Characteristics of the prokaryotic endosymbionts of the genus *Euplotes*.

Symbiont designation	Euplotes species[a]	Site	Killing type	References
Polynucleobacter necessarius	*E. aediculatus (15)*	*Cy*	*NK*	Heckmann and Schmidt, 1987
Omikron-like symbiont	*E. eurystomus (25)*	*Cy*	*NK*	Heckmann et al., 1983b
Omikron-like symbiont	*E. plumipes (24)*	*Cy*	*NK*	Heckmann et al., 1983b
Omikron-like symbiont	*E. daidaleos (13)*	*Cy*	*NK*	Heckmann et al., 1983b
Omikron-like symbiont	*E. octocarinatus (11)*	*Cy*	*NK*	Heckmann et al., 1983b
Omikron-like symbiont	*E. patella (5)*	*Cy*	*NK*	Heckmann et al., 1983b
Omikron-like symbiont	*E. woodruffi (22)*	*Cy*	*NK*	Heckmann et al., 1983b
Omikron-like symbiont	*E. moebiusi*	*Cy*	*ND*	Foissner, 1978
Omikron-like symbiont	*E. harpa*	*Cy*	*ND*	Petroni et al., 2001
Epsilon	*E. minuta* (K_1,K_2, K_3)	*Cy*	*K*	Heckmann et al., 1967
Epsilon-like	*E. minuta* (VF_{17})	*Cy*	*K + MK*	Heckmann et al., 1967
Eta	*E. crassus*	*Cy*	*K*	Nobili et al., 1976
A	*E. crassus*	*Cy*	*NK*	Rosati et al., 1976
B_1	*E. crassus* (C_8)	*Cy*	*MK*	Dini and Luporini, 1976
B_3	*E. crassus*	*Cy*	*NK*	Rosati et al., 1976
C	*E. crassus*	*Cy*	*NK*	Rosati et al., 1976
D	*E. crassus*	*ND*	*ND*	Rosati et al., 1976
Unnamed	*E. crassus*	*Ma*	*NK*	Rosati and Verni, 1975
Unnamed	*E. crassus*	*Cy*	*K + MK*	Demar-Gervais and Genermont, 1975
Unnamed[b]	*E. aediculatus*	*Cy*	*NK*	Heckmann et al., 1983b
Unnamed[c]	*E. octocarinatus*	*Cy*	*NK*	Heckmann et al., 1983b
Unnamed	*E. harpa*	*Cy*	*ND*	Petroni et al., 2001
Unnamed	*E. magnicirratus*	*Cy*	*ND*	Petroni et al., 2001

Abbreviations: Cy, cytoplasm; Ma, macronucleus; NK, nonkiller; K, killer; MK, mate killer; and ND, not determined.
[a]The typical strain designation is given in parentheses.
[b]Unnamed symbiont that is not omikron-like in *E. aediculatus* stocks 7 and 10.
[c]Unnamed symbiont observed in addition to omikron-like symbionts in stock 11 of *E. octocarinatus*.

organisms that may be collected from ponds. They can be grown in a diluted soil medium (Ruthmann and Heckmann, 1961) or in culture medium for *Euplotes* (CME; Kuhlmann and Heckmann, 1989) and fed with *Chlorogonium elongatum* or *Chilomonas paramecium*. Most *Euplotes* species can also utilize bacteria as food.

Since the symbionts may differ in different strains of a host species, it is advisable to start *Euplotes* cultures from single cells, so that the cultures maintained in the laboratory form clones. In most cases, this will ensure that one is dealing with homogeneous populations of endosymbionts. However, double and triple infections have also been observed.

Polynucleobaeter necessarius is the only endosymbiont of *Euplotes* known so far that has been isolated. In this case, cells of strain 15 of *E. aediculatus* were homogenized mechanically, and the symbionts were then purified by applying the homogenate to an ECTEOLA column, followed by elution with phosphate buffer (Heckmann, 1975; Schmidt, 1982). The procedure basically followed the one developed for the isolation of kappa symbionts from *Paramecium* by Smith-Sonneborn and Van Wagtendonk (1964) and resulted in very clean preparations of symbionts.

Identification of the *Euplotes* Symbionts

The bacterial symbionts of *Euplotes* can be detected by direct observation with a phase contrast microscope, by observation of physiological effects produced on other *Euplotes* cells that lack symbionts, or by a combination of these methods. In the past, identification of symbionts of *Euplotes* has also been done using these methods. Now, at least for *Polynucleobacter necessarius*, formerly omikron particles, identification by FISH using appropriate oligonucleotide probes has become possible.

The Taxa of Prokaryotic Symbionts of *Euplotes*

GENUS *POLYNUCLEOBACTER* (HECKMANN AND SCHMIDT, 1987) These are obligate endosymbiotic bacteria living in the cytoplasm of freshwater ciliates of the genus *Euplotes*. Characterized by multiple nucleoids, these symbionts are essential for their host species and are nonmotile and Gram negative. The type species is *Polynucleobacter necessarius*.

Polynucleobacter was found to belong to the β-subclass of Proteobacteria and shows the closest relationship to *Alcaligenes eutrophus*, *Burholderia solanacearum* and *B. pickettii* (Springer et al., 1996).

Polynucleobacter necessarius *(Heckmann and Schmidt, 1987)* This species was formerly called "omikron." Cells are slightly curved rods, about 0.3 μm in diameter and 2.5–7.5 μm long. If stained with DNA-specific dyes, usually 3–9, but in some cases, up to 12 intensely stained and regularly spaced dots become visible (Fig. 19). They are considered to be nucleoids. When examined with the electron microscope, these nucleoids differ from those of most free-living bacteria by exhibiting an electron-dense central core that resembles the chromatin of eukaryotes (Fig. 20). Whether this core is formed by proteins associated with DNA or some other material is not clear (Heckmann, 1975).

The symbionts are individually contained in vesicles, to which ribosomes are often attached. *Polynucleobacter necessarius* reproduces by transverse binary fission in a typical bacterial manner. However, the fission products have often been found to differ in size. Frequently, a 7 μm long rod containing eight to nine nucleoids was observed to bud off a 2.5 μm piece that contained three nucleoids (Heckmann, 1975).

The G+C content of the DNA of *P. necessarius* is 48 mol% by thermal denaturation. A value that was 2.8 mol% lower was found when the G+C content was calculated from the buoyant density, which was 1.7036 g/cm^3 (Schmidt, 1982). The differences could be caused by rare bases; however, no DNA chemistry has been done for *P. necessarius*. The average DNA content of the symbiont was determined to be 5.8×10^{-3} pg. Taking into account the average number of nucleoids, a DNA content of 0.5×10^9 daltons per nucleoid was calculated. This value is in close agreement with the value of the kinetic complexity of the DNA, which was determined to be 0.57×10^9 daltons when corrected for the G+C content (Schmidt, 1982). Equally small genomes have been reported for *Lyticum flagellatum*, *Pseudocaedibacter conjugatus* and *P. falsus*, endosymbionts of the *P. aurelia* species complex (Soldo and Godoy, 1973a); for xenosomes of *Parauronema acutum* (Figueroa-de Soto and Soldo, 1977); and for chlamydias, rickettsias, and mycoplasmas (Bak et al., 1969). The genomes of these organisms are the smallest known for cells. They code for only about 700–900 proteins.

Heckmann (1975) has shown that it is possible to remove *P. necessarius* from *E. aediculatus* by treating a rapidly growing culture with penicillin (100–500 units/ml) for 5–6 days. Aposymbiotic hosts may undergo one or two fissions but then stop multiplying and die about 15–20 days after the last fission. The same results were obtained with several other antibiotics or with sufficiently high doses of X rays.

Reinfection and rescue of *E. aediculatus* have been achieved either by adding a cell homogenate from symbiont bearers or by injecting symbiont-containing cytoplasm (Heckmann, 1975; Fujishima and Heckmann, 1984a). The type strain is found in ATCC strain 30859 (clone 15 of *E. aediculatus*; see Heckmann and Schmidt, 1987).

THE OMIKRON-LIKE ENDOSYMBIONTS OF *EUPLOTES* These bacteria are very similar in appearance to *P. necessarius*. They were found in six freshwater *Euplotes* species (*E. eurystomus*, *E. plumipes*, *E. octocarinatus*, *E. patella* and *E. woodruffi*) all having a 9 type 1 fronto-ventral cirrus (FVC) pattern (9 FVC double dargyrome; Gates and Curds, 1979) like *E. aediculatus*, the bearer of omikron, and are considered to be closely related. The symbionts may differ from *P. necessarius* in size, shape, and other features, but they always share with *P. necessarius* the characteristic of multiple nucleoids (Heckmann et al., 1983b). Symbionts of this type, but about twice as large as *P. necessarius*, were first noticed by Fauré-Fremiet (1952) in stocks of *E. patella* and *E. eurystomus*. He also observed at that time that small doses of penicillin led to a loss of symbionts and to death of the host ciliates. He therefore suggested that the symbionts might be essential for survival of the ciliate. This was later confirmed when Heckmann et al. (1983b) subjected a large number of stocks of *Euplotes* species with a 9 type 1 cirrus pattern to a penicillin treatment of the kind that had earlier been found to remove *P. necessarius*. They found that all the ciliates stopped dividing and eventually died when their symbionts were removed. Heckmann et al. (1986) suggest that all *Euplotes* species with a 9 type 1 cirrus pattern suffer from a common deficiency that arose in a common ancestor of this group of organisms. This ancestor must have lived in symbiosis with a prokaryote that compensated for the acquired deficiency. *Polynucleobacter necessarius* and the omikron-like symbionts are considered to be progeny of this prokaryote.

SYMBIONTS OF *EUPLOTES* WITHOUT BINOMIAL NAMES As for the symbionts of *Paramecium*, many bacterial symbionts of *Euplotes* have not been given binomial names. For the time being, there is no information about their phylogenetic position. Nevertheless, they are included because they may be identified in further isolations by their host specificities and other distinct biological traits.

THE EPSILON SYMBIONTS OF *EUPLOTES* The epsilon symbionts from killer strains K_1, K_3, and K_7 of *E. minuta*, collected from the Mediterranean Sea at Villefranche-sur-Mer (France), were described by Heckmann et al. (1967). They are small rods, about 0.4 μm in diameter and 0.8–2.5 μm long, when observed in freshly crushed cells with bright phase optics, and appear somewhat darker than free-living bacteria. When the killer strain K_3 was fixed in Schaudinn's fluid, hydrolyzed in 1 N HCl, and stained with basic fuchsin, as described by Dippell and Chao in Sonneborn (1950), several dozen darkly stained bodies were found scattered in the cytoplasm. Their staining properties, size, shape, and cellular distribution resembled that of *Caedibacter* species and other endosymbionts of *Paramecium*. Since epsilon particles were found only in killer strains, it is assumed that they are responsible for the killer phenotype of *E. minuta*.

Siegel and Heckmann (1966) noticed that certain strains of *E. minuta* killed certain other strains of this species when conjugation occurred. It was found that the sensitive cells formed vacuoles, ceased their normal swimming movements, settled to the bottom of the culture vessel, and finally lysed. These changes occurred within 1–4 days after the strains had been mixed. No evidence for killing was observed when cells of the three killer strains were mixed together.

A good example of the difficulties encountered in the identification of endosymbionts is provided by the killer particles of stock VF_{17} of *E. minuta*. Heckmann et al. (1967) had noticed that this strain caused symptoms in sensitives slightly different from those observed in mixtures with the other *E. minuta* killers. Here, vacuole coalescence generally proceeded to the point where only one large vacuole was present in the posterior region of the sensitive, giving the whole affected organism a pear-shaped form. Nevertheless, no morphological differences between these symbionts and epsilon particles could be detected. Later, when stock VF_{17} was employed for cross-breeding experiments in a study of the genetic control of cortical pattern in *E. minuta* (Heckmann and Frankel, 1968), it was found that this stock not only killed sensitive strains via particles liberated into the medium, but also acted as a mate killer. From a pair of conjugants, one generally developed into a healthy looking exconjugant, whereas the other one failed to develop a large macronuclear anlage, became quiescent, and finally died. The surviving exconjugant clones had the same cortical pattern as stock VF_{17} and were all found to act as killers. They were therefore cytoplasmic descendants of the mate-killer parent. That they were true hybrids, i.e., that they had received a gametic nucleus from the sensitive partner, was shown from presence of certain genetic markers followed in these crosses. Whether VF_{17} was host

to a mixed population of symbionts—with one type responsible for the killer trait and the other one for the mate-killer trait, both types being so similar that they could not be distinguished morphologically from each other—or whether a single class of particles present in VF_{17} determined both traits cannot be decided. Both the killer trait and the mate-killer trait were lost concurrently with aging of the stock (Frankel, 1973).

THE ETA SYMBIONT OF *EUPLOTES* The eta symbiont, which was observed only by electron microscopy, is round or oval shaped with an average diameter of 0.9 μm. It is bound by three juxtaposed membranes. The outermost membrane has ribosomes attached to it. The interior of the symbiont is granular with a few scattered fibrous strands (Rosati and Verni, 1977). The symbiont was found in strains of *E. crassus* collected by Luporini (1974) from a site on the coast of Somalia on the Indian Ocean. It was named "eta" by Nobili et al. (1976), who investigated the killer trait conferred on its host by this symbiont. Sensitive testers, when exposed to the culture fluid of killer strains or to a homogenate prepared from cells of these lines, developed a large vacuole in the posterior part of the body within 6–24 hours. Affected cells are gradually transformed into transparent spheres that eventually "explode."

THE B_1 SYMBIONT OF *EUPLOTES* A symbiont very similar in morphology to eta appears to be responsible for the mate-killer properties of strain C_8 of *E. crassus* (Dini and Luporini, 1976); it was named "B_8." When cells of this strain were treated with penicillin, both the mate-killer trait and the symbiont disappeared. Dini and Luporini (1982) provided evidence for the existence of specific host genes required for maintenance of the B_8 particles.

INCONSPICUOUS SYMBIONTS OF *EUPLOTES* The other symbionts of *Euplotes* listed in Table 3 (A, B_3, C, D, and the unnamed ones) have been discovered by chance during electron microscopic investigations of certain strains. No special functions of these symbionts are known, nor have any effects of the symbionts on their hosts been reported. Interestingly, in two cases they have been observed in addition to omikron-like symbionts (Heckmann et al., 1983b; Petroni et al., 2001).

Ectosymbionts of *Euplotidium*

Peculiar ectosymbionts have been observed on the surface of the marine ciliate *Euplotidium itoi* (Fig. 21) and other species of the genus (Verni and Rosati, 1990). Though they have been studied in detail, their phylogenetic position remained obscure over years until their relationship to *Verrucomicrobia* was revealed (Petroni et al., 2000). It is the only ciliate symbiont belonging to *Verrucomicrobia*, while most symbionts of ciliates belong to the Proteobacteria. The authors found two morphologically different forms of the particles. At stage I of the life cycle, the organisms are spherical (1 μm in diameter; Fig. 22) and have a simple bacteria-like organization of their cell. These cells apparently divide by binary fission. Stage I epixenosomes may transform into stage II by gradually acquiring a more complex structure. Fully developed, stage II epixenosomes are larger (2.5 μm long) and egg-shaped (Fig. 23). They contain a sophisticated extrusive structure of a ribbon coiled around a central core surrounded by a basket built of bundles of regularly arranged tubules. The tubules have a number of features in common with eukaryotic microtubules (Rosati et al., 1993a). On top of the extrusive structure in the distal part of the cell body, an electrondense, dome-shaped structure lies underneath the cell membrane (Fig. 23). The dome-shaped structure contains DNA and basic proteins, and its ultrastructure resembles eukaryotic chromatin. The extrusive apparatus may be ejected upon appropriate stimuli (Rosati et al., 1993b).

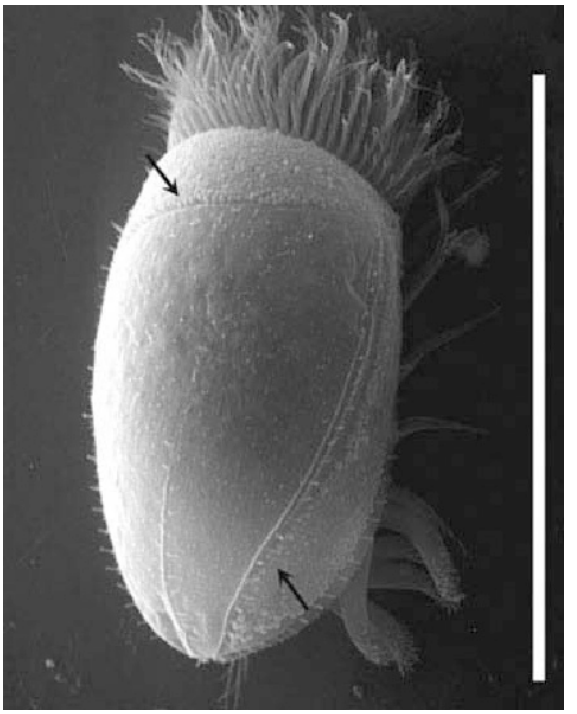


Fig. 21. Scanning electron micrograph of *Euplotidium itoi*, dorsal view. Epixenosomes (arrows) are arranged in a band on the cortex of the ciliate. Bar = 100 μm. From Petroni et al. (2000). © 2000 National Academy of Sciences, USA.

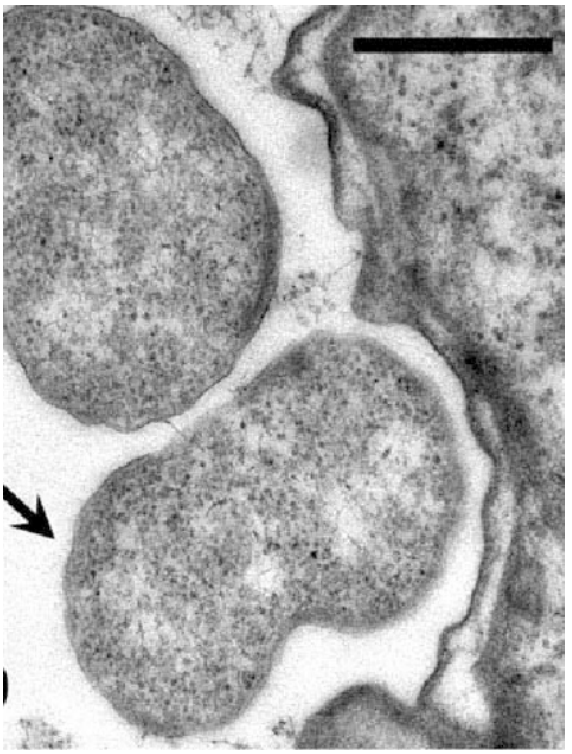


Fig. 22. Epixenosomes of stage I. Transmission electron micrograph of thin section. Bar = 1 µm. From Petroni et al. (2000). © 2000 National Academy of Sciences, USA.

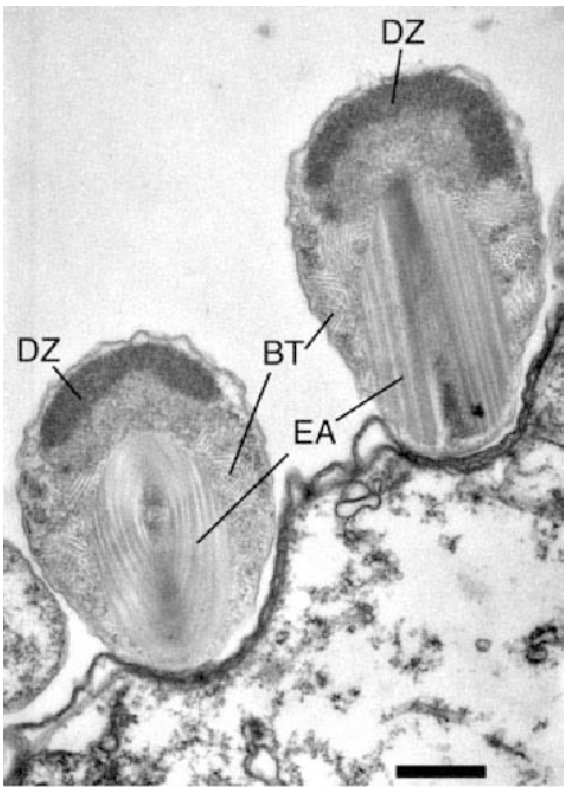


Fig. 23. Epixenosomes of stage II. Sections of two bacteria at different levels. DZ, apical (dome-shaped) zone; EA, extrusive apparatus; and BT, microtubule-like elements forming a basket around the extrusive apparatus. Bar = 1 µm. From Petroni et al. (2000). © 2000 National Academy of Sciences, USA.

Habitat and Biology of Epixenosomes of *Euplotidium*

The ciliate hosts of epixensomes, *Euplotidium itoi* and *E. arenarium*, were collected from tide pools along the rocky shore of the Ligurian Sea. As has been shown by Rosati, Verni and collaborators, epixenosomes have defensive function for the host cell (Rosati et al., 1999b). Predators such as the ciliate *Litonotus* easily ingested *Euplotidium* without epixenosomes, whereas they were not able to ingest *Euplotidium* with epixenosomes unless the ejecting capacity of the bacteria was inhibited.

Epixenosomes are arranged in a broad cortical band on the surface of host cells (Fig. 21). Individual bacteria appear fixed in cup-shaped indentations of the ciliate cortex. At these sites, the cortex of host cells is different in symbiont-bearing ciliates compared to symbiont-free cells (Verni and Rosati, 1990; Rosati, 1999a).

Identification of Epixenosomes of *Euplotidium*

While it appears easy to identify epixenosomes of *Euplotidium* as such by their unique site specificity, arrangement on the host cell, and ultrastructure, their phylogenetic position long remained obscure. Earlier attempts of classification of these organisms did not give definite results. Only by using a Eukaryotes forward primer and a backward primer designed for Archaea did amplification of the small ribsomal subunit (SSU) rRNA gene become possible (Petroni et al., 2000). A probe designed for part of the sequence labeled epixenosomes on the host *E. arenarium*.

The Xenosomes of *Parauronema acutum*

The term "xenosome" was coined by Soldo to denote the infectious particles that he found in the cytoplasm of the marine ciliate *Parauronema acutum* (Soldo et al., 1974c). They were later shown to be small bacteria that possess multicopy genomes and resemble *Caedibacter taeniospiralis* (kappa) and other cytoplasmic symbionts of *Paramecium* in many respects; see The Prokaryotic Symbionts of *Paramecium*. Two types of xenosomes were distinguished: killer xenosomes, which inhibit growth when taken up

by susceptible ciliates, especially those of the genus *Uronema* (Soldo and Brickson, 1978), and non-killer xenosomes (Soldo et al., 1987b). The xenosomes have not yet received binomial names.

Habitat of the Xenosomes of *Parauronema acutum*

Parauronema acutum is a small hymenostome marine ciliate that can be maintained axenically. The culture medium is basically that developed for species of the *Paramecium aurelia* complex by Soldo and Van Wagtendonk (1969), distilled water being replaced by seawater (density 1.015–1.026 g/ml) and modified to contain asolectin (500 μg/ml) as the sole source of lipid (Soldo et al., 1974c). The association with xenosomes is stable. Symbiont-bearing strains have now been maintained for more than 15 years.

Xenosomes released from host cells can infect symbiont-free strains of *P. acutum*. A single xenosome is capable of infecting a susceptible cell. However, a threshold of 100–200 xenosomes appears to be required before a single xenosome can infect a potential host (Soldo, 1983a). While phagocytosis is the usual way infectious bacteria enter host ciliates, Soldo and Brickson (1978) found that the symbionts in *Parauronema acutum*, termed "xenosomes" by those authors, penetrate the cell membrane at sites of the cortex other than the oral apparatus.

Soldo and coworkers measured the oxygen consumption of purified xenosome preparations and reported a rate of oxygen uptake of 1.3 nmoles O_2/min/mg of protein, which is higher than the oxygen uptake of *Bdellovibrio* and of the same order of magnitude as that of rickettsiae and *Caedibacter taeniospiralis*. Oxygen uptake is about 20 times lower than that of *E. coli*. The rate of oxygen consumption was found to be stimulated by various fatty acids, by intermediates of the glycolytic pathway, and by intermediates of the citrate cycle with the exception of citrate itself, which had no effect. Cyanide was found to be a potent inhibitor of oxygen consumption of xenosomes (Soldo, 1983a).

Isolation of Xenosomes from *Parauronema acutum*

Xenosomes can be isolated from *Parauronema acutum* by the same procedures as those used for the isolation of *Paramecium* symbionts (Soldo and Godoy, 1974b). It has been shown that killer xenosomes isolated in this way and purified with the help of Percoll gradients retain their ability to infect *P. acutum* and remain capable of killing sensitive *Uronema* strains (Soldo et al., 1986a). Results obtained from experiments in which isolated killer xenosomes lost their capacity to kill after they had been treated with various enzymes or had been coated with antibodies directed against the xenosomes indicate that the toxic principle of the xenosomes is a protein present at or near the surface (Soldo, 1987a).

Identification of Xenosomes from *Parauronema acutum*

These symbionts are rod-shaped, Gram-negative bacteria, 0.3 μm in diameter and 0.8 μm long. The G+C content of the DNA is 33.9 mol%. The symbionts occur in the cytoplasm of the marine ciliate *Parauronema acutum* in numbers ranging from 50 to 300 per cell. Negative staining reveals the presence of flagella, which provide the symbionts with a spinning and darting motility when released from the host (Soldo, 1987a). The xenosomes were found to contain inclusions in the form of helical arrays. These structures (called "H bodies") are about 0.6 μm long and 0.026 μm wide. They occur singly and in multiples and extend almost the entire length of the symbiont. The H bodies were found in over 50% of killer- and non-killer xenosomes (Soldo et al., 1987b).

The genome size of xenosomes was found to be only 515 kbp, which is much smaller than that of free-living bacteria. Analytical measurements and data from sedimentation rate analyses led to the conclusion that the chromosomal DNA exists in the form of nine circularly permuted double-stranded DNA molecules, each about 515 kbp in length (Soldo et al., 1983b). Both the small size and the multicopy nature of the genome are typical of bacterial symbionts rather than for free-living bacteria (Soldo and Godoy, 1973a; Soldo, 1987a).

Killer and non-killer xenosomes both contain plasmids (Soldo et al., 1986b; Soldo, 1987a). The non-killer plasmids consist of two circular DNA duplexes, each 63 kbp in length. The killer plasmid consists of four circular 63-kbp DNA duplexes. When a ciliate, which previously harbored killer xenosomes but had been freed of them, is infected with non-killer xenosomes, it becomes a killer. Soldo et al. (1986b) report that together with this transformation, the restriction pattern of the extrachromosomal DNA is altered. The mechanism by which this transformation takes place is unknown.

Prokaryotic Symbionts of Ciliates from Anaerobic Environments

Ciliates living in an anaerobic habitat, such as the rumen or sewage sludge rich in hydrogen sulfide,

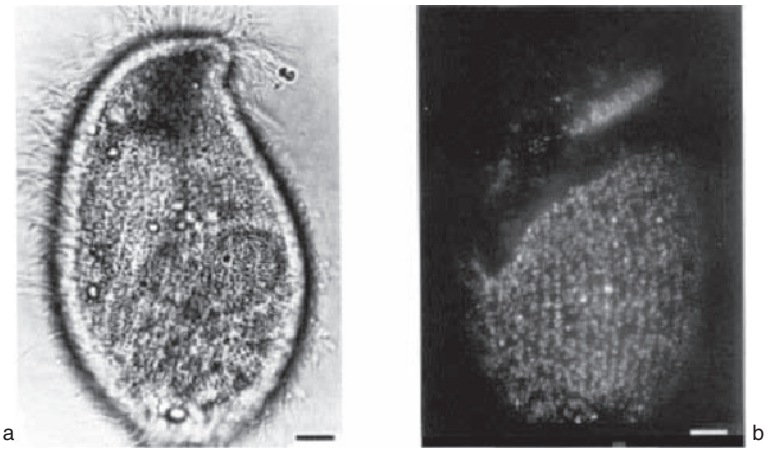


Fig. 24. *Metopus contortus* containing methanogenic endosymbionts: The methanogenic bacteria are located parallel to the kineties (ciliary rows) or the inner side of the cell. Cells are fixed with 1.2% formaldehyde and 0.3% glutaraldehyde. (a) Bright field micrograph. (b) Epifluorescence micrograph. Bars = 10 µm. From Van Bruggen et al. (1986).

show special adaptations. They usually lack mitochondria, bear hydrogenosomes, and are often associated with methanogenic bacteria (Finlay and Fenchel, 1989; Stumm and Vogels, 1989; Fenchel and Finlay, 1991a). It has been shown that anaerobic ciliates generate their energy by converting carbohydrates to lactate, acetate, and butyrate and that they can remove reducing equivalents in the form of H_2 (Müller, 1988). Production of H_2 by proton reduction involves the enzyme hydrogenase. However, this enzyme functions well only if the concentration of H_2 is kept low ($<10^{-5}$ atm). This requirement is apparently achieved by methanogenic bacteria, which consume H_2 and tend to be abundant in these habitats.

Habitat of Prokaryotic Symbionts of Ciliates from Anaerobic Environments

An episymbiotic association with methanogenic bacteria was described for 11 species of rumen ciliates of the family Ophryoscolecidae (Vogels et al., 1980). The attached bacteria were rods 0.9–3.8 µm long and 0.6–0.7 µm wide that occurred as clusters or long chains. The symbionts were identified as methanogens (probably *Methanobrevibacter ruminantium*) on the basis of specific fluorescent coenzymes (F_{350} and F_{420}; Figs. 24 and 25). Experiments with a fistulated sheep revealed a decrease of the association frequency of methanogenic bacteria with rumen ciliates when the hydrogen concentration in the rumen fluid increased and the reverse when hydrogen became scarce (Stumm et al., 1982). This is interpreted in favor of an interspecies transfer of hydrogen. The finding that rumen ciliates have hydrogenosomes, showing strong hydrogenase activity, supports this view (Yarlett et al., 1981). Stumm and Vogels (1989) propose that the attachment of hydrogen-consuming methanogens to rumen ciliates facilitates hydrogen

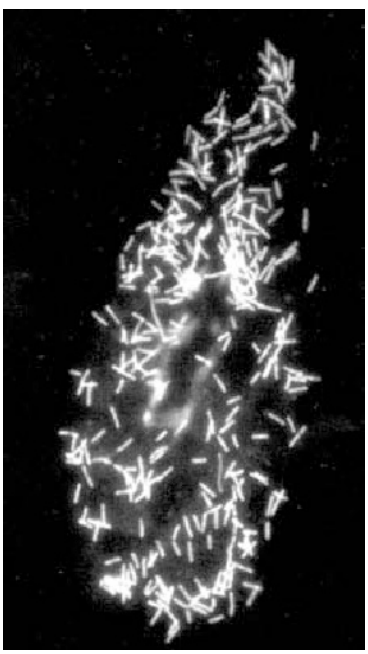


Fig. 25. Autofluorescence of endosymbiotic methanogens in *Metopus palaeformis*. From Finlay and Fenchel (1992).

removal, which is advantageous to both the ciliates and to their episymbionts. Fenchel and Finlay (Fenchel and Finlay, 1990; Fenchel and Finlay, 1991a), however, have argued that the symbiosis is not of great advantage for the ciliates, since the external hydrogen tension is low and diffusion pathways are very short, the problem being greater for the bacteria to get hold of the H_2 that is produced than it is for the ciliate to get rid of it. Fenchel and Finlay (1991) state that the presence of methanogens does not seem to be vital to the ciliates in any case that has been studied.

Episymbiotic bacteria have been known from sand-dwelling ciliates of anaerobic habitats for many years (Sauerbrey, 1928; Kahl, 1933; Kahl,

1935; Fauré-Fremiet, 1950b; Fauré-Fremiet, 1951). They are firmly attached to the cell surface and appear to differ in size, shape, pigmentation, and wall structure from species to species (Fenchel et al., 1977; Fenchel and Finlay, 1991a). Fauré-Fremiet (1950a) carried out a study of the symbionts of two species of the genus *Kentrophoros* (formerly *Centrophorella*). He investigated their symbionts and found them to be attached to the nonciliated dorsal side of these ribbon-shaped ciliates. The oblong symbionts were densely packed and protruded perpendicularly, so that they appeared like bristles of a brush. They were Gram-negative, nonmotile rods, and were observed to divide by longitudinal fission. Besides containing rather large quantities of polysaccharides (indicated by iodine treatment), they contained dark refractive sulfur globules, so that the host appeared black. The dark pigmentation is a strong indication that the symbionts are purple sulfur bacteria (Chromatiaceae). Fauré-Fremiet, who found the symbionts exclusively on the ciliates and neither on accompanying sand grains nor on glass slides that had been in the sand in which these ciliates lived for several days, considered the symbionts as obligately episymbiotic. Electron microscope studies by Raikov (Raikov, 1971; Raikov, 1974) confirmed Fauré-Fremiet's observations and led to the discovery that the episymbiotic bacteria are phagocytized. He studied *Kentrophoros fistulosum* and *K. latum* and found that these ciliates take up the episymbiotic bacteria by pseudopodia-like cytoplasmic protrusions and enclose them in food vacuoles. This may occur at any place on the nonciliated body side of these ciliates, which have no special mouth structures. The unique form of phagocytosis discovered in these species has been named "random phagocytosis." Based on the content of the food vacuole of these ciliates, their epizoic bacteria appear to be their main nutrition source. Fourteen *Kentrophoros* species are now known. They all live in the interstices of sandy marine sediments.

Fenchel et al. (1977) examined marine sediment-dwelling ciliates for cytochrome oxidase activity and for fine-structural details. They found that many of the ciliates of this habitat lacked cytochrome oxidase activity and mitochondria but contained microbodies that were identified as hydrogenosomes (Van Bruggen et al., 1986). In addition, these ciliates harbored a species-specific flora of epi- and endosymbiotic bacteria. Large numbers of symbionts per cell were observed, ranging from about 1000 bacteria per ciliate, corresponding to less than 1% of the host's biomass, to about 100,000 bacteria per ciliate, corresponding to about 20% of the ciliate's biomass. Some of these anaerobic ciliates were found to contain both epi- and endosymbionts, and often more than one type of episymbiont was recognized. Fenchel et al. (1977) hypothesized that the bacteria and the ciliates interact metabolically.

Anaerobic conditions also exist in sediments that are rich in decaying plant material, such as those of freshwater ponds, lakes, ditches and swamps. This habitat, also called "Faulschlamm" in German, was named "Sapropel" by Lauterborn (1901). Endosymbiotic bacteria in sapropelic ciliates were first described by Fauré-Fremiet (1909). Later, Liebmann (Liebmann, 1937; Liebmann, 1938) noted that all the sapropelic ciliates that he investigated contained rod-like bacteria in their cytoplasm. In recent years, this habitat was thoroughly investigated (Stumm and Vogels, 1989; Fenchel and Finlay, 1991a; Hackstein and Stumm, 1994), and it is now, next to the rumen, the best-studied anaerobic habitat. It is inhabited by large numbers of anaerobic protozoa and methanogenic bacteria. A survey of sapropelic ciliates by means of fluorescent microscopy revealed the presence of methanogenic bacteria inside the cells; they were spread throughout the cytoplasm in considerable quantities (Van Bruggen et al., 1983).

Electron microscopic investigations of sapropelic ciliates revealed the absence of mitochondria and the presence of microbodies. Because the bacteria were found to consume hydrogen, it has been hypothesized that the symbiont-associated microbodies are hydrogenosomes (Van Bruggen et al., 1986). In *Metopus striatus*, a Gram-positive rod-shaped bacterium was regularly found to be in close association with a hydrogenosome consisting of a granular matrix surrounded by a membrane (Van Bruggen et al., 1984). The bacterium was isolated and identified as *Methanobacterium formicicum*. A similar or even closer association of hydrogenosomes and methanogenic bacteria is reported for *Metopus contortus* (Van Bruggen et al., 1986; Finlay and Fenchel, 1989; Fig. 26), *Plagiopyla nasuta* (Goosen et al., 1988) and *Plagiopyla frontata* (Embley and Finlay, 1994). A direct proof was provided by Zwart et al. (1988), who demonstrated hydrogenase activity in microbodies of *Plagiopyla nasuta* and *Trimyema compressum*. Whereas methanogens are tightly packed with hydrogenosomes in ciliates such as *M. contortus* and *Plagiopyla frontata* (Fig. 27) and *Trimyema* species (e.g., Finlay and Fenchel, 1989; Fenchel and Finlay, 1991a), the long methanogens found in *Metopus palaeformis* are not closely associated (Finlay and Fenchel, 1991; Fig. 28). Symbionts in this ciliate appear to divide simultaneously and growth rates of ciliates and methanogens are approximatly equivalent (Fenchel and Finlay, 1991b; Finlay and Fenchel, 1992). A comparison of the phylogenetic diversity of

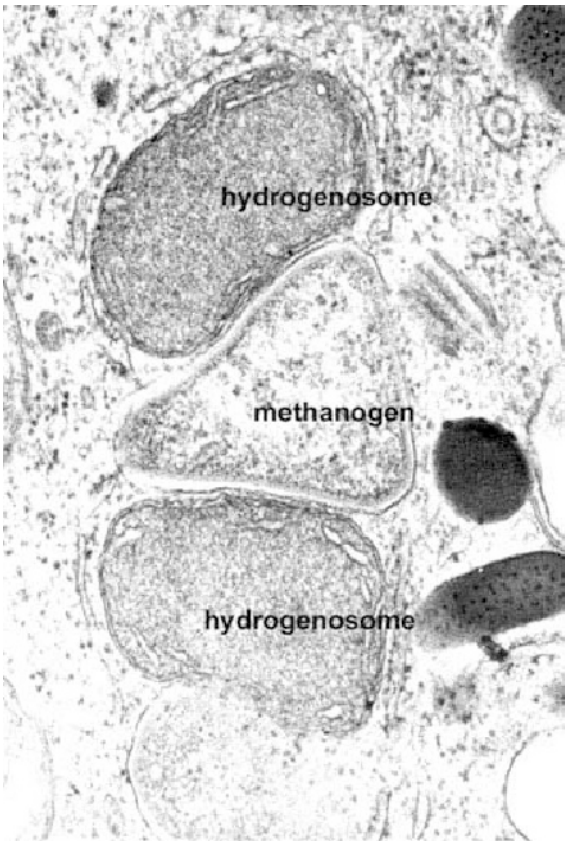


Fig. 26. Symbiotic methanogen with hydrogenosomes in a transmission electron micrograph of the ciliate *Metopus contortus*. From Finlay and Fenchel (1989). Reprinted from Finlay and Fenchel, 1989, © 1989, with permission from Elsevier Science.

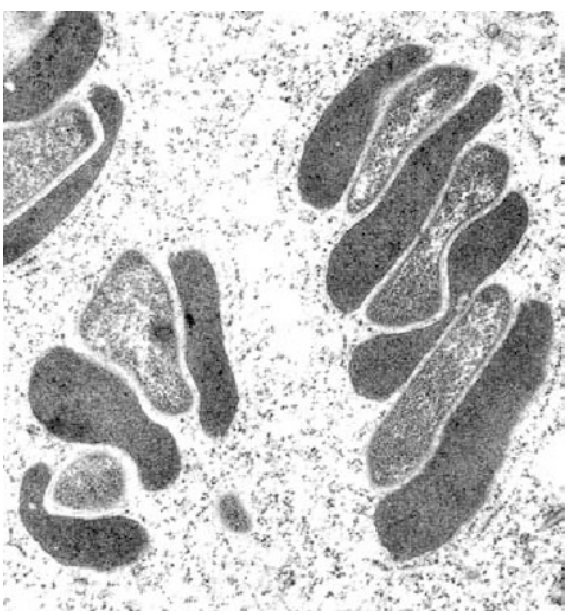


Fig. 27. Stacks of alternating hydrogenosomes (dark bodies) and methanogens in a transmission electron micrograph of *Plagiopyla frontata*. From Embley and Finlay (1994).

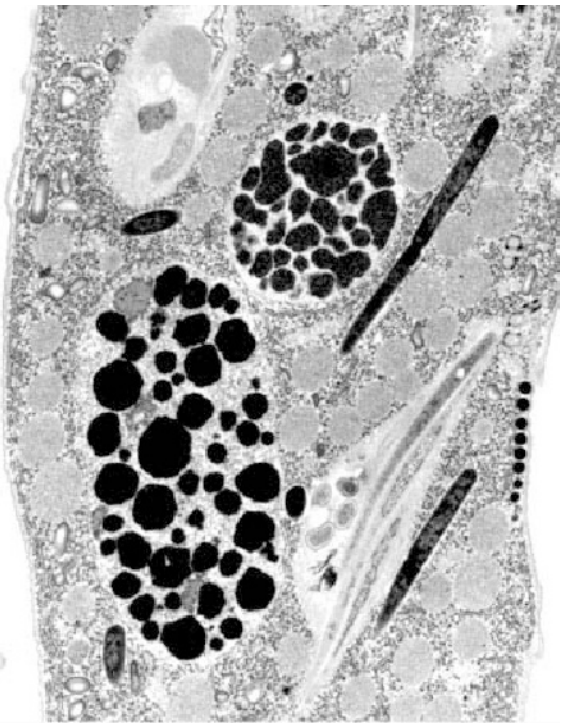


Fig. 28. A transmission electron micrograph of *Metopus palaeformis* with long rod-shaped methanogens. h, hydrogenosomes; and dv, digestive vacuole. Bar = 1 μm. From Finlay and Fenchel (1991).

endosymbiotic methanogens and anaerobic ciliates revealed that symbioses have formed repeatedly and independently (Finlay et al., 1993; Embley and Finlay, 1994) with endosymbionts from the genus *Methanobacterium* and from the Methanomicrobiales. The authors found no congruence between the host and endosymbiont trees and thus no evidence of parallel speciation. Congeneric hosts in the genus *Plagiopyla* and *Metopus* contain endosymbionts which are more closely related to free-living methanogens than to each other (Embley and Finlay, 1994).

While many marine anaerobic ciliates have endosymbiotic methanogens, ectosymbiotic bacteria that are often found in addition to the endosymbionts are never methanogens (Fenchel et al., 1977). Fenchel and Finlay (1991) assumed that the ectosymbionts are sulfate-reducing bacteria. Epibiotic bacteria have been reported also on the peritrichous ciliate *Zoothamnium niveum* collected in about 0.5–2.0 m depth on the vertical walls of a tidal channel cut into mangrove peat off the island of Twin Cays (Belize Barrier Reef, Carribean Sea; Bauer-Nebelsick et al., 1996a; Bauer-Nebelsick et al., 1996b). The association appeared to be obligatory (the ciliate being unable to survive without its symbiont), and it was also shown that oxygen and sulfide were

needed simultaneously. The white color of the bacteria is assumed to represent inclusions of elemental sulfur used as storage within the sulfide-oxidizing process. The bacteria were proven to be autotrophic. Epibiotic bacteria have been reported for a number of different species of *Zoothamnium* (cited in Bauer-Nebelsick et al., 1996a) but have not been further investigated.

Isolation of Prokaryotic Symbionts of Ciliates from Anaerobic Environments

For enrichment and culture of sapropelic ciliates, samples from anoxic natural sediments are introduced into mineral media under reducing conditions. Two media which have successfully been used for this purpose are given by Wagener and Pfennig (1987). They also reported the first monoxenic large-scale culture of an anaerobic ciliate. They enriched the ciliate *Trimyema compressum* from anoxic mud samples and then established a pure culture. The ciliate was fed with a bacterial strain isolated from the enrichment culture that proved capable of serving as food. During continued cultivation, *T. compressum* gradually lost its endosymbionts.

An isolation method for methanogenic bacteria from sapropelic ciliates has been described by Van Bruggen et al. (1986): A small number of ciliates is collected with a pipette and is washed free of potential contaminants in an isolation medium under a dissection microscope. The microscope is covered with a plastic hood flushed with nitrogen. The ciliates are then homogenized in an anaerobic glove box. The homogenate with the symbionts is plated on solid isolation medium containing penicillin G or lysozyme, which does not affect methanogenic bacteria. The homogenate is incubated at 22–37°C under an atmosphere of H_2/CO_2 (80/20). In liquid media, growth can be followed by measuring the methane production.

Identification of Prokaryotic Symbionts of Ciliates from Anaerobic Environments

The symbionts of several sapropelic ciliates have been identified as methanogenic bacteria. The endosymbionts of species of the ciliate *Metopus* (Fig. 24) were first recognized to be methanogens by their autofluorescence when irradiated with short-wavelength blue light (Van Bruggen et al., 1983; Van Bruggen et al., 1986). According to Doddema and Vogels (1978), epifluorescence microscopy can detect the presence of the deazaflavin coenzyme F_{420} and the pterin compound F_{342}, both of which are specific for methanogens. The methanogenic character of the endosymbionts has subsequently been proven by studies of the isolated symbionts and measurements of their methane production in situ. The symbionts were finally identified as *Methanobacterium formicicum* (Van Bruggen et al., 1984). The methanogenic symbionts of *Plagiopyla nasuta* were also identified as *M. formicicum* (Goosen et al., 1988). This is a bacterium that often occurs in the sapropel; it is a slender, nonmotile rod, with a diameter of 0.4 μm and a length of 2–7 μm. It was identified by colony form, cell morphology, temperature and pH optimum, substrate specificity, DNA base composition, and type of coenzyme F_{420}. It appears likely that *M. formicicum* is preadapted for endosymbiosis, because symbiont-free lines of the ciliate *Trimyema compressum* could also successfully be infected with this bacterium (Wagener et al., 1990).

Methanoplanus endosymbiosus was isolated from the marine ciliate *Metopus contortus* (Van Bruggen et al., 1986). In liquid media, the isolated bacteria were irregular, nonmotile, nonsporeforming discs with a diameter of 1.6–3.4 μm. In a side view, they appeared as rods that were sometimes branched. The symbionts were osmotically fragile and lysed immediately when suspended in water. Growth and methanogenesis were observed with H_2/CO_2 or formate as substrates, with generation times of 7 or 12 h, respectively. The temperature range from growth was between 16 and 36°C, with an optimum at 32°C. The optimal pH range for growth was 6.8–7.3. Tungsten and NaCl at a concentration of 0.25 M were required for optimal growth. The G+C content of the DNA is 38.7 mol%.

For detecting methanogenic endosymbionts, epifluorescence microscopy proved to be a powerful method. Even when symbionts are inside cells, their blue autofluorescence may be visible. Endosymbionts that fluoresce have been observed in the sapropelic ciliates *Brachonella spiralis*, *Caenomorpha medusula*, *C. universalis*, *Lacrymaria cucumis*, *Metopus es*, *M. laminarius* (Van Bruggen et al., 1983), *Parablepharisma pellitum*, *Metopus palaeformis* and others (Fenchel and Finlay, 1991b). They have not been found in ciliates from aerobic habitats.

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Literature Cited

Allen, S. L., and T. A. Nerad. 1978. Method for the simultaneous establishment of many axenic cultures of Paramecium. J. Protozool. 25:134–139.

Amann, R., N. Springer, W. Ludwig, H.-D. Görtz, and K.-H. Schleifer. 1991. Identification in situ and phylogeny of

uncultured bacterial endosymbionts. Nature 351:161–164.

Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.

Bak, A. L., F. T. Black, C. Christiansen, and E. A. Freundt. 1969. Genome size of mycoplasmal DNA. Nature 224: 1209–1210.

Ball, G. H. 1969. Organisms living on and in protozoa. *In:* T. T. Chen (Ed.) Research in Protozoology. Pergamon Press. Oxford, UK. 565–718.

Bauer-Nebelsick, M., C. F. Bardele, and J. Ott. 1996a. A symbiosis between Zoothamnium niveum (Hemprich and Ehrenberg 1831) Ehrenberg 1838 (Oligohymenophorea, Peritrichida) and thiotrophic bacteria. I: Redescription of the species based on light and scanning electron microscopic techniques. Eur. J. Protistol. 32:18–30.

Bauer-Nebelsick, M., C. F. Bardele, and J. Ott. 1996b. Electron microscopic studies of Zoothamnium niveum (Hemprich & Ehrenberg 1831) Ehrenberg 1838 (Oligohymenophorea, Peritrichida), a ciliate with ectosymbiotic, chemmoautotrophic bacteria. Eur. J. Protistol. 32: 202–215.

Beale, G. H., and A. Jurand. 1966. Three different types of mate-killer (mu) particle in Paramecium aurelia (syngen 1). J. Cell Sci. 1:31–34.

Beale, G. H., A. Jurand, and J. R. Preer Jr. 1969. The classes of endosymbionts of Paramecium aurelia. J. Cell Sci. 5:65–91.

Bedingfield, G., I. Gibson, and R. W. Horne. 1984. A comparative study of the structure of isolated refractile bodies (R-bodies) from Paramecium. 1: The effect of treatment with EDTA and EGTA. Micron. Microsc. Acta 15:235–246.

Beier, C. L., Horn, M., Michel, R., Schweikert, M., Görtz, H.-D., and Wagner, M. 2002. The genus Caedibacter comprises endosymbionts of Paramecium spp. related to the Rickettsiales (Alphaproteobacteria) and to Francisella tularensis (Gammaproteobacteria). Appl. Environ. Microbiol. 68(12):6043–6050.

Borchsenius, O. N., Skoblo, I. I., and Ossipov, D. V. 1983. Holospora curviuscula—a new species of macronuclear symbiotic bacteria of Paramecium bursaria. Cytologia 25:91–97.

Boss, A. O.-L., O. N. Borchsenius, and D. V. Ossipov. 1987. Pseudolyticum multiflagellatum n. g., n. sp.—a new symbiotic bacterium in the cytoplasm of Paramecium caudatum (Ciliata, Protozoa). Cytologia 29:94–97.

Bütschli, O. 1876. Studien über die ersten Entwicklungsvorgänge der Eizelle, die Zelltheilung und die Conjugation der Infusorien. Abh. Senckenb. Naturforsch. Ges. 10:213–464.

Bütschli, O. 1889. Protozoa. Abt. 111: Infusoria und System der Radiolaria. *In:* H. G. Bronn (Ed.) Klassen und Ordnungen des Thierreichs. C. F. Winter. Leipzig, Germany. 1:1098–2035.

Chen, T. T. 1955. Paramecin 34, a killer substance produced by Paramecium bursaria. Proc. Soc. Exp. Biol. Med. 88:541–543.

Corliss, J. O. 1985. Concept, definition, prevalence, and host-interactions of xenosomes (cytoplasmic and nuclear endosymbionts). J. Protozool. 32:373–376.

Demar-Gervais, C., and J. Genermont. 1976. Recherches génétiques sur Euplotes vannus: Les charactères "monster" et "killer." J. Protozool. 23 (Suppl.):8A.

Dilts, J. A. 1976. Covalently closed, circular DNA in kappa endosymbionts of Paramecium. Genet. Res. 627:161–170.

Dilts, J. A. 1986a. The importance of the refractile body in expression of the killer trait in Paramecium. *In:* R. B. Wickner, A. Hinnebusch, A. M. Lambowitz, I. C. Gunsalus, and A. Hollaender (Eds.) Extrachromosomal Elements in Lower Eukaryotes. Plenum Press. New York, NY.

Dilts, J. A., and R. L. Quackenbush. 1986b. A mutation in the R body-coding sequence destroyes expression of the killer trait in P. tetraurelia. Science 232:641–643.

Dini, F., and P. Luporini. 1976. The mate-killer trait in a stock of Euplotes crassus (Dujardin) (Ciliata, Hypotrichida). Monitore Zool. Ital. (N.S.) 10:15–24.

Dini, F., and P. Luporini. 1982. The inheritance of the mate-killer trait in Euplotes crassus (Hypotrichida, Ciliophora). Protistologica 18:179–184.

Doddema, H. J., and G. D. Vogels. 1978. Improved identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 36:752–754.

Dohra, H., M. Fujishima, and K. Hoshide. 1994. Monoclonal antibodies specific for periplasmic materials of the macronuclear specific bacterium Holospora obtusa of the ciliate Paramecium caudatum. Eur. J. Protistol. 30:288–294.

Dohra, H., K. Yamamoto, M. Fujishima, and H. Ishikawa. 1997. Cloning and sequencing of gene coding for a periplasmic 5.4 kDa peptide of the macronucleus-specific symbiont Holospora obtusa of the ciliate Paramecium caudatum. Zool. Sci. 14:69–75.

Dohra, H., M. Fujishima, and H. Ishikawa. 1998. Structure and expression of a GroE-homologous operon of a macronucleus-specific symbiont Holospora obtusa of the ciliate Paramecium caudatum. J. Eukaryot. Microbiol. 4:71–79.

Dohra, H., and M. Fujishima. 1999a. Cell structure of the infectious form of Holospora, an endonuclear symbiotic bacterium of the ciliate Paramecium. Zool. Sci. 16:93–98.

Dohra, H., and M. Fujishima. 1999b. Effects of antibiotics on the early infection process of the macronuclear endosymbiotic bacterium Holospora obtusa of Paramecium caudatum. FEMS Microbiol. Lett. 179:473–477.

Dorner, R. W. 1957. Stability of paramecin 34 at different temperatures and pH values. Science 126:1243–1244.

Ehrsam, E., and H.-D. Görtz. 1999. Surface proteins of the gram-negative bacterium Holospora obtusa bind to macronuclear proteins of its host Paramecium caudatum. Eur. J. Protistol. 35:304–308.

Embley, T. M., and B. J. Finlay. 1994. The use of small subunit rRNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. Microbiology 140:225–235.

Estève, J.-C. 1957. Une population de type "killer" chez Paramecium caudatum (Ehrenberg). Protistologica 14:201–207.

Euzeby, J. P. 1997. Revised nomenclature of specific or subspecific epithets that do not agree in gender with generic names that end in-bacter. Int. J. Syst. Bacteriol. 47(2):585–592.

Fauré-Fremiet, E. 1909. Sur un cas de symbiose par un infusoir cilié. C.R. Soc. Biol. 67:113–114.

Fauré-Fremiet, E. 1950a. Caulobactéries épizoiques associées aux Centrophorella (Cilies holotriches). Bull. Soc. Zool. France 75:134–137.

Fauré-Fremiet, E. 1950b. Ecologie des ciliés psammophiles litteraux. Bull. Biol. France-Belgique 84:35–75.

Fauré-Fremiet, E. 1951. The marine sand dwelling ciliates of Cape Cod. Biol. Bull. 100:59–70.

Fauré-Fremiet, E. 1952. Symbionts bacteriens des ciliés du genre Euplotes. C.R. Acad. Sci. 235:402–403.

Favinger, J., R. Stadtwald, and H. Gest. 1989. Rhodospirillum centenum, sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium. Ant. v. Leeuwenhoek 55:291–296.

Fenchel, T., T. Perry, and A. Thane. 1977. Anaerobiosis and symbiosis with bacteria in free-living ciliates. J. Protozool. 24:154–163.

Fenchel, T., and B. J. Finlay. 1990. Anaerobic free-living protozoa: growth efficiencies and the structure of communities. FEMS Microbiol. Ecol. 74:269–276.

Fenchel, T., and B. J. Finlay. 1991a. The biology of free-living anaerobic ciliates. Eur. J. Protistol. 26:200–216.

Fenchel, T., and B. J. Finlay. 1991b. Symchronous division of an endosymbiotic methanogenic bacterium in the anaerobic ciliate Plagiopyla frontata Kahl. J. Protozool. 38:22–28.

Figueroa-de Soto, V. E., and A. T. Soldo. 1977. Characterization and base sequence homologies of the DNA of xenosomes and related particles. Int. Congr. Protozool. 5:339.

Finlay, B. L., and T. Fenchel. 1989. Hydrogenosomes in some anaerobic protozoa resembling mitochondria. FEMS Microbiol. Lett. 65:187–190.

Finlay, B. L., and T. Fenchel. 1991. An anaerobic protozoon, with symbiotic methanogens, living in municipal landfill material. FEMS Microbiol. Lett. 65:169–180.

Finlay, B. L., and T. Fenchel. 1992. An anaerobic ciliate as a natural chemostat for the growth of endosymbiotic methanogens. Eur. J. Protistol. 28:127–137.

Finlay, B. J., T. M. Embley, and T. Fenchel. 1993. A new polymorphic methanogen, closely related to Methanocorpusculum parvum, living in stable symbiosis within the anaerobic ciliate Trimyema sp. J. Gen. Microbiol. 139:371–378.

Foissner, W. 1978. Euplotes moebiusi f. quadricirratus (Ciliata, Hypotrichida) II. The fine structure of some cytoplasmic organelles. Naturk. Jahrb. Stadt Linz 23:17–24.

Fok, A., and R. D. Allen. 1979. Axenic Paramecium caudatum. 1: Mass culture and structure. J. Protozool. 6:463–470.

Fok, A., and R. D. Allen. 1988. The lysosome system. *In:* H.-D. Görtz (Ed.) Paramecium. Springer-Verlag. Berlin, Germany. 301–324.

Fokin, S. I., and D. V. Ossipov. 1986. Pseudocaedibacter glomeratus n. sp.—A cytoplasmic symbiont of the ciliate Paramecium pentaurelia. Cytologia 28:1000–1004.

Fokin, S. I., D. V. Ossipov, I. I., Skoblo, M. S. Rautian, and E. V. Sabaneyeva. 1987a. Nonospora macronucleata g. n., sp. n.–a vegetative nucleus symbiont of the ciliate Paramecium caudatum. Cytologia 29:963–970.

Fokin, S. I., A. O.-L. Boss, and D. V. Ossipov. 1987b. Virus-containing cytoplasmic symbiont of the ciliate Paramecium woodruffi. Cytologia 29:1303–1306.

Fokin, S. I. 1988. A bacterial symbiont of the macronucleus perinuclear space in the ciliate Paramecium duboscqui. Cytologia 30:632–635.

Fokin, S. I. 1991. Holospora recta sp. nov., a micronucleus-specific endobiont of the ciliate Paramecium caudatum (Russian with English summary). Cytologia 33:135–141.

Fokin, S. I., and E. V. Sabaneyeva. 1993. Bacterial endocytobionts of the ciliate Paramecium calkinsi. Eur. J. Protistol. 29:390–395.

Fokin, S., and S. Karpov. 1995. Bacterial endocytobionts inhabiting the perinuclear space of Protista. Endocyt. Cell Res. 11:81–94.

Fokin, S. I., T. Brigge, J. Brenner, and H.-D. Görtz. 1996. Holospora species infecting the nuclei of Paramecium appear to belong into two groups of bacteria. Eur. J. Protistol. 32 (Suppl. 1):19–24.

Fokin, S. I., and E. Sabaneyeva. 1997. Release of endonucleobiotic bacteria Holospora bacillata and Holospora curvata from the macronucleus of their host cells Paramecium woodruffi and Paramecium calkinsi. Endocyt. Cell Res. 12:49–55.

Fokin, S. I., and Chivilev, S. M. 1999. Brackish water Paramecium species and Paramecium polycaryum: Morphometric analysis and some biological peculiarities. Acta Protozool. 38:105–117.

Frankel, J. 1973. Dimensions of control of cortical patterns in Euplotes: The role of preexisting structure, the clonal life cycle, and the genotype. J. Exp. Zool. 183:71–94.

Freiburg, M. 1985. Isolation and characterization of macronuclei of Paramecium caudatum infected with the macronucleus-specific bacterium Holospora obtusa. J. Cell Sci. 73:389–398.

Fujishima, M., and K. Heckmann. 1984a. Intra- and interspecific transfer of endosymbionts in Euplotes. J. Exp. Zool. 230:339–345.

Fujishima, M., and K. Nagahara. 1984b. Isolation of endonuclear symbiont Holospora obtusa from mass cultures of Paramecium caudatum. *In:* Proceedings of the Annual Meeting of the Zoological Societyof Japan. Toryokosku Press. Morioka, Japan. 55:36–40.

Fujishima, J., and M. Fujita. 1985. Infection and maintenance of Holospora obtusa, a macronuclear-specific bacterium of the ciliate Paramecium caudatum. J. Cell Sci. 76:179–187.

Fujishima, J., K. Nagahara, and K. Kojima. 1990a. Changes in morphology, buoyant density and protein composition in differentiation from the reproductive short form to the infectious long form of Holospora obtusa, a macronucleus-specific symbiont of the ciliate Paramecium caudatum. Zool. Sci. 7:849–860.

Fujishima, M., H. Sawabe, and K. Iwatsuki. 1990b. Scanning electron microscopic observation of differentiation from the reproductive short form to the infectious long form of Holospora obtusa. J. Protozool. 37:123–128.

Fujishima, M. 1993. Control of morphological changes of the endonuclear symbiont Holospora of the ciliate paramecium. *In:* S. Sato, M. Ishida, and H. Ishikawa (Eds.) Endocytobiology. Tübingen University Press. Tübingen, Germany. V:505–508.

Fujishima, M., H. Dohra, and M. Kawai. 1997. Quantitative changes in periplasmic proteins of the macronucleus-specific bacterium Holospora obtusa in the infection process of the ciliate Paramecium caudatum. J. Euk. Microbiol. 44:636–642.

Gates, M. A., and C. R. Curds. 1979. The dargyroome of the genus Euplotes. Bull. Br. Mus. Nat. Hist. (Zool.) 35:127–200.

Gellissen, G., and G. Michaelis. 1987. Gene transfer: mitochondria to nucleus. *In:* J. J. Lee and R. Fredrick (Eds.) Endocytobiology. NY Academy of Science. New York, NY. III:391–401.

Gibson, I., and G. H. Beale. 1961. Genic basis of the mate-killer trait in Paramecium aurelia stock 540. Genet. Res. 2:82–91.

Gibson, I. 1974. The endosymbionts of Paramecium. Crit. Rev. Microbiol. 3:243–273.

Gibson, I. 1984. A comparison of the refractile bodies (R-bodies) of certain bacteria. III. Nucleotide sequence homologies and R-body function. Micron. Microsc. Acta 15:253–273.

Goosen, N. K., A. M. C. Hormenans, S. J. W. Hillebrand, C. K. Stumm, and G. D. Vogels. 1988. Cultivation of the sapropelic ciliate Plagiopyla nasuta Stein and isolation of the endosymbiont Methanobacterium formicicum. Arch. Microbiol. 150:165–170.

Görtz, H.-D., and J. Dieckmann. 1980. Life cycle and infectivity of Holospora elegans Hafkine, a micronucleus-specific symbiont of Paramecium caudatum (Ehrenberg). Protistologica 16:591–603.

Görtz, H.-D. 1981. Ein neues symbiontisches Bakterium in Paramecium sexaurelia. Verh. Dtsch. Zool. Ges. 74: 227.

Görtz, H.-D. 1983. Endonuclear symbionts in ciliates. *In:* K. W. Jeon (Ed.) Intracellular Symbiosis. International Review of Cytology, Supplement 14. Academic Press. New York, NY. 145–176.

Görtz, H.-D., and M. Freiburg. 1984. Bacterial symbionts in the micronucleus of Paramecium bursaria. Endocyt. Cell Res. 1:37–46.

Görtz, H.-D. 1987a. Different endocytobionts simultaneously colonizing ciliate cells. Ann. NY Acad. Sci. 503:261–268.

Görtz, H.-D., and M. Wiemann. 1987. Colonization of the ciliate Stentor multiformis by three different endocytobionts. Endocyt. Cell Res. 4:177–184.

Görtz, H.-D., M. Freiburg, and M. Wiemann. 1988. Polypeptide differences between infectious and reproductive forms of Holospora obtusa, an endonucleobiotic bacterium in Paramecium caudatum. Endocyt. Cell Res. 5:233–244.

Görtz, H.-D., and M. Wiemann. 1989a. Route of infection of the bacteria Holospora elegans and Holospora obtusa into the nuclei of Paramecium caudatum. Europ. J. Protistol. 24:101–1090.

Görtz, H.-D., N. Ahlers, and H. Robenek. 1989b. Ultrastructure of the infectious and reproductive forms of Holospora obtusa, a bacterium infecting the macronucleus of Paramecium caudatum. J. Gen. Microbiol. 135:3079–3085.

Görtz, H.-D., S. Lellig, O. Miosga, and M. Wiemann. 1990. Changes in fine structure and polypeptide pattern during development of Holospora obtusa, a bacterium infecting the macronucleus of Paramecium caudatum. J. Bacteriol. 172:5664–5669.

Görtz, H.-D., and G. Maier. 1991. A bacterial infection in a ciliate from sewage sludge. Endocyt. Cell Res. 8:45–52.

Görtz, H.-D. 1992a. Bactéries endosymbiotiques de Paramecium. Année Biologique. 73:41–49.

Görtz, H.-D., J. Benting, I. Ansorge, and M. Freiburg. 1992b. Cell surface proteins of the infectious forms of the symbiotic bacterium Holospora obtusa. Symbiosis 14:391–397.

Görtz, H.-D. 2001. Intracellular bacteria in Ciliates. Internatl. Microbiol. 4(3):143–150.

Grimes, G. W., and J. R. Preer Jr. 1971. Further observations on the correlation between kappa and phage-like particles in Paramecium. Genet. Res. 18:115–116.

Gromov, B. V., and D. V. Ossipov. 1981. Holospora (ex Hafkine 1890) nom. rev., a genus of bacteria inhabiting the nuclei of paramecia. Int. J. Syst. Bacteriol. 31:348–352.

Hackstein, J. H. P., and C. K. Stumm. 1994. Methane production in terrestrial arthropods. Proc. Natl. Acad. Sci. 91:5441–5445.

Hafkine, M.-W. 1890. Maladies infectieuses des paramécies. Ann. Inst. Pasteur (Paris) 4:148–162.

Hausmann, K., and P. C. Bradbury (Eds.). 1996. Ciliates—cells as organisms. Gustav Fischer-Verlag. Stuttgart, Germany.

Heatherington, S. 1934. The sterilization of protozoa. Biol. Bull. 67:315–321.

Heckmann, K. 1963. Paarungstypsystem und genabhängige Paarungstypdifferenzierung bei dem hypotrichen Ciliaten Euplotes vannus O. F. Müller. Arch. Protistenk. 106:393–421.

Heckmann, K., J. R. Preer Jr., and W. H. Straetling. 1967. Cytoplasmic particles in the killers of Euplotes minuta and their relationship to the killer substance. J. Protozool. 1:360–363.

Heckmann, K., and J. Frankel. 1968. Genic control of cortical pattern in Euplotes. J. Exp. Zool. 168:11–38.

Heckmann, K. 1975. Omikron, ein essentieller Endosymbiont von Euplotes aediculatus. J. Protozool. 22:97–104.

Heckmann, K. 1983a. Endosymbionts of Euplotes. *In:* K.-W. Jeon (Ed.) Intracellular Symbiosis. International Review of Cytology, Supplement 14. Academic Press. New York, NY. 111–144.

Heckmann, K., R. ten Hagen, and H.-D. Görtz. 1983b. Fresh-water Euplotes species with a 9 type 1 cirrus pattern depend upon endosymbionts. J. Protozool. 30:284–289.

Heckmann, K., H. J. Schmidt, and M. Fujishima. 1986. Omikron and omikron-like endosymbionts of Euplotes. *In:* R. B. Wickner, A. Hinnebusch, A. M. Lambowitz, I. C. Gunsalus, and A. Hollaender (Eds.) Extrachromosomal Elements of Lower Eukaryotes. Plenum Press. New York, NY. 327–335.

Heckmann, K., and H. J. Schmidt. 1987. Polynucleobacter necessarius gen. nov., spe. nov., an obligately endosymbiotic bacterium living in the cytoplasm of Euplotes aediculatus. Int. J. Syst. Bacteriol. 37:456–457.

Heckmann, K., and H.-D. Görtz. 1991. Prokaryotic symbionts of ciliates. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-D. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3865–3890.

Henry, S. M. 1966. Symbiosis. Academic Press. New York, NY.

Heruth, D. P., F. R. Pond, J. A. Dilts, and R. L. Quackenbush. 1994. Characterization of genetic determinants for R body synthesis and assembly in Caedibacter taeniospiralis 47 and 116. J. Bacteriol. 176:3559–3567.

Holtzman, H. E. 1959. A kappa-like particle in a non-killer stock of Paramecium aurelia, syngen 5. J. Protozool. 6 (suppl.):26.

Jenkins, R. A. 1970. The fine structure of a nuclear envelope associated endosymbiont of Paramecium. J. Gen. Microbiol. 61:355–359.

Kahl, A. 1933. Ciliata libera et ectocommensalia. *In:* G. Grimpe and E. Wagler (Eds.) Die Tierwelt der Nord- und Ostsee, Teil II. Akademische Verlagsgesellschaft Becker & Erler. Leipzig, Germany. 29–146.

Kahl, A. 1935. Urtiere oder Protozoa I: Wimpertiere oder Ciliata (Infusoria). 4: Peritricha und Chonotricha. Nachtrag. *In:* F. Dahl (Ed.) Die Tierwelt Deutschlands. Gustav Fischer. Jena, Germany. 806–842.

Kanabrocki, J. A., J. Lalucat, B. J. Cox, and R. L. Quackenbush. 1986. Comparative study of refractile (R) bodies and their genetic determinants: relationship of type 51 R bodies to R bodies produced by Pseudomonas taeniospiralis. J. Bacteriol. 168:1019–1022.

Kawai, M., and M. Fujishima. 2000. Invasion of the macronucleus of Paramecium caudatum by the bacterium Holospora obtusa: Fates of the bacteria and timing of invasion steps. Eur. J. Protistol. 36:46–52.

Kirby Jr., H. 1941. Organisms living on and in protozoa. *In:* G. N. Calkins and F. M. Summers (Eds.) Protozoa in Biological Research. Columbia University Press. New York, NY. 1009–1113.

Kuhlmann, H.-W., and K. Heckmann. 1989. Adolescence in Euplotes octocarinatus. J. Exp. Zool. 251: 316–328.

Kusch, J., M. Stremmel, M. Schweikert, V. Adams, and H. J. Schmidt. 2000. The toxic symbiont Caedibacter caryophila in the cytoplasm of Paramecium novaurelia. Microb. Ecol. 40:330–335.

Kusch, J., L. Czubatinski, S. Wegmann, M. Hübner, M. Alter, and P. Albrecht. 2001. Competetive advantages of Caedibacter-infected paramecia. Protist 153:47–58.

Lalucat, J., O. Meyer, F. Mayer, R. Pares, and H. G. Schlegel. 1979. R bodies in newly isolated free-living hydrogen-oxydizing bacteria. Arch. Microbiol. 121:9–15.

Lalucat, J., B. Wells, and I. Gibson. 1986. Relationships between R bodies of certain bacteria. Micron Microsc. Acta 17:243–245.

Landis, W. G. 1981. The ecology, role of the killer trait, and interactions of five species of the Paramecium aurelia complex inhabiting the littoral zone. Can. J. Zool. 59:1734–1743.

Landis, W. G. 1987. Factors determining the frequency of the killer trait within populations of the Paramecium aurelia complex. Genetics 115:197–206.

Lauterborn, R. 1901. Die "sapropelische" Lebewelt. Zool. Anz. 24:50–55.

Laval, M. 1970. Présence de bactéries intranucléaires chez Zoothamnium pelagicum. Congr. Intern. Microsc. Electron., Grenoble 7th:403–404.

Liebmann, H. 1937. Bakteriensymbiose bei Faulschlammziliaten. Biol. Zbl. 57:442–445.

Liebmann, H. 1938. Biologie und Chemismus der Bleilochsperre. Arch. Hydrobiol. 33:1–81.

Luporini, P. 1974. Killer and autogamous strains of Euplotes crassus Dujardin from the Somalien coast. Monit. Zool. Ital. (N.S.) 5 (Suppl.):129–132.

Margulis, L., and R. Fester. 1991. Symbiosis as the source of evolutionary innovation. MIT Press. Cambridge, MA.

Meenaghan, H., K. Reilly, B. Wells, and I. Gibson. 1984. A comparison of the refractile bodies (R-bodies) of certain bacteria. II: Effects of pH on the structure. Micron Microsci. Acta 15:247–252.

Mueller, J. A. 1963. Separation of kappa particles with infective activity from those with killing activity and identification of the infective particles in Paramecium aurelia. Exp. Cell Res. 30:492–508.

Müller, J. 1856. Beobachtungen an Infusorien. Monatsberichte der Berliner Akademie 389–393.

Müller, M. 1988. Energy metabolism of protozoa without mitochondria. Ann. Rev. Microbiol. 42:465–488.

Nobili, R., G. Rosati, and F. Verni. 1976. The killer-trait in Euplotes crassus. Boll. Zool. 43:251–258.

Ossipov, D. V. 1973. Specific infectious specificity of the omega-particles, micronuclear symbiotic bacteria of Paramecium caudatum. Cytologia 15:211–217.

Ossipov, D. V., I. I. Skoblo, and M. S. Rautian. 1975. Iota-particles, macronuclear symbiotic bacteria of the ciliate Paramecium caudatum clone M 115. Acta Protozool. 14:263–280.

Ossipov, D. V., and S. A. Podlipaev. 1977. Electron microscope examinations of early stages of infection of Paramecium caudatum by bacterial symbionts of the macronucleus (Iota-bacteria). Acta Protozool. 16:289–308.

Ossipov, D. V., I. I. Skoblo, O. N. Borchsenius, M. S. Rautian, and S. A. Podlipaev. 1980. Holospora acuminata—a new species of symbiotic bacterium from the micronucleus of the ciíate Paramecium bursaria Focke. Cytologia 22:922–929.

Ossipov, D. V. 1981. Problems of nuclear heteromorphism in the unicellular organisms. NAUKA. Leningrad.

Ossipov, D. V., I. I. Skoblo, O. N. Borchsenius, and N. A. Lebedeva. 1993. Interactions between Paramecium bursaria (Protozoa, Ciliophora, Hymenostomatida) and their nuclear symbionts. I: Phenomenon of symbiogenic lysis of the bacterum Holospora acuminata. Eur. J. Protistol. 29:61–71.

Paulin, J. J. 1996. Morphology and cytology of ciliates. *In:* K. Hausmann and P. C. Bradbury (Eds.) Ciliates: Cells as Organisms. Gustav Fischer-Verlag. Stuttgart, Germany. 1–40.

Petroni, G., S. Spring, K.-H. Schleifer, F. Verni, and G. Rosati. 2000. Defensive extrusive ectosymbionts of Euplotidium (Ciliophora) that contain microtubule-like structures are bacteria related to Verrucomicrobia. Proc. Natl. Acad. Sci. USA 97:1813–1817.

Petroni, G., F. Dini, F. Verni, and G. Rosati. 2001. A molecular approach to the tangled intragenic relationships underlying phylogeny in Euplotes (Ciliophora, Spirotricha). Molec. Phylogenet. Evol. 22(1):118–130.

Pond, F. R., I. Gibson, J. Lalucat, and R. L. Quackenbush. 1989. R-body producing bacteria. Microbiol. Rev. 53:25–67.

Preer Jr., J. R. 1948a. A study of some properties of the cytoplasmic factor "kappa" in Paramecium aurelia, variety 2. Genetics 33:349–404.

Preer Jr., J. R. 1948b. The killer cytoplasmic factor kappa: Its rate of reproduction, the number of particles per cell, and its size. Am. Naturalist 82:35–42.

Preer Jr., J. R. 1950. Microscopically visible bodies in the cytoplasm of the "killer" strains of Paramecium aurelia. Genetics 35:344–362.

Preer Jr., J. R., and P. Stark. 1953. Cytological observations on the cytoplasmic factor "kappa" in Paramecium aurelia. Exp. Cell Res. 5:478–491.

Preer Jr., J. R., L. A. Hufnagel, and L. B. Preer. 1966. Structure and behavior of "R" bodies from killer paramecia. J. Ultrastr. Res. 15:131–143.

Preer Jr., J. R., and L. B. Preer. 1967. Virus-like bodies in killer-paramecia. Proc. Natl. Acad. Sci. USA 58:1774–1781.

Preer Jr., J. R., and A. Jurand. 1968. The relationship between virus-like particles and R bodies of Paramecium aurelia. Genet. Res. 12:331–340.

Preer Jr., J. R., L. B. Preer, B. Rudman, and A. Jurand. 1971. Isolation and composition of bacteriophage-like parti-

cles from kappa of killer paramecia. Molec. Gen. Genet. 111:202–208.
Preer Jr., J. R., L. B. Preer, and A. Jurand. 1974. Kappa and other endosymbionts of Paramecium aurelia. Bacteriol. Rev. 38:113–163.
Preer Jr., J. R. 1977. The killer system in Paramecium-kappa and its viruses. Microbiology 1977:576–578.
Preer Jr., J. R., and L. B. Preer. 1982. Revival of names of protozoan endosymbionts and proposal of Holospora caryophila nom. nov. Int. J. Syst. Bacteriol. 32:140–141.
Preer Jr., J. R., and L. B. Preer. 1984. Endosymbionts of protozoa. *In:* N. R. Krieg (Ed.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:795–811.
Preer, L. B., and J. R. Preer Jr. 1964. Killing activity from lysed particles of Paramecium. Genet. Res. 5:230–239.
Preer, L. B. 1969. Alpha, an infectious macronuclear symbiont of Paramecium aurelia. J. Protozool. 16:570–578.
Preer, L. B., A. Jurand, J. R. Preer Jr., and B. Rudman. 1972. The classes of kappa in Paramecium aurelia. J. Cell Sci. 11:581–600.
Preer, L. B., B. Rudman, and J. R. Preer Jr. 1974. Induction of R bodies by ultraviolet light in killer paramecia. J. Gen. Microbiol. 80:209–215.
Preer, L. B. 1981. Prokaryotic symbionts of Paramecium. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes. Springer. New York, NY. 2127–2136.
Przybosz, E., and S. I. Fokin. 1997. Species of the Paramecium aurelia complex Sonneborn in Germany. Arch. Protistenkd. 148:167–172.
Quackenbush, R. L. 1978. Genetic relationships among bacterial endosymbionts of Paramecium aurelia: polynucleotide sequence relationship among members of Caedobacter. J. Gen. Microbiol. 108:181–187.
Quackenbush, R. L. 1982. In: Validation of the publication of new names and new combinations previously published outside the IJSB. List No. 8. Int. J. Syst. Bacteriol. 32:266–268.
Quackenbush, R. L. 1983a. Plasmids from bacterial endosymbionts of hump-killer paramecia. Plasmid 9:298–306.
Quackenbush, R. L., and J. A. Burbach. 1983b. Cloning and expression of DNA sequences associated with the killer trait of Paramecium tetraurelia stock 47. Proc. Natl. Acad. Sci. USA 80:250–254.
Quackenbush, R. L., B. J. Cox, and J. A. Kanabrocki. 1986. Extrachromosomal elements of extrachromosomal elements of Paramecium and their extrachromosomal elements. *In:* R. B. Wickner, A. Hinnebusch, A. M. Lambowitz, I. C. Gunsalus, and A. Hollaender (Eds.) Extrachromosomal Elements in Lower Eukaryotes. Plenum Press. New York, NY. 265–278.
Quackenbush, R. L. 1988. Endosymbionts of killer paramecia. *In:* H.-D. Görtz (Ed.) Paramecium. Springer-Verlag. Berlin, Germany. 406–418.
Raikov, I. B. 1971. Bactéries épizoiques et mode de nutrition du cilié psammophile Kentrophorus fistulosum Fauré-Fremiet (étude au microscope electronique). Protistologica 7:365–378.
Raikov, I. B. 1974. Etude ultrastructurale des bactéries épizoiques et éndozoiques de Kentrophorus latum Raikov, cilié holotriche mésopsammique. Cahiers de Biologie Marine 15:379–393.
Read, C. P. 1970. Parasitism and Symbiology. Ronald Press. New York, NY.
Roller, C., W. Ludwig, and K.-H. Schleifer. 1992. Gram-positive bacteria with a high DNA G-C content are characterized by a common insertion with their 23 S rRNA genes. J. Gen. Microbiol. 138:1167–1175.
Rosati, G., and F. Verni. 1975. Macronuclear symbionts in Euplotes crassus (Ciliata, Hypotrichida). Bull. Zool. 42:231–232.
Rosati, G., F. Verni, and P. Luporini. 1976. Cytoplasmic bacteria-like endosymbionts in Euplotes crassus (Dujardin) (Ciliata, Hypotrichida). Monitore Zool. Ital. (N.S.) 10:449–460.
Rosati, G., and F. Verni. 1977. Bacteria-like endosymbionts in Euplotes crassus. *In:* Hutner, S. H. (Ed.) 5th Int. Congr. Protozool., Abstract Society of Protozoology. New York, NY. 443.
Rosati, G., P. Lenzi, and F. Verni. 1993a. "Epixenosomes": Peculiar epibionts of the protozoon ciliate Euplotidium itoi. Do their cytoplasmic tubules consist of tubulin?. Micron 24:465–471.
Rosati, G., F. Verni, and P. Lenzi. 1993b. "Epixenosomes": Peculiar epibionts of the ciliate Euplotidium itoi. The formation of the extrusive apparatus and the ejecting mechanism. Eur. J. Protistol. 29:238–245.
Rosati, G. 1999a. Epixenosomes: symbionts of the hypotrich ciliate Euplotidium itoi. Symbiosis 26:1–24.
Rosati, G., G. Petroni, S. Quochi, L. Modeo, and F. Verni. 1999b. Epixenosomes: peculiar epibionts of the hyprotrich ciliate Euplotidium itoi defend their host against predators. J. Eukaryot. Microbiol. 46:278–282.
Ruthmann, A., and K. Heckmann. 1961. Formwechsel und Struktur des Makronukleus von Bursaria truncatella. Arch. Protistenkd. 105:313–340.
Sauerbrey, E. 1928. Beobachtungen über einige neue oder wenig bekannte marine Ciliaten. Arch. Protistenkd. 61: 355–407.
Schmidt, H. J. 1982. Isolation of omikron-endosymbionts from mass cultures of Euplotes aediculatus and characterization of their DNA. Exp. Cell Res. 140:417–425.
Schmidt, H. J. 1984. Studies on protein synthesis in kappa particles. J. Gen. Microbiol. 130:1517–1523.
Schmidt, H. J., M. Freiburg, and H.-D. Görtz. 1987a. Comparison of the infectious forms of two bacterial endonucleobionts, Holospora elegans and H. obtusa, from the ciliate Paramecium caudatum. Microbios 49: 189–197.
Schmidt, H. J., H.-D. Görtz, and R. L. Quackenbush. 1987b. Caedibacter caryophila sp. nov., a killer symbiont inhabiting the macronucleus of Paramecium caudatum. Int. J. Syst. Bacteriol. 37:459–462.
Schmidt, H. J., F. R. Pond, and H.-D. Görtz. 1987c. Refractile bodies (R bodies) from the macronuclear killer particle Caedibacter caryophila. J. Cell Sci. 88:177–184.
Schmidt, H. J., H.-D. Görtz, F. R. Pond, and R. L. Quackenbush. 1988. Characterization of Caedibacter endonucleobionts from the macronucleus of Paramecium caudatum and the identification of a mutant with blocked R-body synthesis. Exp. Cell Res. 174:49–57.
Schneller, M. V. 1958. A new type of killing action in a stock of Paramecium aurelia from Panama. Proc. Indian Acad. Sci. 67:302.
Schneller, M. V., T. M. Sonneborn, and J. A. Mueller. 1959. The genetic control of kappa-like particles in Paramecium aurelia. Genetics 44:533–534.
Schönefeld, U., A. W. Alfermann, and J. E. Schultz. 1986. Economic mass cultivation of Paramecium tetraurelia on a 200-liter scale. J. Protozool. 33:222–225.

Siegel, R. W. 1953. A genetic analysis of the mate-killer trait in Paramecium aurelia, variety 8. Genetics 38:550–560.

Siegel, R. W., and K. Heckmann. 1966. Inheritance of autogamy and the killer trait in Euplotes minuta. J. Protozool. 13:34–48.

Simon, E. M., and M. V. Schneller. 1973. The preservation of ciliated protozoa at low temperature. Cryobiology 10: 421–426.

Sitte, P. 1993. Symbiogenetic evolution of complex cells and complex plastids. Eur. J. Protistol. 29:131–143.

Skoblo, I. I., O. N. Borchsenius, N. A. Lebedeva, and D. V. Ossipov. 1985. A new species of symbiotic bacteria of Paramecium bursaria, Ciliophora, Protozoa. Cytologia 27:1292–1297.

Skoblo, I. I., and N. A. Lebedeva. 1986. Infection of the nuclear apparatus of Paramecium bursaria (Ciliata) by the symbiotic bacterium Holospora curviuscula. Cytologia 28:367–372.

Skoblo, I. I., O. N. Borchsenius, N. A. Lebedeva, and D. V. Ossipov. 1990. Symbiogenic lysis of bacteria Holospora acuminata in the generative nucleus of the ciliate Paramecium bursaria. Cytologia 32:515–519.

Smith, I. E. 1961. Purification of kappa particles of Paramecium aurelia, stock 51. Am. Zool. 1:390.

Smith-Sonneborn, J. E., and W. J. Van Wagtendonk. 1964. Purification and chemical characterization of kappa of stock 51, Paramecium aurelia. Exp. Cell Res. 33:50–59.

Soldo, A. T. 1963. Axenic culture of Paramecium: Some observations on the growth behavior and nutritional requirements of a particle-bearing strain of Paramecium aurelia 299 lambda. Ann. NY Acad. Sci. 108:380–388.

Soldo, A. T., G. A. Godoy, and W. J. Van Wagtendonk. 1966. Growth of particle-bearing and particle-free Paramecium aurelia in axenic culture. J. Protozool. 13:492–497.

Soldo, A. T., and W. J. Van Wagtendonk. 1969. The nutrition of Paramecium aurelia, stock 299. J. Protozool. 16:500–506.

Soldo, A. T., W. J. Van Wagtendonk, and G. A. Godoy. 1970. Nuclei acid and protein content of purified endosymbiote particles of Paramecium aurelia. Biochim. Biophys. Acta 204:325–333.

Soldo, A. T., and G. A. Godoy. 1973a. Molecular complexity of Paramecium symbiont lambda deoxyribonucleic acid: evidence for the presence of a multicopy genome. J. Mol. Biol. 73:93–108.

Soldo, A. T., and G. A. Godoy. 1973b. Observations on the production of folic acid by symbiont lambda particles of Paramecium aurelia stock 299. J. Protozool. 20:502.

Soldo, A. T. 1974a. Intracellular particles in Paramecium. *In:* W. J. Van Wagtendonk (Ed.) Paramecium: A Current Survey. Elsevier. Amsterdam, The Netherlands. 377–442.

Soldo, A. T., and G. A. Godoy. 1974b. The molecular complexity of mu and pi symbiont DNA of Paramecium aurelia. Nuclei Acid Res. 1:387–396.

Soldo, A. T., G. A. Godoy, and S. A. Brickson. 1974c. Infectious particles in a marine ciliate. Nature 249:284–286.

Soldo, A. T., and S. A. Brickson. 1978. Observation on the ultrastructure, mode of infectivity and host range of xenosomes. Tissue and Cell 10:609–618.

Soldo, A. T. 1983a. The biology of the xenosome, an intracellular symbiont. *In:* K. W. Jeon (Ed.) Intracellular Symbiosis. International Review of Cytology, Supplement 14. Academic Press. New York, NY. 79–109.

Soldo, A. T., S. A. Brickson, and F. Larin. 1983b. The size and structure of the DNA genome of symbiont xenosome particles in the ciliate Parauronema acutum. J. Gen. Microbiol. 129:1317–1325.

Soldo, A. T., S. A. Brickson, G. A. Castiglione, and A. F. Freytag. 1986a. Association of transformation of xenosomes from nonkiller to killer with extrachromosomal DNA. J. Bacteriol. 169:96–102.

Soldo, A. T., S. A. Brickson, and A. Freytag. 1986b. Structure and characterization of extrachromosomal DNA of killer xenosomes, intracellular symbionts of a marine protozoan. *In:* R. B. Wickner, A. Hinnebusch, A. M. Lam, I. C. Gunsalus, and A. Hollaender (Eds.) Extrachromosomal Elements in Lower Eukaryotes. Plenum Press. New York, NY. 291–302.

Soldo, A. T. 1987a. Parauronema acutum and its xenosomes, a model system. J. Protozool. 34:447–451.

Soldo, A. T., S. A. Brickson, and G. A. Castiglione. 1987b. Inclusion bodies in xenosomes purified on percoll gradients as revealed by electron microscopy. J. Protozool. 34:6–9.

Sonneborn, T. M. 1938. Mating types, toxic interactions and heredity in Paramecium aurelia. Science 88:503.

Sonneborn, T. M. 1943. Gene and Cytoplasm. I: The determination and inheritance of the killer character in variety 4 of P. aurelia. II: The bearing of determination and inheritance of characters in P. aurelia on problems of cytoplasmic inheritance, pneumococcus transformation, mutations and development. Proc. Natl. Acad. Sci. USA 29:329–343.

Sonneborn, T. M. 1950. Methods in the general biology and genetics of Paramecium aurelia. J. Exp. Zool. 113:87–148.

Sonneborn, T. M. 1959a. Kappa and related particles in Paramecium. Adv. Virus Res. 6:229–356.

Sonneborn, T. M., J. A. Mueller, and M. V. Schneller. 1959b. The classes of kappa-like particles in Paramecium aurelia. Anat. Rec. 134:642.

Sonneborn, T. M. 1970. Methods in Paramecium research. *In:* M. Prescott (Ed.) Methods in Cell Physiology. Academic Press. New York, NY. 4:241–339.

Sonneborn, T. M. 1975. The Paramecium aurelia complex of fourteen sibling species. Trans. Am. Microsci. Soc. 94:155–178.

Springer, N., W. Ludwig, R. Amann, H. J. Schmidt, H.-D. Görtz, and K.-H. Schleifer. 1993. Occurrence of fragmented 16S rRNA in an obligate bacterial endosymbiont of Paramecium caudatum. Proc. Natl. Acad. Sci. USA 90:9892–9895.

Springer, N., R. Amann, W. Ludwig, K.-H. Schleifer, and H. J. Schmidt. 1996. Polynucleobacter necessarius, an obligate bacterial endosymbiont of the hypotrichous ciliate Euplotes aediculatus, is a member of the beta-subclass of Proteobacteria. FEMS Microbiol. Lett. 135:333–336.

Stumm, C. K., H. J. Gijzen, and G. D. Vogels. 1982. Association of methanogenic bacteria with bovine rumen ciliates. Br. J. Nutr. 47:95–99.

Stumm, C. K., and G. D. Vogels. 1989. Relations of autotrophic bacteria to protozoa. *In:* H. G. Schlegel and B. Bwien (Eds.) Autotrophic Bacteria. Springer-Verlag. Berlin, Germany. 177–191.

Takayanagi, T., and S. Hayashi. 1964. Cytological and cytogenetical studies on Paramecium polycarium. V: Lethal interactions in certain stocks. J. Protozool. 11:128–132.

Thiele, J., O. Honer-Schmid, J. Wahl, G. Kleefeld, and J. E. Schultz. 1980. A new method for axenic mass cultivation of Paramecium tetraurelia. J. Protozool. 27:118–121.

Van Bruggen, J. J. A., C. K. Stumm, and G. D. Vogels. 1983. Symbiosis of methanogenic bacteria and sapropelic protozoa. Arch. Microbiol. 136:89–95.

Van Bruggen, J. J. A., K. B. Zwart, R. M. Van Assema, C. K. Stumm, and G. D. Vogels. 1984. Methanobacterium formicicum, an endosymbiont of the anaerobic ciliate Methopus striatus McMurrich. Arch. Microbiol. 139:1–7.

Van Bruggen, J. J. A., K. B. Zwart, J. G. F. Hermans, E. M. Van Hove, C. K. Stumm, and G. D. Vogels. 1986. Isolation and characterization of Methanoplanus endosymbiosus sp. nov., an endosymbiont of the marine sapropelic ciliate Methopus contortus Quennerstedt. Arch. Microbiol. 144:367–374.

Van Wagtendonk, W. J., and A. T. Soldo. 1970. Methods used in the axenic cultivation of Paramecium aurelia. *In:* M. Prescott (Ed.) Methods in Cell Physiology. Academic Press. New York, NY. 4:117–130.

Verni, F., and G. Rosati. 1990. Peculiar epibionts in Euplotidium itoi (Ciliata, Hypotrichida). J. Protozool. 37:337–343.

Vogels, G. D., W. F. Hoppe, and C. K. Stumm. 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ. Microbiol. 40:608–612.

Wagener, S., and N. Pfennig. 1987. Monoxenic culture of the anaerobic ciliate Trimyema compressum Lackey. Arch. Microbiol. 149:4–11.

Wagener, S., C. F. Bardele, and N. Pfennig. 1990. Functional integration of Methanobacterium formicicum into the anaerobic ciliate Trimyema compressum. Arch. Microbiol. 153:496–501.

Wells, B., and R. W. Horne. 1983. The ultrastructure of Pseudomonas avenae. II: Intracellular refractile (R body) structure. Micron Microsc. Acta 14:329–344.

Wichterman, R. 1953. The biology of Paramecium. Plenum Press. New York, NY.

Wiemann, M. 1989a. The release of Holospora obtusa from Paramecium caudatum observed with a new device for extended in vivo microscopy. J. Protozool. 36:176–179.

Wiemann, J., and H.-D. Görtz. 1989b. Release of the endonucleobiotic bacterium Holospora elegans from its host cell Paramecium caudatum. Eur. J. Protistol. 25:100–108.

Wiemann, M., and H.-D. Görtz. 1991. Identification and localization of major stage-specific polypeptides of infectious Holospora obtusa with monoclonal antibodies. J. Bacteriol. 173:4842–4850.

Yarlett, N., A. C. Hann, D. Lloyd, and A. Williams. 1981. Hydrogenosomes in the rumen protozoon Dasytricha ruminantium Schuberg. Biochem. J. 200:365–372.

Zwart, K. B., N. K. Goosen, M. W. Van Schijndel, C. A. M. Broers, C. K. Stumme, and G. D. Vogels. 1988. Cytochemical localization of hydrogenase activity in the anaerobic protozoa Trichomonas vaginalis, Plagiopyla nasuta and Trimyema compressum. J. Gen. Microbiol. 134:2165–2170.

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CHAPTER 2.3

Bacteriocyte-Associated Endosymbionts of Insects

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Introduction

Intracellular associations between bacteria and insects are widespread in nature (Baumann and Moran, 1997; Buchner, 1965; Dasch et al., 1984; Douglas, 1989; Houk and Griffiths, 1980). Extensive studies of the natural history of such associations have led to the conclusion that they are commonly found in insects that utilize diets containing an excess of one class of compounds but a deficiency of some essential nutrients (Buchner, 1965; Dadd, 1985). It was thought that the function of the endosymbionts was to rectify this imbalance by the synthesis of these essential nutrients for the host. Extensive compilations of the occurrence of endosymbionts in different groups of insects are found in Buchner (1965) and Dasch et al. (1984). Because most of the prokaryotes involved in such associations are not cultivable on common laboratory media, their characterization had to await the development of recombinant DNA methodology. The past 10 years have witnessed the initiation of studies on the intracellular association of prokaryotes with a variety of insect hosts. In this chapter we will provide an overview of the evolution, and where possible, genetics and physiology of such recently studied associations. A summary of some of their features is presented in Table 1, and the phylogeny of the endosymbionts based on 16S rDNA is presented in Fig. 1.

The diversity of symbiotic associations and problems of definitions have been previously discussed and will not be considered here (Smith and Douglas, 1987; Werren and O'Neill, 1997). Some of the phylogenetic studies have included few host taxa and are thus not entirely conclusive; nevertheless, current results suggest that most of the associations considered in this chapter have common features and represent a relatively well-defined type. To aid presentation we will describe these common features, which are established from recent, largely molecular, studies as well as from older investigations based on morphological analyses. References to the earlier studies are found in Buchner (1965), who arrived at similar conclusions. References to recent studies are given as each association is considered.

The associations listed in Table 1 and Fig. 1 are the results of infections of various insect lineages with different prokaryotes. These associations became stable, resulting in the emergence of a new composite (of host and endosymbiont) organism. The endosymbiont became heritable through the acquisition of mechanisms ensuring vertical, maternal transmission to progeny. The association also became obligate, or beneficial, for host growth. Because the host depended on the association, and because horizontal or infectious transmission between hosts did not occur, the phylogeny of the endosymbionts is congruent with the phylogeny of the hosts. With some exceptions, heritable associations tend to become mutualistic (Lipsitch et al., 1995; Werren and O'Neill, 1997). In most cases, the host cannot survive without the endosymbiont, or the elimination of the endosymbiont has a deleterious effect. Although the advantage for the host is in most cases apparent, the advantage for the endosymbiont is not always clear. Perhaps it is more correct to think that the host domesticates the endosymbiont for its own welfare, utilizing functions that are present in the prokaryote but lacking in the host (Douglas and Smith, 1989; Maynard Smith and Szathmáry, 1995). In this chapter, the organism which is present in all the species of an insect group and which appears to be of essential value to the host is designated by either its scientific name or, if one is lacking, by the term primary (P-) endosymbiont.

Superimposed on this fundamental association may be associations with additional endosymbionts. Although these are heritable, they appear to be the result of multiple independent infections, horizontal transmission, or both. Since these endosymbionts may be absent in some hosts, their contribution to the welfare of the organism may not be major or essential. These organisms are designated as secondary (S-) endosymbionts. In this connection it is relevant that some bacterial strains may exist within the body cavity of insects for long periods without obvious deleterious effects, thus serving as

Table 1. General properties of the considered endosymbiotic associations.[a]

Host category[b]	Principal host food source	Symbiont designation	16S rRNA group or other taxonomic desgnation
Order: Homoptera Suborder: Sternorrhyncha Superfamily: Aphidoidea			
Aphids	Phloem sap	*Buchnera aphidicola* S-endosymbiont	γ-*Proteobacteria* *Enterobacteriaceae*[c]
Superfamily: Psylloidea			
Psyllids	Phloem sap	P-endosymbiont S-endosymbiont	γ-*Proteobacteria* γ-*Proteobacteria*
Superfamily: Aleyrodoidea			
Whiteflies	Phloem sap	P-endosymbiont S-endosymbiont	γ-*Proteobacteria* *Enterobacteriaceae*
Superfamily: Coccoidea Family: Pseudococcidae			
Mealybugs	Phloem sap	P-endosymbiont	β-*Proteobacteria*
Order: Diptera Family: Muscidae Genus: *Glossina*			
Tsetse flies	Vertebrate blood	*Wigglesworthia glossinidia* (P-endosymbiont) *Sodalis glossinidius* (S-endosymbiont)	γ-*Proteobacteria* *Enterobacteriaceae*
Order: Coleoptera Family: Curculionidae Genus: *Sttophilus*			
Weevils	Stored grain	P-endosymbiont	*Enterobacteriaceae*
Order: Hymenoptera Family: Formicidae Genus: *Camponotus*			
Carpenter ants	Plantnectar, honeydew, detritus and other sources	P-endosymbiont	γ-*Proteobacteria*
Order: Orthoptera Superfamily: Blattoidea			
Cockroaches	Universalists	*Blattabacterium cuenoti*	Flavobacterium-Bacteroides group
Order: Isoptera Family: Mastotermitidae Genus: *Mastotermes*			
Termites	Dead wood	*Blattabacterium cuenoti*	Flavobacterium-Bacteroides group

[a]See text for references.
[b]Taxonomy of the host according to Borror et al. (1989).
[c]As defined by Brenner (1984).

potential endosymbiont precursors of endosymbiotic associations (Boman and Hultmark, 1987; Faye, 1978).

These conclusions are tentative and, with the possible exception of the *Sitophilus*-endosymbiont association, are probably applicable to most of the endosymbiotic associations considered in this chapter. One well-studied, contrasting association is between many arthropods and the intracellular prokaryote, *Wolbachia* (O'Neill et al., 1997). Although this organism is typically heritable, being transmitted maternally, results of phylogenetic studies imply some incidence of horizontal exchange between very different lineages. *Wolbachia* causes a number of different reproductive alterations favoring the spread of infected host lineages and has properties characteristic of a parasite (O'Neill et al., 1997). Recent work may necessitate a modification of this conclusion because newly discovered *Wolbachia* in filarial worms appear to be essential for host survival and may show phylogenetic congruence with their hosts, indicating vertical evolution (Bandi et al., 1998, 1999).

Symbionts of Insects Which Utilize Plant Sap as Food

Aphids, psyllids, whiteflies and mealybugs share a number of common structural and nutritional

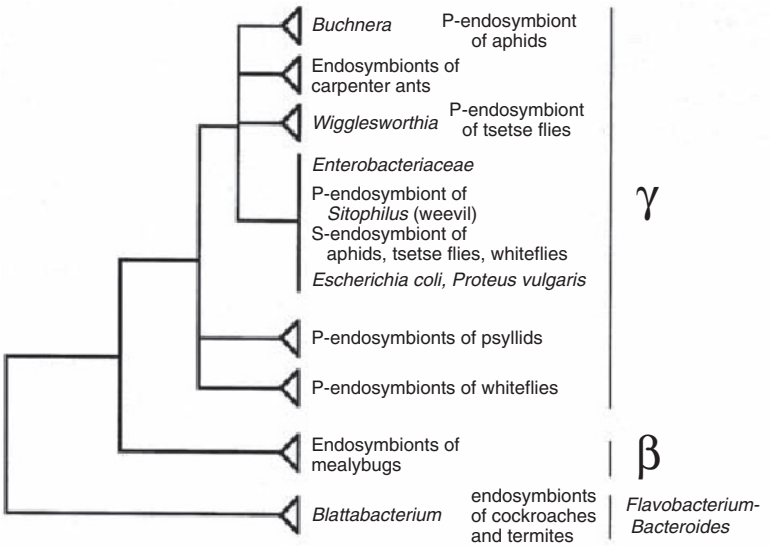

Fig. 1. Phylogenetic tree resulting from parsimony analysis of insect endosymbionts based on 16S rDNA sequence analysis. P-, primary endosymbiont; S, secondary endosymbiont; Greek letters, subdivisions of the Proteobacteria. References given in text.

properties (Borror et al., 1989) and constitute four separate lineages within the suborder Sternorrhyncha (order Homoptera; Campbell et al., 1994; von Dohlen and Moran, 1995). All of these groups feed predominantly or exclusively on plant phloem sap. This mode of life necessitates the penetration of plant tissue by flexible tubular mouthparts (stylets) and the ingestion of plant phloem sap. This diet is unbalanced, as it is rich in carbohydrates but deficient in amino acids and other nitrogenous compounds (Dadd, 1985; Sandström and Pettersson, 1994). Because of the low concentrations of nitrogenous compounds, phloem-feeding insects ingest a large amount of plant sap and then excrete the excess sugar as honeydew. This mode of feeding is conducive to the transmission of plant viruses, and members of the Homoptera are important vectors of viral plant pathogens (Blackman and Eastop, 1984; Gray and Banerjee, 1999; Sylvester, 1985). In addition, these insect populations may reach enormous numbers on plants, causing nutrient deprivation, leaf curling, and gall formation (Borror et al., 1989).

In spite of these common properties, aphids, whiteflies, psyllids, and mealybugs have different prokaryotic P-endosymbionts (Table 1, Fig. 1). These insects, like other animals, require ten essential amino acids, and endosymbionts are thought to upgrade the diet by synthesizing these essential amino acids and providing them to the host (Baumann et al., 1995, 1997a, b; Douglas, 1989; Moran and Telang, 1998). Of these four symbiotic associations, the most extensively studied is that between *Buchnera* (the P-endosymbiont) and aphids. This association will be considered in some detail and followed by a brief discussion of three other associations.

Aphid Endosymbionts

Buchnera—the Primary Endosymbiont of Aphids

Phylogeny

The initial characterization of the 16S-rDNA gene of *Buchnera* involved the use of an *Escherichia coli* 16S rDNA hybridization probe to perform a restriction enzyme and Southern blot analysis on total *Acyrthosiphon pisum* DNA, which established that this gene was present as a single copy (Unterman et al., 1989). Subsequently three overlapping DNA fragments were cloned and the 16S-rDNA gene was sequenced. In addition, bacteriomes were dissected from the aphid and the DNA purified. Restriction enzyme and Southern blot analysis gave the same results with whole aphid DNA and DNA obtained from dissected bacteriomes, indicating that the bacteriomes were the source of endosymbiont DNA. In all subsequent studies, the 16S rDNA was obtained by PCR amplification using whole aphid DNA preparations cloned into plasmid vectors and then sequenced (Munson et al., 1991b).

Based on 16S rDNA analysis, *Buchnera* is a distinct lineage within the γ-3 subgroup of the Proteobacteria (Fig. 1; Moran et al., 1993; Munson et al., 1991b; Unterman et al., 1989; van Ham et al., 1997). The closest known organisms are the endosymbionts of carpenter ants, endosymbionts of tsetse flies (*Wigglesworthia*), and members of the Enterobacteriaceae as defined by Brenner (Brenner, 1984; Aksoy, 1995a, b; Schröder et al., 1996). Phylogenetic analyses based on 16S rDNA indicate that these organisms are four separate lineages but do not permit

firm conclusions regarding their relationships to one another. *Buchnera* contains a single copy of rRNA genes which are arranged as two transcription units, 16S rRNA and tRNAGlu-23S rRNA-5S rRNA (Munson et al., 1993; Rouhbakhsh and Baumann, 1995). This organization of the rRNA genes into two transcription units is somewhat rare but also has been found in *Wolbachia* (Bensaadi-Merchermerk et al., 1995) and *Rickettsia* (Andersson et al., 1998), organisms which are in the α-subdivision of the Proteobacteria and also associated with insects. In the endosymbionts of carpenter ants, the rRNA genes are also split into two transcription units (C. Sauer and R. Gross, personal communication) whereas in *Wigglesworthia* (Aksoy, 1995b) and the Enterobacteriaceae the order is 16S–23S (Berlyn, 1998). The organization of the rRNA genes into two transcription units suggests a possible relationship between *Buchnera* and the endosymbionts of carpenter ants.

The results of phylogenetic analyses involving all of the currently available *Buchnera* sequence information are presented in Fig. 2. Most of the characterized endosymbionts are from the family Aphididae. Based on 16S rDNA, *Buchnera* forms one clade within which two well-supported subclades are apparent. These are the aphids of the Aphididae and the Sc and Mr from the tribe Fordini in the family Pemphigidae. Additional studies using a portion of *trpB* (Fig. 2b) confirmed some of these relationships and provided further resolution within the genus *Uroleucon*. These relationships are in broad agreement with the results of evolutionary studies of plasmid-associated *trpE*, *leuBCD*, and *repA1* (Fig. 2c, d, e; Baumann et al., 1997b, 1999b; Bracho et al., 1995; Rouhbakhsh et al., 1996, 1997; Silva et al., 1998; van Ham et al., 1997, 1999). Within the genus *Uroleucon*, the phylogeny based on *trpB* is in good agreement with the more extensive analysis of host phylogeny based on mitochondrial and nuclear genes (Clark et al., 2000; Moran et al., 1999).

The congruence of phylogenies derived from *Buchnera* chromosomal and plasmid genes, as well as host mitochondrial and nuclear genes, is strong evidence for a vertical mode of evolution with no exchange of either bacteria or plasmids among host lineages (Moran and Baumann,

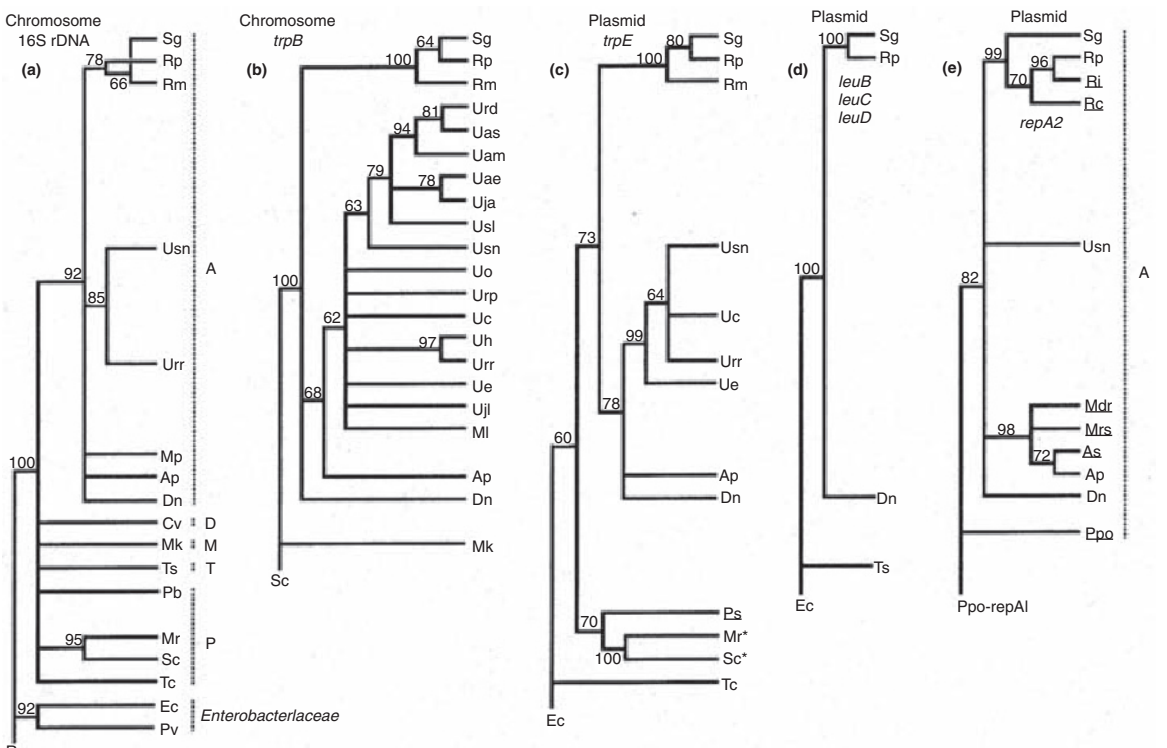

Fig. 2. Phylogenetic trees resulting from parsimony analyses using Buchnera (a) 16S rDNA, (b) trpB, (c)trpE, (d) leuB, leuC, and leuD, and (e) repA1. Numbers at nodes are bootstrap percentages from parsimony searches (1000 replicates). Abbreviations designating the insect hosts and are given in Table 2. In (a), Enterobacteriaceae: Ec(E. coli), Pv(P. vulgaris); Ra(Ruminobacter amylophilus). Dashed lines in (a) designate aphid species within one family: A, Aphididae; D, Drepanosiphidae; M, Mindaridae; T, Thelaxidae; P, Pemphigidae. Dashed line in (e) designates aphids within one family. Underlined abbreviations in (c) and (e) refer to aphid species not included in the other analyses. * in (c) designates chromosomal genes. For references see text.

Table 2. Abbreviations of aphid species used in this chapter.

Abbreviation	Aphid species	Family	Tnbe
Rc	*Rhopalosiphum cerasifoliae*	Aphididae	Aphidini
Ri	*Rhopalosiphum insertum*	Aphididae	Aphidini
Rm	*Rhopalosiphum maidis*	Aphididae	Aphidini
Rp	*Rhopalosiphum padi*	Aphididae	Aphidini
Sg	*Schizaphis graminum*	Aphididae	Aphidini
Ap	*Acyrthosiphon pisum*	Aphididae	Macrosiphini
As	*Aulacorthum solani*	Aphididae	Macrosiphini
Dn	*Diuraphis noxia*	Aphididae	Macrosiphini
Ml	*Macrosiphoniella ludovicianae*	Aphididae	Macrosiphini
Mp	*Myzus persicae*	Aphididae	Macrosiphini
Mdr	*Metopolophium dirhodum*	Aphididae	Macrosiphini
Mrs	*Macrosiphum rosae*	Aphididae	Macrosiphini
Uae	*Uroleucon aeneum*	Aphididae	Macrosiphini
Uam	*Uroleucon ambrosiae*	Aphididae	Macrosiphini
Uas	*Uroleucon astronomus*	Aphididae	Macrosiphini
Uc	*Uroleucon caligatum*	Aphididae	Macrosiphini
Ue	*Uroleucon erigeronense*	Aphididae	Macrosiphini
Uh	*Uroleucon helianthicola*	Aphididae	Macrosiphini
Uja	*Uroleucon jaceae*	Aphididae	Macrosiphini
Ujl	*Uroleucon jaceicola*	Aphididae	Macrosiphini
Uo	*Uroleucon abscurum*	Aphididae	Macrosiphini
Urd	*Uroleucon rudbeckiae*	Aphididae	Macrosiphini
Urp	*Uroleucon rapunculoidis*	Aphididae	Macrosiphini
Urr	*Uroleucon rurale*	Aphididae	Macrosiphini
Usl	*Uroleucon solidaginis*	Aphididae	Macrosiphini
Usn	*Uroleucon sonchi*	Aphididae	Macrosiphini
Ppo	*Pterocomma populeum*	Aphididae	Pterocommatinae
Cv	*Chaitophorus vininalis*	Drepanosiphidae	Chaitophorini
Mk	*Mindarus kinseyi*	Mindaridae	Mindarini
Mr	*Melaphis rhois*	Pemphigidae	Fordini
Sc	*Schlechtendalia chinensis*	Pemphigidae	Fordini
Pb	*Pemphigus betae*	Pemphigidae	Pemphigini
Ps	*Pemphigus spyrothecae*	Pemphigidae	Pemphigini
Tc	*Tetraneura caerulescens*	Pemphigidae	Eriosomatini
Ts	*Thelaxes suberi*	Thelaxidae	

1994). An implication of the congruence between the phylogenies of *Buchnera* and corresponding aphid hosts is that dates for branch points inferred from fossil aphids can be extended to ancestral *Buchnera* (Moran et al., 1993). A further implication is that modern *Buchnera* descend from an infection of a common ancestor of all modern aphids. From the aphid fossil record, we can infer that this infection by a free-living bacterium must have occurred at least 150–250 million years ago. The divergence in 16S rDNA of modern *Buchnera* is consistent with this hypothesis of an ancient infection followed by cospeciation of *Buchnera* and hosts.

Buchnera shows a rate of base substitution in its 16S rDNA that is about twice as great as that in related free-living bacteria based both on relative rate comparisons with free-living taxa and on comparisons of rates calibrated with respect to absolute time (Clark et al., 1999b; Moran, 1996). The elevated substitution rate of *Buchnera* relative to that of related free-living bacteria extends to genes encoding proteins (Brynnel et al., 1998; Clark et al., 1999b; Moran, 1996; Wernegreen and Moran, 1999). Based on calibrated rates for protein-coding genes, synonymous sites evolve about twice as fast and nonsynonymous sites about six times as fast in *Buchnera* as in *E. coli*/*Salmonella typhimurium*, based on an absolute time scale (Table 3). The rate differences are considerably greater on a scale based on generations, since *Buchnera* appears to have fewer generations per year than do natural populations of enteric bacteria (Clark et al., 1999b).

The most plausible explanation for the faster rate of substitution in *Buchnera* is that the population structure of *Buchnera*, involving strictly vertical transmission of a small inoculum between hosts, results in greater levels of genetic drift, which can increase the fixation rate of mildly deleterious mutations. Several observations support this explanation. First, the rate increase is found at all genes and is concentrated at sites, such as nonsynonymous sites, that are

Table 3. Substitution rates in *Buchnera* and enteric bacteria.[a]

Species pair	Estimated time of divergence	Synonymous rate		Nonsynonymous rate	
		Absolute[b]	Generation[c]	Absolute[b]	Generation[c]
Buchnera (Sg/Dn)	50–70MY	6.8–9.5	0.14–0.19	1.0–1.4	0.02–0.03
Buchnera (Sc/Mr)	50–70MY	5.1–7.2	0.17–0.24	1.1–1.6	0.04–0.05
E. coli/S. typhimurium	100–150MY	2.9–4.4	0.03–0.04	0.1–0.2	0.001–0.002

[a]Based on comparisons of over 5100 codons (Clark et al., 1999b).
[b]Substitutions/site/10° years.
[c]Substitutions/site/10° generations.

expected to be under selective constraint (Moran, 1996; Wernegreen and Moran, 1999). Second, polypeptide compositions are consistently biased towards amino acids that allow more adenine and thymine in the DNA sequence, indicating that mutational bias has affected protein evolution. Third, faster substitution rates in 16S rDNA are observed in other insect endosymbionts that share a similar transmission mode, suggesting that the endosymbiotic lifestyle has repeatedly produced the same changes in patterns of molecular evolution. Finally, the 16S rRNA secondary structure of *Buchnera* and other endosymbionts has lower thermal stability than that of related free-living bacteria, as expected if the DNA base substitutions are mildly deleterious (Lambert and Moran, 1998).

The 16S rDNA substitution rate of *Buchnera* is about 35 times greater than that of homologous regions of 18S rDNA of hosts, based on comparisons of pairwise divergences of corresponding aphid and *Buchnera* taxa (Moran et al., 1995). Thus, the hypothesis of a universal rate of substitution in rDNA is not even approximately true.

Taxonomy

The genus *Buchnera* contains one species, *Buchnera aphidicola*, and the type strain is the endosymbiont of the aphid *Schizaphis graminum* (Munson et al., 1991a). Currently this species name designates the lineage consisting of the P-endosymbionts of aphids. There are over 4,000 species of aphids (Blackman and Eastop, 1984; Remaudière and Remaudière, 1997) of which only 35 have been characterized by molecular methods. Consequently our conclusions are based on a very small sample of aphid species. Although 16S rDNA has been useful for showing the monophyletic origin of aphid endosymbiosis and the establishment of major aphid subgroups, it is far too conserved to be useful for defining relationships among endosymbionts of closely related aphids. Some success has been obtained by the use of other, less conserved, molecules (Fig. 2). The 16S rDNA sequence difference of *Buchnera* in Sg and Sc (the most distantly related aphids) is about the same as that between *E. coli* and *Proteus vulgaris*. Thus, subsequent studies using less conserved molecules will probably indicate that *Buchnera* should be subdivided into new species. So far, no studies have addressed the range of variation within endosymbionts of a single aphid species.

Habitat

Location and Ultrastructure During their reproductive phase, aphids contain within their body cavity a bilobed structure called a bacteriome consisting of 60–90 uninucleate, polyploid cells called bacteriocytes (Douglas and Dixon, 1987). These cells are filled with host-derived vesicles containing *Buchnera* (Fig. 3a). This organism is spherical or oval in shape, 2–4 m in diameter, with a cell wall consisting of two-unit membranes, as is characteristic of Gram-negative bacteria (Fig. 3b; Akhtar and van Emden, 1994; Griffiths and Beck, 1973; Hinde, 1971b; McLean and Houk, 1973). A thin layer corresponding to peptidoglycan has been detected (Houk et al., 1977). The presence of peptidoglycan also is indicated by penicillin-induced alterations of the cell wall as well as by chemical analysis (Griffiths and Beck, 1974; Houk et al., 1977).

Number of Endosymbionts *Buchnera* contains one copy of the 16S rRNA gene (Munson et al., 1991b, 1993; Unterman et al., 1989) and one copy of *groEL* (Ohtaka et al., 1992; Hassan et al., 1996). The number of copies of *Buchnera* 16S rRNA genes in the aphid Sg was studied by quantitative PCR (Baumann and Baumann, 1994) and was found to be $0.5–1.2 \times 10^7$ mg^{-1} aphid wet weight. Using quantitative hybridization of a *Buchnera groEL* probe, the number of genome copies in Ap was estimated at $1–2 \times 10^7$ mg^{-1} aphid wet weight (Humphreys and Douglas, 1997). In both of these studies the number of *Buchnera* cells was assumed to be identical to the number of genome copies. However a recent study has demonstrated that *Buchnera* (Ap) is polyploid, containing an average of about 120 genomes per cell (Komaki and Ishikawa, 1999). If the average number of genomes per

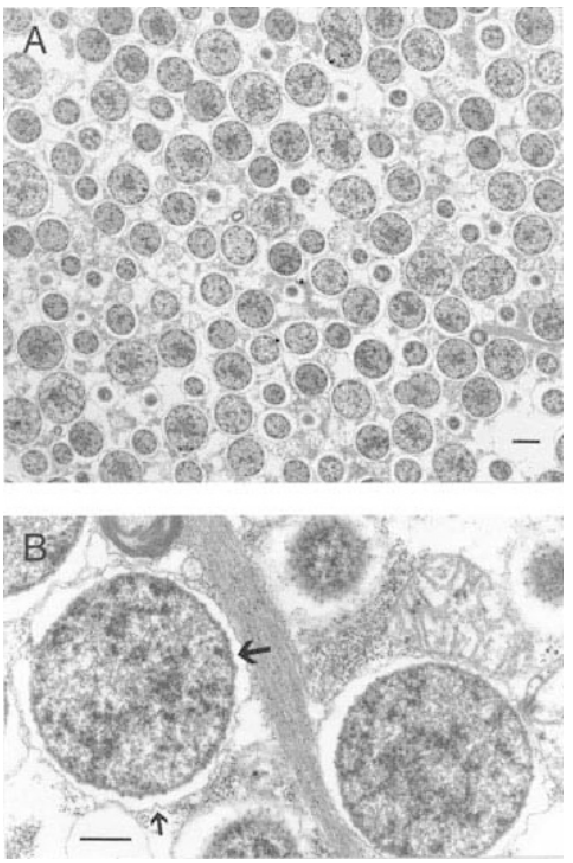

Fig. 3. Electron micrographs of *Buchnera*, the P-endosymbiont of aphids. (a) Endosymbionts within a bacteriocyte, bar = 1 m. (b) Higher magnification showing the Gram-negative cell-wall (large arrow) and the vesicle membrane (small arrow), bar = 0.5 m. Photos courtesy of Marv Kinsey and Don McLean.

endosymbiont is relatively constant, then the number of endosymbionts is about 100-fold less than estimated previously, or about 105 mg^{-1} aphid wet weight. This value is considerably lower than the estimates for Ap of $1.6–1.8 \times 10^6$ endosymbionts mg^{-1} aphid wet weight, based on microscopic enumeration of the endosymbionts (Harrison et al., 1989).

Growth and Reproduction In their most active stage, aphids are wingless females, which reproduce by parthenogenesis, giving birth to live young. There is telescoping of generations in that the mother aphid contains embryos that, in turn, may contain other embryos (Dixon, 1973, 1992). Studies on the growth of Sg (Baumann and Baumann, 1994) have indicated that newly born aphids weigh 24 μg and contain 2×10^5 copies of the *Buchnera* genome. The increase in the number of endosymbiont genomes parallels the increase in the weight of the aphid. The maximum weight is reached in 9–10 days at which time the aphid weighs 540 μg and contains 5.6×10^6 *Buchnera* genomes. The endosymbionts are partitioned between maternal and embryonic bacteriocytes. In a mature aphid, most of the *Buchnera* genomes are found in the embryos (Humphreys and Douglas, 1997). The first young are born in 8–9 days; each aphid can produce 60 to 80 live young during its lifetime. Douglas and Dixon (1987) showed that, during the period of growth, there is a concomitant increase in the maternal bacteriocyte volume as well as a small drop in bacteriocyte number. In the adult aphid the number of maternal bacteriocytes in the bacteriome undergoes a sharp decrease probably due, in part, to their dispersion within the abdomen, their degradation, as well as the degradation of *Buchnera* (Brough and Dixon, 1990; Douglas and Dixon, 1987; Griffiths and Beck, 1973; Hinde, 1971a).

Aphids may also produce sexual forms with the females depositing eggs that overwinter and hatch in the spring. *Buchnera* is maternally transmitted (transovarial transmission) to both developing embryos and eggs. Maternal bacteriocytes adjacent to an embryo near the blastoderm stage form a small opening through which the endosymbionts pass. *Buchnera* then moves through the intervening hemolymph and enters a nearby opening on the oocyte surface. During early embryonic development, the presumptive bacteriocytes form, and the endosymbionts migrate into these cells (Buchner, 1965; Blackman, 1987; Hinde, 1971a). Symbiont invasion of eggs also occurs from the dispersed bacteriocytes and they can be observed as an aggregate at the posterior pole of the mature egg (Buchner, 1965; Brough and Dixon, 1990). *Buchnera* and bacteriomes appear to be nearly universal in aphids (Buchner, 1965). However, some species of the tribe Cerataphidini lack both and instead contain yeast-like extracellular symbionts within their body cavity (Buchner, 1965; Fukatsu and Ishikawa 1992a, 1996). Some species of aphids may produce dwarf males and/or sterile female soldiers that may also lack endosymbionts (Buchner, 1965; Fukatsu and Ishikawa, 1992b; Fukatsu et al., 1994).

Physiology

Nutrition and Metabolism Plant sap, the diet of aphids, has an excess of carbohydrate relative to amino acids and other nitrogenous compounds (Dadd, 1985; Douglas, 1998; Sandström and Pettersson, 1994; Sandström and Moran, 1999). Aphids, like other insects, are thought to require 10 preformed amino acids and these essential amino acids are present in low amounts in plant sap. Some species of aphids can grow on synthetic diets even in the absence of essential amino acids. Adding antibiotics to such diets

results in the elimination of endosymbionts and the failure of the aphids to reproduce. There is some sparing effect when the essential amino acids are included in the antibiotic-containing diet. These experiments have generally been interpreted as indicating that one of the functions of *Buchnera* is the synthesis of essential amino acids for the aphid host (reviewed by Baumann et al., 1995; Douglas, 1998). A major problem is that compared with growth on plants, growth on artificial diets is poor and generally limited to a few generations. In addition, aphid growth on complete synthetic diets in the presence of antibiotics is even worse, indicating that *Buchnera* provides nutrients or functions that cannot be provided by the artificial diets. The effects of antibiotics on a number of aphid properties have been recently reviewed (Wilkinson, 1998). There is a further complication with some of the nutritional studies, in which physiological effects have been attributed to the removal of *Buchnera*. The aphid strain used may also contain S-endosymbionts, as is true of the strain of the aphid Ap used in the studies of A. E. Douglas and her collaborators (Douglas and Prosser, 1992; Wilkinson 1998). The S-endosymbiont probably does not perform any essential functions for the host (see section on S-endosymbionts of aphids in this Chapter); nevertheless, the use of antibiotics eliminates both endosymbionts, and consequently the observed effects of this loss may not be attributable solely to the loss of *Buchnera*.

Currently one of the more complete studies involves the essential amino acid tryptophan. Using a strain of Ap containing an S-endosymbiont, Douglas and Prosser (1992) have shown a sparing effect of tryptophan in chlortetracycline-containing synthetic diets on aphid growth. In addition they detected low levels of tryptophan synthase in *Buchnera* and found that activity was absent in chlortetracycline-treated and thus endosymbiont-free aphids. The assays used (Smith and Yanofsky, 1962) crude extracts of whole aphids as well as preparations enriched in endosymbionts and measured the disappearance of the substrate indole and not the appearance of the product tryptophan. Indole or indole derivatives may be substrates for a variety of reactions catalyzed by enzymes found in crude extracts of insects. No information was provided about the linearity of increasing enzyme activity with increasing crude extract concentration. In spite of possible difficulties with this assay, the dependence of the reaction on "the substrate [sic] pyridoxal phosphate" (Douglas and Prosser, 1992) is consistent with it being a measure of tryptophan synthase activity.

Using synthetic diets containing radiolabelled sulfate, it was shown that *Buchnera* can reduce this compound to the level of hydrogen sulfide and incorporate it into methionine and cysteine and that these amino acids are found in aphid tissue (Douglas, 1988). Using ^{14}C-radiolabelled amino acids, it was found that the synthesis of the essential amino acids arginine, threonine, isoleucine and lysine was reduced or eliminated by the inclusion of rifampicin in the diet (Liadouze et al., 1996). Sasaki and Ishikawa (1995) also showed that treatment of aphids with rifampicin eliminated the incorporation of dietary ^{15}N-glutamine into the essential amino acids arginine, histidine, isoleucine and/or leucine, phenylalanine, threonine, and valine.

Glutamine is the predominant amino acid in phloem and also in aphid hemolymph (Sandström and Pettersson, 1994; Sasaki et al., 1990). Isolated bacteriocytes were found to take up glutamine and convert it to glutamate, which subsequently was taken up by *Buchnera* (Sasaki and Ishikawa, 1995). Isolated endosymbionts incorporated the nitrogen of glutamine into the essential amino acids isoleucine, leucine, valine, and phenylalanine as well as a number of other amino acids, and these amino acids were excreted into the suspending medium. Whitehead and Douglas, however, could not find any evidence for excretion of amino acids by *Buchnera* (cited in Douglas, 1997).

Using synthetic diets, Nakabachi and Ishikawa (1999) demonstrated a requirement for riboflavin by rifampicin-treated aphids. These results indicate that *Buchnera* is required for the synthesis of at least one vitamin for the aphid host.

Whitehead and Douglas (1993) isolated vesicles containing *Buchnera* and showed that they readily took up acetic, glutamic and aspartic acid as well as tricarboxylic acid cycle intermediates and oxidized them to CO_2. Oxygen consumption was also detected and was greatly reduced by KCN. These results suggest the presence of a tricarboxylic acid cycle in the endosymbionts and indicate a respiratory metabolism. The latter conclusion is consistent with the presence of a gene for a subunit of NADH dehydrogenase I, an enzyme involved in the generation of a proton motive force during respiration, and of all the genes for ATP synthase, a membrane-associated enzyme which utilizes the proton motive force for the synthesis of ATP (Table 4).

Gene Expression *Buchnera* messenger RNA (mRNA) has been detected for a variety of genes encoding proteins involved in amino acid biosynthesis (Table 4). This includes genes for amino acids of the glutamate (*argA*) and aspartate (*thrB*) families (Nakabachi and Ishikawa, 1997), the shikimate pathway (*aroH*), as well as the biosynthetic pathway for tryptophan (*trpE*, *trpD*,

Table 4. Genes of *Buchnera* from the aphid *S. graminum*.[a]

Gene symbol	Gene product description	Linkage group[b]
I. SMALL-MOLECULE METABOLISM		
B. Energy metabolism		
1. Glycolysis		
gap A	Glyceraldehyde-3-phosphate dehydrogenase	13
tpiA	Triosephosphate isomerase	3
5. Pentose phosphate pathway		
a. Oxidative branch		
gnd	Gluconate-6-phosphate dehydrogenase	2
7. Respiration		
a. Aerobic		
nuoC(D)[c]	NADH dehydrogenase I, subunits cd	
c. Electron transport		
fdx	Ferredoxin	1
fpr	Ferredoxin-NADP reductase	12
9. ATP proton motive force		
atp A	ATP synthase, α-subunit	1
atp B	ATP synthase, subunit a	1
atp C	ATP synthase, ε-subunit	1
atp D	ATP synthase, β-subunit	1
atp E	ATP synthase, subunit c	1
atp F	ATP synthase, subunit b	1
atp G	ATP synthase, γ-subunit	1
atp H	ATP synthase, -subunit	1
D. Amino acid biosynthesis		
1. Glutamate family		
argA[d]	N-acetylglutamate synthase	
argH	Argininosuccinate lyase	7
2. Aspartate family		
dapD	Succinyl-diaminopimelate aminotransferase	4
thrA	Aspartokinase I	
thrB[d]	Homoserine kinase	
3. Serine family		
cysE	Serine acetyltransferase	7
serC	Phosphoserine amino transferase	3
4. Aromatic amino acid family		
aroA	5-Enolpyruvylshikimate-3-phosphate synthase	3
aroC	Chorismate synthase	2
aroE	Dehydroshikimate reductase	8
aroH	3-deoxy-*D-arabino*-heptulosonate-7-phosphate synthetase (DAHP synthetase)	10
trpA	Tryptophan synthase, A protein	5
trpB	Tryptophan synthase, B protein	5
trpC(F)	Indole-3-glycerolphosphate synthetase and *N*-(5-phosphoribosyl) anthranilate isomerase	5
trpD	Phosphoribosylanthranilate transferase	5
trpE (p)[e]	Anthranilate synthase, A subunit	16
trpG (p)[e]	Anthranilate synthase, B subunit (glutamine amidotransferase)	16
5. Histidine		
hisA	*N*-(5′-phospho-L-ribosyl-formimino)-5-amino-1-(5′-phosphoribosyl)-4-imidazolecarboxamide isomerase	2
hisB	Imidazoleglycerolphosphate dehydratase and histidinol phosphate phosphatase	2
hisC	Histidinol-phosphate aminotransferase	2
hisD	Histidinol dehydrogenase	2
hisF	Imidazoleglycerol phosphate synthase subunit (with HisH)	2
hisG	ATP phosphoribosyl transferase	2
hisH	Glutamine amidotransferase subunit (with HisF)	2
hisI	Phosphoribosyl-AMP cyclohydrolase and phosphoribosyl-ATP pyrophosphatase	2
7. Branched-chain family		
ilvC	Acetohydroxy acid isomeroreductase	1
ilvD	Dihydroxyacid dehydratase	1
ilvH	Acetohydroxyacid synthase, small subunit	4
ilvI	Acetohydroxyacid synthase, large subunit	4
leuA (p)[e]	2-Isopropylmalate synthase	15
leuB (p)[e]	3-Isopropylmalate dehydrogenase	15
leuC (p)[e]	Isopropylmalate isomerase subunit	15
leuD (p)[e]	Isopropylmalate isomerase subunit	15

(Continued)

Table 4. *Continued*

Gene symbol	Gene product description	Linkage group[b]
F. Purines, pyrimidines, nucleosides, and nucleotides		
3.2′-Deoxyribonucleotide metabolism		
dcd	2′-Deoxycytidine 5′-triosephosphate deaminase	2
trxB	Thioredoxin reductase	3
G. Biosynthesis of cofactors, prosthetic groups, and carriers		
9. Riboflavin		
ribE[d]	Riboflavin synthase, β-chain	
10. Thioredoxin, glutaredoxin, and glutathione		
trxA	Thioredoxin	1
II. BROAD REGULATORY FUNCTIONS		
rpoD	RNA polymerase, σ^{70} subunit	7
rpoH[d]	RNA polymerase, σ^{32} subunit, regulation of proteins induced at high temperature	
III. MACROMOLECULE METABOLISM		
A. Synthesis and modification of macromolecules		
1. rRNA and "stable" RNAs		
rrf	5S rRNA	8
rrl	23S rRNA	8
rrs	16S rRNA	11
2. Ribosomal protein synthesis and modification		
rplL	50S ribosomal protein L7/L 12	9
rplT	50S ribosomal protein L20	10
rpmH	50S ribosomal protein L34	1
rpmI	50S ribosomal protein L35	10
rpsA	30S ribosomal protein S1	3
rpsD	30S ribosomal protein S4	14
rpsK	30S ribosomal protein S11	14
4. tRNAs		
tRNA^{Glu}	Glutamate-tRNA	8
tRNA^{Phe}	Phenylalanine-tRNA	1
tRNA^{Trp}	Tryptophan-tRNA	1
5. Aminoacyl tRNA synthetases and their modification		
argS	Arginine tRNA synthetase	11
aspS	Aspartic tRNA synthetase	3
cysS	Cysteine tRNA synthetase	8
metG	Methionine tRNA synthetase	2
serS	Serine tRNA synthetase	3
thrS	Threonine tRNA synthetase	10
trmE	tRNA methyltransferase	1
7. DNA replication, restriction/modification, and recombination		
dnaA	DNA biosynthesis, initiation of chromosome replication, global transcription regulator	1
dnaG	DNA biosynthesis, DNA primase	7
dnaN	DNA polymerase III holoenzyme, β-subunit	1
dnaQ	DNA polymerase III holoenzyme, ε-subunit	11
gidA	Chromosome replication?	1
gyrB	DNA gyrase subunit B	1
himD	Integration host factor, β-subunit	3
rep	Rep helicase, ssDNA-dependent ATPase	1
8. Protein translation and modification		
efp[f]	Elongation factor EF-P	
infC	Initiation factor IF-3	10
tuf[g]	Elongation factor EF-Tu	
9. RNA synthesis, RNA modification, and DNA transcription		
rho	Transcription termination factor Rho	1
rpoA	RNA polymerase, α-subunit	14
rpoB	RNA polymerase, β-subunit	9
rpoC	RNA polymerase, β′-subunit	9
11. Phospholipids		
clh[h]	Cardiolipin synthase	
B. Degradation of macromolecules		
1. RNA		
rnh	RNase H	11
rnpA	RNase P	1
3. Proteins, peptides, and glycopeptides		
hslU	Heat shock protein, protease?	12
htrA	Periplasmic serine protease and heat shock protein	4
soh[i]	Periplasmic protease	

Table 4. *Continued*

Gene symbol	Gene product description	Linkage group[b]
C. Cell envelope		
2. Surface polysaccharides, lipopolysaccharides, and antigens		
kdtB	Putative enzyme of lipopolysaccharide synthesis	12
4. Murein sacculus and peptidoglycan		
ddlB	D-alanine-D-alanine ligase	6
murC	L-alanine-adding enzyme, UDP-*N*-acetyl-muramate-alanine ligase	6
murE	*meso*-Diaminopimelate-adding enzyme	4
IV. CELL PROCESSES		
B. Chaperones		
dnaJ[d]	Heat shock protein	
dnaK[d]	Heat shock protein (Hsp 70), DNA biosynthesis	
groEL	Heat shock protein (Hsp 60)	1
groES	Heat shock protein (Hsp 10)	1
hscA	Cold shock protein (Hsp 70)	1
hscB	Cold shock protein	1
C. Cell division		
ftsA	Cell division protein, complexes with FtsZ	6
ftsI	Septum formation; penicillin binding protein 3; peptidoglycan synthase	4
ftsL	Cell division protein; ingrowth of wall at septum	4
ftsZ	Cell division, forms circumferential ring	6
E. Protein and peptide secretion		
secB	Protein export, molecular chaperone	7
V. OTHER		
F. Adaptations and atypical conditions		
ibp	Heat shock protein, HSP20 family	12
ADDITIONAL ORFs		
bcp	*E. coli homolog* Bacterioferritin comigratory protein 1788825	2
nifS	1788879	1
ybbN	1790040	7
yjEA	Hypothetical lysine tRNA synthase homolog 1790599	12
10kDA	YIDD_ECOLI	1
39kDa	1790589	1
60kDa	1790140	1
ORF113	1786351	6
ORF128	1788878	1
ORF177	1788671	2
ORF194	1788860	1
ORF217	1787362	13
ORF235	1786354	6
ORF312	1786270	4
ORF340	1788543	4
ORF453	1788858	1
ORFI	1786406	11
ORFV	1787508	5
ORFVI	1787507	5
P14	1787506	5
ORFA	1788269 Transmembrane protein?	5
ORFB	1787524	5
ORFC	1787361 ABC transporter protein? ATP-binding site?	13
ORFD	1789158	13
pLeu		
repA1 (p)[e]	Related to RepA protein of IncFII plasmids	15
repA2 (p)[e]	Related to RepA protein of IncFII plasmids	15
ORF1 (p)[e]	Related to *E. coli* 1789376	15

[a]Gene list arranged according to the classification of gene products of Riley and Labedan (1996). Genes from *Buchnera* from other species of aphids are indicated.
[b]See Table 5.
[c]From *Buchnera* (Usn).
[d]From *Buchnera* (Ap) (Nakabachi and Ishikawa, 1997, 1999; Sato and Ishikawa, 1997a, b).
[e]p, plasmid associated gene.
[f]From *Buchnera* (Mp) (Hogenhout et al., 1998).
[g]From Brynnel et al. (1998).
[h]From *Buchnera* (Dn, Usn).
[i]From *Buchnera* (Sc) (Lai et al., 1995).

trpA), branched-chain amino acids (*ilvI*, *ilvD*, *leuA*), and histidine (*hisG*; Baumann et al., 1999a). *Buchnera* mRNA has been detected also for a gene involved in the biosynthesis of riboflavin (*ribE*; Nakabachi and Ishikawa, 1999) as well as for genes involved in the heat shock response (*groEL*, *groES*, *dnaK*, *dnaJ*; Table 4; Sato and Ishikawa, 1997a, b). Numerous *Buchnera* proteins have also been detected by immunological methods. These include *GroEL*, *GroES*, and *DnaK* (Kakeda and Ishikawa, 1991; Sato and Ishikawa, 1997b) as well as ribosomal protein S1 (the product of *rpsA*; Clark et al., 1996) and the protein involved in septum formation during cell division (the product of *ftsZ*; Table 4; Baumann and Baumann, 1998).

In bacteria, rRNA genes are transcribed from strong promoters. Comparisons of the regions upstream of rRNA genes from six species of *Buchnera* indicated conservation of sequences resembling the –35 and –10 regions of σ^{70} promoters as well as boxA and boxC (Munson et al., 1993; Rouhbakhsh, 1995). Similar putative –35 and –10 regions were found in *Buchnera* plasmids containing genes for tryptophan and leucine biosynthesis (Baumann et al., 1999b; Rouhbakhsh et al., 1996; Silva et al., 1998).

GroEL Overproduction and Its Significance In *Buchnera* the chaperonin, GroEL, constitutes a major fraction of the total protein (Sato and Ishikawa, 1997a). In addition, GroEL is present in aphid hemolymph (van den Heuvel et al., 1994). Overproduction of GroEL is a characteristic of some endosymbionts and pathogens in the intracellular environment (Hogenhout et al., 1998). This protein mediates the folding of peptides into their functional forms as well as the repair of damaged proteins (Gross, 1996). *Buchnera* GroEL is able to complement *E. coli* mutants (Ohtaka et al., 1992). GroEL has been localized in maternal and embryonic *Buchnera* by immunohistochemistry (Fukatsu and Ishikawa, 1992c). Electron micrographs indicate that the purified *Buchnera* GroEL has the characteristic double-ring appearance observed with the *E. coli* protein (Filichkin et al., 1997; Hara and Ishikawa, 1990). The endosymbiont protein has ATPase activity and in the presence of *E. coli*, GroES could reconstitute denatured *Rhodospirillum rubrum* ribulose-1,5-biphosphate carboxylase (Kakeda and Ishikawa, 1991).

The *Buchnera groESL* operon organization resembles that of *E. coli* (Hassan et al., 1996; Hogenhout et al., 1998; Ohtaka et al., 1992). Upstream of *groES* are nucleotide sequences characteristic of the –35 and –10 regions of σ^{32} promoters. A message of 2.1 kb (containing both *groES* and *groEL*) is made by the endosymbiont using only this promoter (Sato and Ishikawa, 1997a). It is not understood why GroES is low in the endosymbiont, in contrast to the high quantities of GroEL (Kakeda and Ishikawa, 1991). The genes for σ^{32} (*rpoH*) as well as *dnaKJ* have been cloned and sequenced (Sato and Ishikawa, 1997a, b). The latter also are transcribed solely from a σ^{32} promoter. In *E. coli* as well as other organisms, transcription of the *groESL* operon and the *dnaKJ* operon is part of the σ^{32} regulon, and their synthesis is increased by heat shock (Gross, 1996). It would appear that this mode of regulation is modified in *Buchnera* (Sato and Ishikawa, 1997a, b). Synthesis of *groESL* and *dnaKJ* mRNA is constitutive and is not increased by heat shock. This conclusion is supported by the observation that there is no increase in the level of total GroEL in aphids shifted from 23 to 33°C for one day (Baumann et al., 1996).

Baumann et al. (1996) arrived at an estimate of the amount of GroEL per *Buchnera* cell, based on the premise that the endosymbiont contained only one genome copy. Recently it has been shown that *Buchnera* is polyploid, containing an average of 120 genome copies per cell (Komaki and Ishikawa, 1999). If this result is incorporated into the calculation, an impossible excess of GroEL would be present in each endosymbiont cell. Since protein extracts of whole aphids were used in the estimation of GroEL content, this result could be explained by the finding that GroEL is also present in the hemolymph (van den Heuvel et al., 1994).

Perhaps the major economic effect of aphids on agriculture is their ability to transmit plant viruses (Blackman and Eastop, 1984; Gray and Banerjee, 1999; Sylvester, 1985). *Buchnera*-derived GroEL has been implicated in the survival of luteoviruses in the hemolymph (Filichkin et al., 1997; van den Heuvel et al., 1994; Hogenhout et al., 1998). These viruses replicate in the plant and are ingested by aphids when they feed on phloem sap. Subsequently they are transported from the digestive tract into the hemolymph and from there, into the salivary gland for transmission to plants via salivary secretions. The viruses are retained in an infective form (without replication) in the hemolymph throughout the life span of the aphid. There is evidence that the GroEL that is found in the hemolymph coats the virus particles and protects them from host defenses. A region in *Buchnera* GroEL has been identified which is essential for binding to the virus (Hogenhout et al., 1998), and similarly a portion of a viral capsid protein has been identified as the region to which the endosymbiont GroEL binds (van den Heuvel et al., 1997). Transmission of plant viruses may be advantageous to the aphid because infected plants have higher levels of nutrients in their sap (Blua et al., 1994).

An additional reason for the constitutive synthesis of high amounts of GroEL by *Buchnera* may be to compensate for the accumulated amino acid substitutions which have occurred at a high rate in this endosymbiont (Moran, 1996; Table 3). These slightly deleterious changes may result in proteins of decreased stability, and the high levels of GroEL may compensate for these changes, allowing proper folding and retention of function. A similar role for chaperones in masking deleterious mutations has been recently suggested on the basis of work on *Drosophila* heat shock protein (Pennisi, 1998; Rutherford and Lindquist, 1998).

Genetics

Genome Analysis The guanine + cytosine (G+C) content of *Buchnera* is about 28 mol% (Clark et al., 1998a; Ishikawa, 1987). The genome size of the endosymbiont from the aphid Ap has been found to be 0.657 Mb (Charles and Ishikawa, 1999). This is considerably below such free-living organisms as *E. coli* (4.6 Mb; Blattner et al., 1997) and *Haemophilus influenzae* (1.8 Mb; Fleischmann et al., 1995) and the intracellular pathogens, *Chlamydia trachomatis* (1.0 Mb; Stephens et al., 1998) and *Rickettsia prowazekii* (1.1 Mb; Andersson et al., 1998). The *Buchnera* genome is somewhat larger than that of the pathogen *Mycoplasma genitalium* (0.58 Mb; Fraser et al., 1995). An unusual property of the *Buchnera* genome is that it appears to be present as about 120 copies per cell (Komaki and Ishikawa, 1999).

Approximately 130 kb of DNA have been sequenced from *Buchnera* (from Sg) (Baumann and Baumann, 1998; Baumann et al., 1995; Clark et al., 1998a, b; Thao and Baumann, 1998). The choice of this aphid was predicated on the fact that it contains only one endosymbiont as indicated by morphological examinations as well as extensive restriction enzyme and Southern blot analysis of whole aphid DNA, using probes for many different genes. The latter results indicated that, with the exception of plasmid amplified DNA, only one copy of the targeted genes was present in the endosymbiont genome. In many cases, the DNA that was used for cloning was also endosymbiont-enriched. There were many independently cloned DNA fragments with identical overlapping sequences, indicating that the aphids did not contain several closely related endosymbionts. At least 20 kb of DNA also have been sequenced from *Buchnera* of each of the aphids Dn, Sc and Mr (Baumann et al., 1998a; Clark et al., 1999b; Lai et al., 1995, 1996). Table 4 lists, under different functional categories, the genes found in *Buchnera*, primarily in (Sg). The order of these genes in the DNA fragments is presented in Table 5. A total of 126 open reading frames were detected, of which 101 corresponded to *E. coli* genes with known function. The remaining 25 open reading frames all had homologs of no known function in the *E. coli* chromosome (Blattner et al., 1997). Table 6 presents the codon usage of the *Buchnera* structural genes. As expected from the G+C content, there is a strong bias for A and T, especially in the third codon position. This bias also affects the composition of proteins, favoring amino acids for which

Table 5. Order of genes on DNA fragments of *Buchnera* from the aphid *S. graminum*.[a]

Chromosomal genes

1) (34.7kb, AF008210) *39 kDa-groEL-groES-tRNA^{Phe}-trmE-60 kDa-rnpA-rpmH-dnaA-dnaN-gyrB-atpCDGAHFEB-gidA-ORF194-0RF453-fdx-hscA-hscB-ORF128-nifS-tRNA^{Trp}-ilvD-ilvC-rep-trxA-rho*
2) (12.8 kb, AF067228) *bcp-aroC-ORF177-hisG-hisD-hisC-hisB-hisH-hisA-hisF-hisI-gnd-dcd-metG*
3) (11.5 kb, L43549) *aspS-trxB-serS-serC-aroA-rpsA-himD-tpiA*
4) (9.7 kb, AF060492) *dapD-htrA-ORF340-IlvI-ilvH-ORF312-ftsL-ftsI-murE*
5) (8.4 kb, Z19055) *ORFB-ORFA-trpD-trpC(F)-trpB-trpA-ORFV-ORFVI-P14*
6) (6.8 kb, AF012886) *murC-ddlB-ftsA-ftsZ-ORF113-ORF235*
7) (6.5 kb, M90644) *dnaG-rpoD-cysE-secB-yibN-argH*
8) (6.1 kb, U09230) *aroE-tRNA^{glu}-rrl-rrf-cysS*
9) (5.0 kb, Z11913) *rplL-rpoB-rpoC*
10) (4.5 kb, U11066) *aroH-thrS-infC-rpmI-rplT*
11) (4.4 kb, L18927) *argS-rrs-ORF1-rnh-dnaQ*
12) (4.1 kb, AF108665) *hslU-ibp-fpr-yjeA-kdtB*
13) (3.9 kb, U11045) *ORFC-ORF217-gapA-ORFD*
14) (0.9 kb, M74510) *rpsK-rpsD-rpoA*

Plasmid-associated genes

15) (8.0 kb, AF041836) *leuA-leuB-leuC-leuD-repA1-ORF1-repA2*
16) (3.6 kb, Z21938) *trpEG*

[a]Numbers followed by parenthesis indicate linkage groups, numbers within parentheses indicate size of fragment and GenBank number. See Table 4 for description of gene products.

Table 6. Codon usage of *Buchnera* from the aphid *S. graminum*.[a]

AA	Codon	Fraction	AA	Codon	Fraction
Phe	UUU -	0.933	Ala	GCA -	0.470
Phe	UUC -	0.067	Ala	GCG -	0.052
Leu	UUA -	0.663	Tyr	UAU -	0.854
Leu	UUG -	0.091	Tyr	UAC -	0.146
Leu	CUU -	0.132	His	CAU -	0.865
Leu	CUC -	0.011	His	CAC -	0.135
Leu	CUA -	0.087	Gln	CAA -	0.887
Leu	CUG -	0.016	Gln	CAG -	0.113
Ile	AUU -	0.576	Asn	AAU -	0.863
Ile	AUC -	0.075	Asn	AAC -	0.137
Ile	AUA -	0.349	Lys	AAA -	0.918
Met	AUG -	1.000	Lys	AAG -	0.082
Val	GUU -	0.474	Asp	GAU -	0.875
Val	GUC -	0.054	Asp	GAC -	0.125
Val	GUA -	0.407	Glu	GAA -	0.913
Val	GUG -	0.065	Glu	GAG -	0.087
Ser	UCU -	0.448	Cys	UGU -	0.826
Ser	UCC -	0.039	Cys	UGC -	0.174
Ser	UCA -	0.273	Trp	UGG -	1.000
Ser	UCG -	0.034	Arg	CGU -	0.348
Ser	AGU -	0.177	Arg	CGC -	0.045
Ser	AGC -	0.029	Arg	CGA -	0.148
Pro	CCU -	0.456	Arg	CGG -	0.013
Pro	CCC -	0.069	Arg	AGA -	0.416
Pro	CCA -	0.420	Arg	AGG -	0.030
Pro	CCG -	0.055	Gly	GGU -	0.464
Thr	ACU -	0.474	Gly	GGC -	0.042
Thr	ACC -	0.041	Gly	GGA -	0.446
Thr	ACA -	0.433	Gly	GGG -	0.048
Thr	ACG -	0.052	Ter	UAA -	0.887
Ala	GCU -	0.426	Ter	UAG -	0.094
Ala	GCC -	0.052	Ter	UGA -	0.019

[a]Based on 19037 codons (Clark et al., 1998a).

codons contain more A and T (Moran, 1996; Clark et al., 1999b).

Buchnera was found to contain *dnaA*, encoding a protein which initiates bidirectional chromosome replication, and *ftsZ*, encoding a protein involved in septum formation during cell division (Baumann and Baumann, 1998; Lai et al., 1992a). Among other genes that were found are those encoding proteins for: peptidoglycan synthesis, cell division, DNA replication, DNA transcription, ribosomal proteins, amino acid tRNA synthases, ATP synthase, electron transport, protein secretion and glycolysis. In addition, genes for three tRNAs were detected. Genes encoding homologs of proteins involved in the *E. coli* heat shock response (*groEL*, *groES*, *htrA*, *dnaK*, *dnaJ*) and the cold shock response (*hscA*, *hscB*) were also detected (Clark et al., 1998a; Hassan et al., 1996; Ohtaka et al., 1992; Sato and Ishikawa, 1997b). Nakabachi and Ishikawa (1999) detected a gene (*ribE*) encoding a protein involved in riboflavin biosynthesis. In addition some of the genes encoding enzymes for the biosynthesis of aromatic amino acids (shikimate pathway, tryptophan branch), branched-chain amino acids (isoleucine, valine, leucine), lysine, cysteine and serine as well as genes for the complete pathway of histidine biosynthesis were found (Clark et al., 1998a, b; Thao and Baumann, 1998). The presence of genes for enzymes of amino acid biosynthesis is in marked contrast to the obligate intracellular pathogens *Rickettsia prowazekii* and *Chlamydia trachomatis*, as well as such fastidious organisms as *Mycoplasma genitalium* and *Borrelia burgdorferi*, all of which lack genes encoding enzymes of amino acid biosynthesis (Andersson et al., 1998; Fraser et al., 1995, 1997; Stephens et al., 1998). Retention of amino acid biosynthetic genes by *Buchnera* probably reflects the role of these pathways in the mutualistic association with the host aphids. Overall, these results on gene content indicate that *Buchnera* has many of the properties of free-living bacteria and would appear to be, in many respects, a self-contained, physiologically autonomous unit enclosed within bacteriocyte-derived vesicles.

Currently the most interesting comparison of the *Buchnera* genome is with the recently sequenced obligate intracellular pathogen *R. prowazekii*, an organism which is a member of the α-subdivision of the Proteobacteria (Andersson et al., 1998). Compared to the sequenced bacterial genomes, this organism is unusual in that 24% of its DNA is noncoding. In addition it has a number of pseudogenes. These findings are interpreted as a stage in the adaptation of *R. prowazekii* to an intracellular lifestyle, involving the loss of genes encoding metabolic pathways for products that are provided by the host. Currently about 20% of the *Buchnera* genome has been sequenced, and the organization of the genes in these genome fragments is highly compact and similar to that of other bacteria (Clark et al., 1998a). These findings indicate that if, as seems probable, *Buchnera* originated from an organism with a larger genome (Charles and Ishikawa, 1999), then the reduction in the genome size has already been accomplished and what has been retained is the essential gene complement required for the endosymbiotic association.

Plasmid-Associated Amplification of Biosynthetic Genes Some species of aphids have *Buchnera* in which genes for enzymes of amino acid biosynthesis are amplified on plasmids (Bracho et al., 1995; Lai et al., 1994; van Ham et al., 1999). This plasmid-associated gene amplification has been interpreted as an adaptation of *Buchnera* to an endosymbiotic association in which one of its functions is the overproduction of amino acids. This interpretation is based on analogies with other prokaryotic systems in which gene amplification is viewed as an attribute of prokaryotic

genome plasticity allowing the organism to adapt to new environments (for recent reviews see Romero and Palacios, 1997; Roth et al., 1996). Currently *Buchnera* has been found to contain three different types of plasmids. The properties of these plasmids are summarized in Table 7. The plasmid-amplified genes encode the first enzyme of the tryptophan biosynthetic pathway (TrpEG) and four enzymes (LeuA, LeuB, LeuC, LeuD) of the leucine portion of the branched-chain amino acid biosynthetic pathway. For purposes of grouping of plasmid types and ease of presentation, we have used the plasmid designations given in Table 7 followed by the abbreviation corresponding to the aphid species (Table 2).

Table 7. Designations and characteristics of *Buchnera* plasmids.[a]

1) **pTrpEG.** Two or more DnaA boxes in a putative origin of replication (Fig. 5a–h). Arrangement of DnaA boxes varies, one conserved pattern is recognized and designated at *ori*-3.6. (Fig. 5a–c). The plasmids usually consist of tandem repeats of identical or similar units containing gene(s) for a putative anthranilate synthase (TrpEG), the first enzyme of the tryptophan biosynthetic pathway.
2) **pTrpEG-R.** Plasmid contains genes for putative replication initiation proteins (*repAC1, repAC2*) which are related to replication initiation proteins of broad host range plasmids of the IncA/C group (Fig. 5i, 7c). Within the DNA encoding the C-terminal end of the replication initiation proteins and/or downstream of this gene are 19 nt-long repeats which correspond to putative interons (Fig. 5i). Plasmid consists of tandem repeats of similar units and contains genes for a putative anthranilate synthase (TrpEG), the first enzyme of the tryptophan biosynthetic pathway.
3) **pLeu.** Plasmid contains genes for putative replication initiation proteins (*repA1, repA2*) which are related to replication initiation proteins of plasmids of the IncFII group (Fig. 9a-c). Putative origin of replication downstream of *repA1*. Plasmids contain one copy of genes encoding for enzymes of leucine biosynthesis (*leuA, leuB, leuC, leuD*).

[a]For references see text.

When first used, the designation is followed by the original plasmid name given in parentheses (if applicable). There are a number of other examples of plasmid amplification of biosynthetic genes. Amplification has been interpreted as a means of increasing the rate of synthesis of endproducts. These include genes of purine biosynthesis in *Borrelia* (Margolis et al., 1994), cysteine biosynthesis in *Synechococcus* (Nicholson et al., 1995), and histamine biosynthesis in *Vibrio* (Barancin et al., 1998).

The trpEG-Containing Plasmids and Gene Silencing Figure 4 is an outline of the aromatic amino acid biosynthetic pathway. It consists of a common portion leading to chorismate (shikimate pathway) and branches to 1) phenylalanine and tyrosine as well as 2) tryptophan. In the shikimate pathway, arrows that have designations correspond to genes detected in *Buchnera* (Sg). The activity of the tryptophan biosynthetic pathway is regulated by anthranilate synthase (TrpEG) which is feedback inhibited by tryptophan (Crawford, 1989). In *Buchnera* from 11 species of aphids, *trpEG* has been found to be plasmid-associated (Fig. 5; Baumann et al., 1997b; Lai et al., 1994, 1996; Rouhbakhsh et al., 1996, 1997; van Ham et al., 1999). The remaining genes of the pathway [*trpDC(F)BA*] have been found to have a chromosomal location in all cases examined [*Buchnera* (Sg, Dn, Sc, Mr)] (Baumann et al., 1998a; Clark et al., 1999b; Lai et al., 1995). In contrast to the situation in Aphididae, in *Buchnera* (Sc, Mr), *trpEG* is not plasmid-associated but is present as one copy on the endosymbiont chromosome (Fig. 4; Clark et al., 1999b; Lai et al., 1995).

The structure of plasmids of the pTrpEG-type usually consists of tandem repeats of a nearly identical unit (Fig. 5a–h). In *Buchnera* (Sg, Rp) the plasmids contain four tandem repeats of a 3.6 kb unit, in *Buchnera* (Rm) the plasmid consists of one 3.6 kb unit, while in *Buchnera* (Ap) plasmids containing 5, 6, or 10 tandem repeats

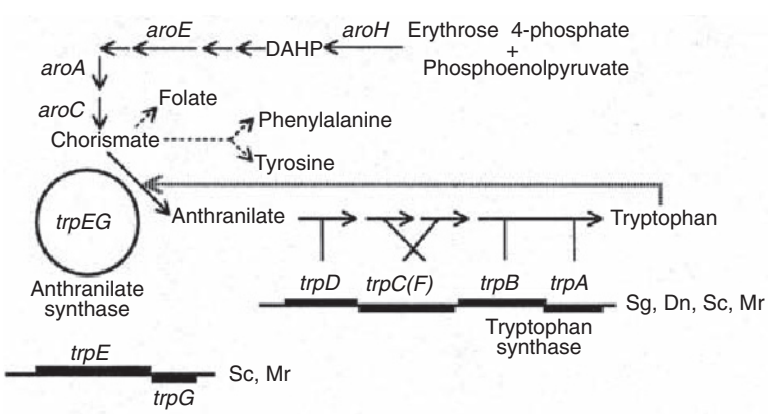

Fig. 4. Outline of the pathway for the biosynthesis of aromatic amino acids. Arrows, single enzymatic reactions; dashed arrows, several enzymatic reactions; striped arrow, feedback inhibition; genetic designations above striped line, genes detected in *Buchnera* (Sg); other genes, detected in *Buchnera* from the designated host aphids; circle, plasmid containing one or multiple copies of trpEG; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate. For a description of genes see Table 4; for references see text.

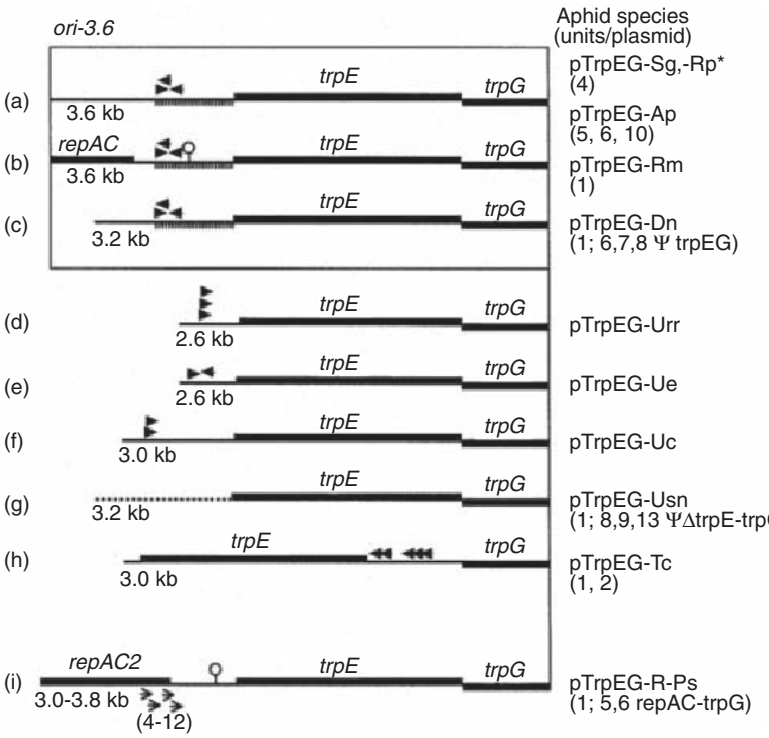

Fig. 5. Genetic maps of the repeated units which constitute trpEG-containing plasmids. Filled arrowheads, position and direction of DnaA boxes which are components of a putative origin of replication; circle on stem in (b) and (i), position of a 19 nt sequence similar to the interon of the broad host range plasmid RA1; ori-3.6, putative origin of replication found primarily in plasmids consisting of 3.6 kb repeat units (boxed); striped line, conserved sequence; dashed line, DNA that has not been sequenced; arrow in (i), 19 nt repeated sequence corresponding to a putative interon; Rp*, TrpEG-Rp also contains a remnant of repAC. For references see text.

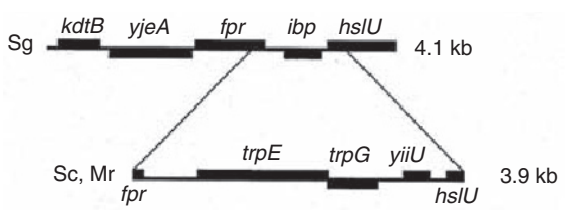

Fig. 6. Genetic map of similar chromosomal DNA fragments from Buchnera (Sg) as well as (Sc, Mr). Buchnera (Sg) lacks trpEG due to its presence on a plasmid while in Buchnera (Sc, Mr) trpEG is chromosomal. For a description of genes see Table 4; for references see text.

are found. *Buchnera* (Sg) contains about 4 plasmids per endosymbiont chromosome resulting in a 16-fold *trpEG* amplification. In *Buchnera* (Sc, Mr), in which *trpEG* is chromosomal, these two genes are found between *fpr* and *hslU* (Fig. 6). In *Buchnera* (Sg), in which *trpEG* is plasmid-associated, *trpEG* is absent from this chromosomal location, consistent with its transfer to the plasmid (Fig. 6). Instead *ibp* is present at this position, suggesting a concomitant or a subsequent acquisition of this gene (Clark et al., 1999a).

The plasmids in Fig. 5a–h all share in common the presence of 2–5 DnaA boxes which are 9 nt-long sequences to which the DnaA protein binds, thereby initiating chromosomal replication (Messer and Weigl, 1996). There is also considerable variation in the length of the repeated units (2.6 to 3.6 kb). Within this plasmid group, a readily recognized subset contains a unique arrangement of three DnaA boxes and a conserved region upstream of *trpEG* which has been designated as *ori-3.6* (Fig. 5a–c; Lai et al., 1996; Rouhbakhsh et al., 1996). *Buchnera* in aphids of the genus *Uroleucon* (which is derived from *Buchnera* within the cluster that has *ori-3.6*-containing plasmids, Fig. 2b, c) have *trpEG* units which show a considerable size range and substantial differences in the arrangement of the DnaA boxes (Fig. 5d–g; Baumann et al., 1997b; Rouhbakhsh et al., 1997). All of these *Buchnera* are from aphid species of the family Aphididae (Fig. 2a). Plasmid pTrpEG-Tc (pBTc2; Fig. 5h) is from an aphid within the family Pemphigidae (van Ham et al., 1999). The predominant form of this plasmid consists of one 3.0 kb unit; a minor form consists of two units. A distinctive feature is the presence of DnaA boxes between *trpE* and *trpG* (Fig. 5h) and not upstream of *trpEG* as is the case of the other plasmids (Fig. 5a–g). However, since there is considerable rearrangement of the DnaA boxes within plasmids in *Buchnera* of *Uroleucon* (Fig. 5d–g), it is plausible that the arrangement of DnaA boxes within pTrpEG-Tc is not a fundamental difference but a variation on the arrangement observed in the other *trpEG*-containing plasmids (Fig. 5a–g).

A totally different *trpEG*-containing plasmid is pTrpEG-R-Ps (pBPs2; Fig. 5i), which does not contain DnaA boxes but instead has putative

replication initiation proteins (RepAC) which are related to those of plasmids of the broad host-range group IncA/C (van Ham et al., 1999). Within the DNA encoding the C-terminal portion of *repAC* and/or downstream of it are 4–12 repeats of a 19 nt-long sequence corresponding to a putative interon. In addition, there is a single copy of a 19 nt-long sequence similar to the interon sequence of IncAC plasmid RA1 (Llanes et al., 1996). Curiously pTrpEG-Rm (Fig. 5b) contains in its DNA a gene for a putative RepAC protein and the 19 nt-long sequence similar to that found in plasmid RA1, while pTrpEG-Rp from a closely related aphid has a remnant of *repAC* (van Ham et al., 1999).

In several pTrpEG plasmids, the expression of most of the *trpEG* copies appears to be silenced (Baumann et al., 1997b; Lai et al., 1996; van Ham et al., 1999). In *Buchnera* (Dn) there are about two copies of pTrpEG-Dn for each endosymbiont genome (Lai et al., 1996). Plasmid pTrpEG-Dn consists of a single 3.2 kb unit containing an open reading frame corresponding to the putative protein TrpEG (Fig. 7a). This is followed by a 2.6 kb unit containing *trpEG* pseudogenes and 5–7 repeats of a 3.2 kb unit also containing *trpEG* pseudogenes. (By pseudogenes, we mean segments of DNA which are clearly recognizable as *trpEG* but which contain numerous frameshifts and stop codons preventing the synthesis of an intact protein.) A comparison of the sequences between the 3.2 kb fragments with and without pseudogenes indicated 244 differences of which 93% were localized in an approximately 900-bp DNA segment which included the putative promoter and the N-terminus of *trpE* (Fig. 7a). These changes should result in the reduction or elimination of mRNA synthesis; if messenger is made, it would be translated into short peptides because of the numerous frame shifts and stop codons in the region of the N-terminus of *trpE*.

Another instance of *trpEG* silencing is illustrated by pTrpEG-Usn (Fig. 7b). This plasmid consists of a 3.2 kb unit of *trpEG* followed by 10–14 2.1 kb units consisting of DNA with a deletion of about 56% of the N-terminal region of *trpE* and an intact trpG. A more remarkable example of gene silencing is found in pTrpEG-R-Ps (Fig. 7c), which consists of a 3.6–3.8 kb unit containing *repAC2-trpEG* followed by 1.8 kb units consisting of *repAC1*, a deletion of *trpE*, and an intact *trpG* (van Ham et al., 1999). Preceding the latter is a short DNA segment which appears to be a remnant of DNA encoding the C-terminus of *trpE*. The structure of this plasmid suggests that, in *Buchnera* (Ps) of the family Pemphigidae, *trpEG* amplification had an origin independent of that of the remaining plasmids shown in Fig. 5. The initial plasmid probably contained tandem repeats of *repAC-trpEG*. Subsequently there was selection pressure for gene silencing resulting in a plasmid with one intact copy of *repAC-trpEG* and tandem repeats of *repAC-trpG* which contain a deletion of the putative promoter region and most of *trpE*.

Speculation Concerning trpEG Amplification

The following is a summary of the results obtained from studies of *trpEG* in *Buchnera*. 1) *trpEG* amplification is widespread in *Buchnera* within the family Aphididae and is also present in at least two members of the family Pemphigidae. In *Buchnera* from two aphid species of the latter family, *trpEG* is chromosomal and is found in the same location. In most cases *trpEG* amplification is affected by plasmids consisting of tandem repeats of the same or similar unit.

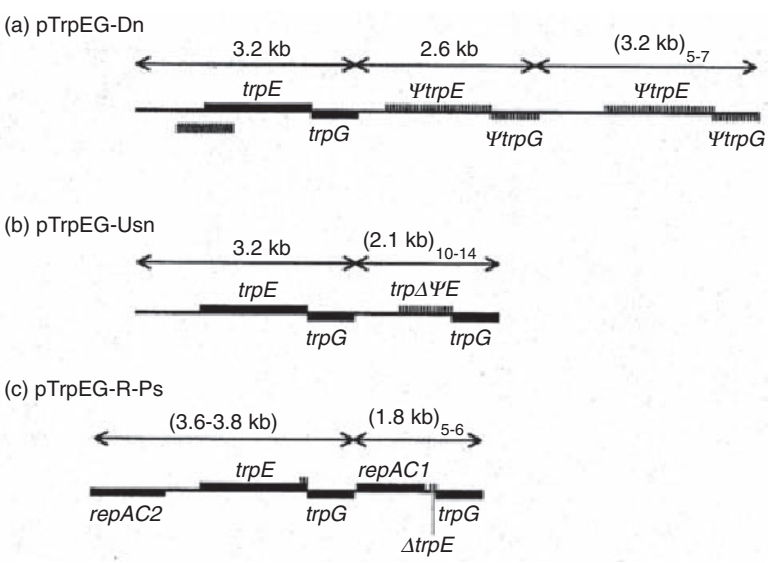

Fig. 7. Genetic map of plasmids which contain silenced trpEG. Stippled line in (a), region which in the pseudogene containing fragments has most of the changes; striped line in (a) and (b), pseudogenes (Ψ); Δ in (b), deletion of the N-terminal portion of trpE; striped line in (c), sequence homologous to the end of trpE. For references see text.

2) Evolution of plasmid-associated *trpEG* is vertical, that is, *Buchnera* from different aphid species do not exchange plasmids. 3) The *trpEG*-containing plasmids constitute at least two replicon types, one of which is based on the presence of DnaA boxes, while the other is based on the presence of interons and replication-initiation proteins related to plasmids of the IncA/C group. *Buchnera* from the related aphids Rm and Rp which belong to the first replicon type also have a gene and/or the remnant of a gene for a replication-initiation protein related to plasmids of the IncA/C group. 4) In *trpEG* plasmids of both replicon types, gene silencing of some of the *trpEG* tandem repeats is observed. 5) In *Buchnera* (Sg) the sequence of two of the 3.6-kb units is virtually identical, as is the sequence of the 2.6 and 3.2-kb *trpEG* pseudogene-containing units of *Buchnera* (Dn). While results above are derived from genetic analyses, the interpretations that follow are speculative and based in large part on analogies with other prokaryotic systems.

Gene amplification is currently viewed as a reversible aspect of genome plasticity which occurs at a frequency considerably higher than that of mutation in structural genes (Romero and Palacios, 1997; Roth et al., 1996). Gene amplification is frequently used by an organism as a means of increasing the amount of a growth-limiting enzyme to levels beyond that achieved by gene regulation of expression. One of the functions of *Buchnera* is the synthesis of essential amino acids (including tryptophan) for the aphid host. In almost all prokaryotes, the limiting enzyme TrpEG (which is feedback inhibited by tryptophan) regulates the activity of the tryptophan biosynthetic pathway. The *Buchnera* enzyme is probably also feedback inhibited by tryptophan since *trpE* has the conserved amino acid residues that are involved in feedback inhibition (Lai et al., 1994). To overproduce tryptophan, the activity of TrpEG must be increased, the potential effect of tryptophan accumulation on activity must be overcome, or both. Since even in high concentrations of tryptophan the activity of TrpEG is not fully inhibited, an increase in enzyme protein will result in increased tryptophan production. This has been the case in experiments on overproduction of tryptophan for industrial purposes; an increase in allosterically inhibitable TrpEG is the primary means of achieving excretion of high amounts of tryptophan into the medium (Katsumata and Ikeda, 1993).

In free-living bacteria, gene amplification is frequently transient. Its persistence depends on a constant selective pressure, the absence of which leads to a rapid decrease in number of repeats primarily by means of RecA-mediated homologous recombination (Roth et al., 1996). There may be differences or fluctuations in the levels of tryptophan in aphid diets; furthermore, the high level of TrpEG protein may impose an energy burden on the endosymbiont. Both of these conditions could provide short-term selective pressure for the elimination of plasmids from *Buchnera* (Baumann et al., 1997a). Consequently some mechanism of stabilization may be necessary for the maintenance of *trpEG*-containing plasmids. The gene *recA* has been cloned and sequenced from many bacterial taxa, and adequate oligonucleotide primers are available for its amplification by PCR (Eisen, 1995). We have made extensive unsuccessful attempts to detect this gene by PCR, suggesting that it may be absent from *Buchnera* or is greatly modified. Once plasmid stabilization occurs, the aphid may encounter conditions in which *trpEG* amplification is no longer necessary due to availability of tryptophan in the diet. If the usual mechanisms (homologous recombination?) which effect a decrease in *trpEG* amplification are absent, then one way of reducing the potentially wasteful synthesis of TrpEG is gene silencing.

In the past we have speculated that *trpEG* amplification is a property of rapidly growing aphids and that gene silencing may occur when following plasmid stabilization, the diet of aphids is nutritionally enriched (Baumann et al., 1997a; Lai et al., 1994, 1995, 1996). Recent studies have indicated that these speculations are overly simplistic. It has been suggested that the lack of *trpEG* amplification in *Buchnera* (Sc, Mr) which are in the Pemphigidae is due to the slow growth rate of these aphids compared to that of aphids within the Aphididae (Lai et al., 1995). There are, however, few studies on the growth rate of aphids, and the finding of amplification in *Buchnera* (Ps, Tc) which are in the family Pemphigidae and presumably also have a slow growth rate makes this explanation questionable. Dn causes major tissue histolysis of plants and it has been suggested that the presence of higher amounts of tryptophan in the diet may be the explanation for gene silencing (Lai et al., 1996). Recent studies on amino acid composition of ingested phloem sap are not strongly supportive of this hypothesis as a sole explanation for the presence of pseudogenes (Sandström and Moran, 1999; Sandström et al., 2000; Telang et al., 1999). *Buchnera* (Rp) and *Buchnera* (Sg) are similar in that both have pTrpEG consisting of four tandem repeats of a 3.6-kb unit (Fig. 5a). In spite of this similarity, Rp causes essentially no modification of the amino acid composition of plant phloem while Sg causes substantial increases (Sandström et al., 2000). Dn contains a plasmid with *trpEG* pseudogenes (Fig. 7a). The ingested diet of Dn has approximately doubled

concentrations of tryptophan, yet the changes it causes in the amino acid composition of phloem are less than those caused by Sg. This suggests that pseudogene formation is not solely the result of increased dietary tryptophan (Sandström et al., 2000; Telang et al., 1999). However, in the case of Usn, the presence of *trpEG* pseudogenes is consistent with the finding of unusually high levels of essential amino acids in the phloem diet (Sandström and Moran, 2000). Thus the availability of nutrients in plant sap may be a partial explanation for *trpEG* amplification and pseudogene formation, but other factors must also be involved.

In this connection it should be mentioned that the past speculations attempt to correlate adaptations of *Buchnera* with properties of the aphid host (growth rate, modification of nutrient content of plant sap). The environment of the endosymbiont is the bacteriocyte vesicle, which harbors the endosymbiont. This environment is a reflection of the activities of the aphid host and is a function of its ability to obtain nutrients from the plant as well as its demands on the biosynthetic attributes of the endosymbiont. Therefore, host properties, such as the efficiency of nutrient uptake from the plant and their transformation and delivery, may determine the nutritional parameters within the bacteriocyte vesicles and impose the selective pressure resulting in *Buchnera* adaptation to the endosymbiotic association.

Some of the phenomena encountered in pTrpEG from *Buchnera* also have been found in other systems. Promoter inactivation by multiple sequence changes is the mechanism used for silencing the expression of the *Bordetella pertussis* toxin gene (Gross and Rappuoli, 1988) and the expression of the *Bordetella* urease gene cluster (McMillan et al., 1998). The changes resemble those observed in gene silencing of *trpEG* in *Buchnera* (Dn; Fig. 7a). Multiple copies of nearly identical chromosomal enzyme-encoding genes have been found in *Thiobacillus ferroxidans* (Kusano et al., 1991) and *Nitrosospira* sp. (Norton et al., 1996). This situation is similar to that found with the repeats of *trpEG* and *trpEG* pseudogenes and has led to the postulation of mechanisms for the preservation of sequence identity of the repeated units (Klotz and Norton, 1998).

The phylogenetic trees constructed on the basis of plasmid-associated genes are congruent with the phylogenetic trees based on *Buchnera* chromosomal genes (Fig. 2a–c). One exception, the basal position of *Buchnera* (Tc; Fig. 2c), is probably an artifact arising from the more rapid change of *trpE* in this lineage (van Ham et al., 1999). These results strongly suggest that there is no exchange of *trpEG*-containing plasmids between endosymbionts of different aphids. Plasmids of the pTrpEG type (Fig. 5a–h) could have an endogenous origin. DnaA boxes are found in other locations of the *Buchnera* genome (Clark et al., 1998c), and their assembly with *trpEG* could generate a separate replicon. In contrast to these plasmids, the *repAC* genes of pTrpEG-R are related to replication initiation proteins of IncA/C plasmids. Thus, this plasmid may be the result of an invasion of *Buchnera* by an exogenous plasmid that recombined with endosymbiont genes, resulting in their amplification. It has been established that some bacteria may persist for a long time in insects; conceivably, such organisms transferred their plasmids to *Buchnera* during the infection of embryos or eggs at a stage at which the endosymbionts are not sequestered within bacteriocytes. It is, however, difficult to explain the presence of *repAC* and its remnant in *Buchnera* (Rm, Rp). One possibility is that it is the result of another invasion by a similar plasmid. However, organization of *ori-3.6* in *Buchnera* (Rm, Rp) closely resembles that of *Buchnera* of related aphids, suggesting a common plasmid origin for this group (Fig. 5a–c). Alternatively, van Ham et al. (1999) suggested that a plasmid of the pTrpEG-R type is the ancestor of all *trpEG* amplification plasmids. This hypothesis requires the subsequent occurrence of multiple losses of *repAC* and the interons in pTrpEG-R type plasmids and the acquisition of DnaA boxes in *Buchnera* of Aphididae.

pLeu Plasmids Figure 8 is an outline of the pathway of branched-chain amino acid biosynthesis. The gene for aspartokinase (*thrA*) has been found in *Buchnera* (Sg). The genes *ilvIH*, *ilvC*, and *ilvD* have been found in *Buchnera* (Sg, Dn, Sc, Mr) and encode three enzymes which func-

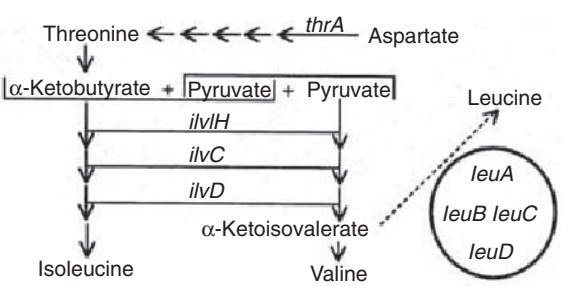

Fig. 8. Outline of the pathway for branched chain amino acid biosynthesis. Arrows, single enzymatic reactions; horizontal lines, enzymatic activities functional in both isoleucine and valine biosynthesis; dashed arrow, four enzymatic reactions; circle, plasmid containing genes for leucine biosynthesis. thrA was detected in *Buchnera* (Sg); ilvIH, ilvC, ilvD were detected in *Buchnera* (Sg, Dn, Sc, Rm); leuACBD were detected in *Buchnera* (Sg, Dn, Rm). For a description of genes see Table 4; for references see text.

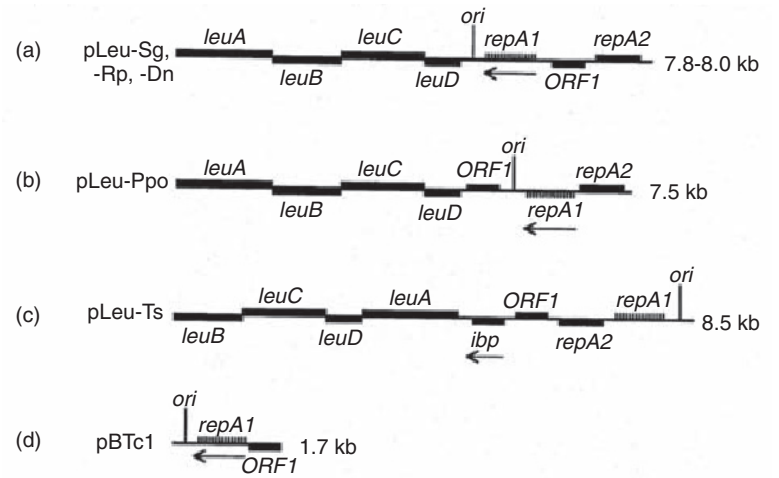

Fig. 9. Genetic maps of pLeu plasmids. Unless indicated by an arrow, transcription is left to right. ori, putative origin of replication; striped line, repA1 downstream of which is ori. For a description of genes see Table 4; for references see text.

tion in both the isoleucine and valine biosynthetic pathways (Clark et al., 1998c, 1999b; Thao and Baumann, 1998). The pathway of leucine biosynthesis is a branch off the valine pathway (Fig. 8). Bracho et al. (1995) found that in *Buchnera* (Rp), the genes for leucine biosynthesis (*leuABCD*) were present on a plasmid (Fig. 9a). This plasmid represents a third type (Table 7), designated pLeu, which is characterized by the presence of genes (*repA1*, *repA2*) encoding putative replication initiation proteins related to those of plasmids of the IncFII incompatibility group. Besides these genes, pLeu also contains *ORF1* encoding a putative membrane-associated protein. The closely related *Buchnera* (Sg, Rp, Dn) all contain very similar plasmids of 7.8 to 8.0 kb in which the genes are arranged in the same order (Fig. 9a; Baumann et al., 1999a; Bracho et al., 1995). In *Buchnera* from the more distantly related pLeu-Ppo (Fig. 2e), there are rearrangements of the *repA* genes and *ORF1* (Fig. 9b; Silva et al., 1998). All of these aphids are within the family Aphididae. In pLeu-Ts (pBTs1; Fig. 9c), which is from an aphid of the family Thelaxidae, there is also a rearrangement of the *leu* genes as well as an acquisition of *ibp* which encodes a heat shock protein (van Ham et al., 1997). All of these plasmids have a conserved region, downstream of *repA1*, which is probably an origin of replication (*ori*) (Baumann et al., 1999; Bracho et al., 1995; van Ham et al., 1997). Remarkably pBTc1 (Fig. 9d), a 1.7 kb plasmid from *Buchnera* (Tc), which is found in an aphid belonging to the family Pemphigidae, contains only *ori*, *repA1*, and *ORF1* and probably constitutes a minimal replicon.

In *Buchnera* (Sg), there are about 24 copies of pLeu per endosymbiont genome while in *Buchnera* (Dn) there are only 2 copies (Thao et al., 1998). This difference in functional gene copy number parallels that observed with pTrpEG in the endosymbionts of these two aphid species. In the case of pLeu, in which only one copy of the genes is present, the reduction in amplification in *Buchnera* (Dn) is achieved by means of a reduction of copy number. In pTrpEG, which contains tandem repeats of the same unit, the reduction in amplification is accomplished by means of pseudogene formation (Fig. 7a).

The similarities of pLeu plasmids suggest a single origin with pBTc1 (Fig. 9d) being the ancestral state (Baumann et al., 1999). In the lineage common to the Aphididae and the Thelaxidae, there was probably a duplication of *repA1* and the acquisition of *leu* genes. This was followed by a rearrangement of the genes and in one lineage the acquisition of *ibp* [for another interpretation, see van Ham et al. (1997)]. Silva et al. (1998) sequenced *repA2* from *Buchnera* of six additional aphids. A phylogeny based on this gene (Fig. 2e) as well as a more limited analysis based on *leu* genes (Fig. 2d) is congruent with trees established on the basis of other *Buchnera* chromosomal and plasmid genes. These results indicate that the pLeu plasmids are not exchanged among endosymbionts from different aphid species and that their evolution is vertical, as is the case with the *trpEG*-containing plasmids.

Unanswered Questions; Other Possible Adaptations One unanswered question is, why are only the *trp* and *leu* genes amplified? The endosymbiont produces other essential amino acids for the aphid host and their overproduction would in principle also be enhanced by plasmid amplification. It has been speculated that aphids make indole acetic acid which is involved in gall formation (Forrest, 1987). In many plant pathogens that cause gall formation, tryptophan is the

precursor of indole acetic acid (Patten and Glick, 1996). Amplification of *trpEG* is, however, found in aphids that do not produce galls (Sg, Dn, Rp, Usn) and is absent in some that do (Sc, Mr). Consequently this does not appear to be a probable explanation for *trpEG* amplification. Leucine, lysine, valine, arginine and threonine are the most common amino acid in aphids (Sandström and Moran, 1999), yet only genes for leucine biosynthesis have been detected on plasmids. It is possible that, in other cases where amino acids are overproduced for the host, an increase in enzyme activity is obtained by increasing the expression of the gene(s) by promoter modification. Alternatively changes of the allosteric properties of regulated enzymes may allow retention of activity in the presence of endproducts.

A possible example of the latter is *cysE* of *Buchnera* (Sg; Lai and Baumann, 1992b). This gene encodes an enzyme of the biosynthetic pathway of cysteine, and its activity is regulated by cysteine feedback inhibition (Kreditch, 1996). It has been established that the amino acids at the C-terminus of the *E. coli* enzyme are involved in cysteine feedback inhibition (Denk and Böck, 1987). The *Buchnera* (Sg) enzyme lacks these C-terminal amino acids and consequently is probably not subject to feedback inhibition by the endproduct; this change would result in cysteine overproduction (Lai et al., 1992b).

Secondary Endosymbionts of Aphids

Besides *Buchnera*, many aphids have additional endosymbionts usually called secondary (S-) endosymbionts (Buchner, 1965; Houk and Griffiths, 1980; Moran and Baumann, 1994). In many cases these endosymbionts are spheres or rod-shaped with different width and length; they were initially recognized by differences in size and shape from the round or oval *Buchnera*. The S-endosymbionts are also maternally inherited. They have not been extensively studied, and most of the available information is for the S-endosymbionts of the aphid Ap. Electron microcopic studies have shown that the rod-shaped S-endosymbionts are located within vesicles found in the flattened, syncytial, sheath cells which surround the bacteriome (Griffiths and Beck, 1973). Using a probe derived from *E. coli* 16S rDNA and restriction enzyme and Southern blot analysis of total aphid DNA, it was found that the S-endosymbiont from Ap contained a single copy of the 16S rDNA-gene (Unterman et al., 1989). DNA obtained from dissected bacteriocytes gave the same restriction pattern, indicating that the S-endosymbiont was located in the bacteriome. Two DNA fragments of 2.3 kb each were cloned and the 16S rDNA sequence determined. A phylogenetic analysis indicated that the S-endosymbiont was a member of the Enterobacteriaceae (Fig. 1). As in other members of this family, the 16S-rDNA gene of the S-endosymbiont was directly upstream of 23S rDNA (Unterman and Baumann, 1990).

Chen and Purcell (1997) found that 88% of the strains of Ap had the S-endosymbiont. In addition, the S-endosymbionts of Ap and *Macrosiphum rosae* were identical, suggesting recent infection or horizontal transmission. Interestingly, it was also found that the hemolymph of 48% of Ap strains contained a rod-shaped organism which had a 16S rDNA sequence nearly identical to that of *Rickettsia bellii*, an organism found in ticks (Chen et al., 1996). As a result of these studies strains of Ap became available which had 1) only the S-endosymbiont, 2) only the *Rickettsia* sp., as well as 3) neither of these two organisms. Chen (1996) injected one or both of these organisms into Ap, which originally lacked both, and observed their effect on fecundity, longevity and the length of the reproductive period. The results were complex in that they were affected by the plant on which the aphids grew and by the temperature of growth. At 20°C both the S-endosymbiont and the *Rickettsia* sp. reduced the fecundity, longevity and reproductive period of Ap on clover but had no significant effects on Ap grown on alfalfa or sweet pea (Chen, 1996). In some cases, both of these organisms appeared to cause an increase in the fitness of Ap when grown at 25°C. These results suggest that both the S-endosymbiont and the *Rickettsia* sp. can have either a deleterious or a beneficial effect on the host, depending on the environmental conditions (Chen, 1996). The S-endosymbiont, upon injection into the closely related species, *Acyrthosiphon kondoi*, was found to be pathogenic. Although the rate of maternal transmission of both the S-endosymbiont and the *Rickettsia* sp. was high, one instance of S-endosymbiont loss was observed (Chen and Purcell, 1997).

Studies based on light and electron microscopy have suggested that some S-endosymbionts in some aphid species may inhabit syncytial cells or, possibly, bacteriocytes that appear similar to those containing *Buchnera* (Buchner, 1965; Hinde, 1971b; Iaccarino and Tremblay, 1973). Fukatsu and Ishikawa (1993) surveyed 61 aphids for the presence of S-endosymbionts. Previously it was found that *Buchnera* (Ap) overproduced GroEL (Kakeda and Ishikawa, 1991). Using anti-*E. coli*-GroES for immunoprecipitation of

Buchnera (Ap) GroES, it was concluded that *Buchnera* produced low levels of this protein (Kakeda and Ishikawa, 1991). This antiserum as well as anti-*E. coli*-GroEL was used to detect GroEL and GroES in immunoblots of whole aphid extracts as well as for histochemical detection in thin sections of aphids. From these experiments it was concluded that the synthesis of substantial amounts of GroES by S-endosymbionts distinguishes them from *Buchnera*, and that this property can be used for the identification of S-endosymbionts (Fukatsu and Ishikawa, 1993). These studies have a number of problems that limit their general applicability to the survey of S-endosymbionts. The principal one is the use of antisera against *E. coli* GroES for the detection of cross reactivity of GroES from organisms that have an unknown relationship to *E. coli*. Since it is probable that many of the S-endosymbionts are members of the Enterobacteriaceae, a stronger cross-reaction would be expected with their proteins than with the proteins from *Buchnera*; consequently, an increased reactivity need not indicate a major difference in the amount of the protein. Conversely, in those cases where the S-endosymbiont is not a member of the Enterobacteriaceae, the distant relationship may preclude a strong cross-reaction (Eremeeva et al., 1998). The cross-reactivity of the *Buchnera* and S-endosymbiont proteins with the anti-*E. coli* protein antisera has not been compared. Finally, the relative production of GroES may vary among S-endosymbionts of different types.

The studies of Fukatsu and Ishikawa (1993, 1998), in which thin sections of aphids were stained by immunohistochemistry and examined by light microscopy, do suggest that in many aphids the S-endosymbionts occupy bacteriocytes distinct from those containing *Buchnera*. The authors also state that the S-endosymbionts have a variety of different shapes. The methods used and the photographs presented do not, however, allow adequate visualization of cell shape and the resolution of bacteriocyte structure. In addition the designation of some of the endosymbionts as *Buchnera* or S-endosymbionts appears to be arbitrary.

Fukatsu et al. (1998) used group-specific oligonucleotide probes for *in situ* detection of aphid P- and S-endosymbionts. A universal eubacterial 16S rRNA probe was used as well as probes specific for the 23S rRNA γ- and β-subdivision Proteobacteria. The sequences of the latter two probes differ by only one nucleotide. *Buchnera* and S-endosymbionts all hybridized with the 16S eubacterial probe, although the intensities of the signal differed considerably. Curiously the putative P-endosymbionts of two out of seven aphids did not hybridize with the 23S γ-subdivision probe. Using the total DNA preparation from these aphids, the 16S rDNA was amplified, cloned and sequenced. Two sequences were detected in each aphid DNA preparation, and one of these was related to *Buchnera* 16S rDNA. Based on this result, it was concluded that these aphids contained *Buchnera* but that their 23S-rDNA gene was changed to such an extent that hybridization with the γ-subdivision probe no longer occurred. This conclusion is questionable since the 23S rDNA of *Buchnera* (Sg, Dn, Sc, Mr), which span the diversity of aphid hosts (Fig. 2), contains the exact sequence complementary to the probe used (Clark et al., 1999b; Rouhbakhsh and Baumann, 1995). The S-endosymbiont of *Tetraneura radicicola* hybridized with the probe to the β-subdivision but not to the γ-subdivision, suggesting that this endosymbiont is a member of the former group. Since there is only a single nucleotide difference between these two probes, a confirmation of this conclusion by sequencing the rDNA from this organism seems desirable. The S-endosymbiont from two other aphid species did not hybridize with either the γ- or β-subdivision probe. In view of the technical difficulties encountered with some of the specimens, these conclusions cannot be interpreted as indicating that the S-endosymbionts of these aphids belong to different bacterial groups.

Absence of a Stable Bacterial Flora in Aphid Guts

Aphids maintained under clean conditions do not appear to have a bacterial gut flora (Douglas, 1990; Grenier et al., 1994; Harada and Ishikawa, 1993). Older aphids as well as aphids reared under crowded conditions may acquire a gut flora which appears to consist of members of the Enterobacteriaceae (*Serratia*, *Erwinia*), *Pseudomonas*, *Staphylococcus*, and *Bacillus* (Grenier et al., 1994; Harada and Ishikawa, 1993). These organisms are frequently associated with plant surfaces. None of these organisms has a close relationship to *Buchnera*, precluding a recent common ancestor from which they and *Buchnera* are descended. The presence of this bacterial flora has a deleterious effect on aphid performance (Grenier et al., 1994). The actual bacterial numbers have not been established. Harada et al. (1996) have isolated 38 bacterial strains from the guts of 20 aphids. This hardly suggests the presence of an indigenous bacterial flora and the numbers are insignificant compared to the numbers of *Buchnera* or the S-endosymbionts. These studies indicate that the guts of aphids are generally sterile but under certain conditions a transient bacterial gut flora may be present.

Endosymbionts of Other Plant Sap-Utilizing Insects

Psyllids, whiteflies and mealybugs are three separate lineages of the suborder Sternorrhyncha and contain P-endosymbionts from three distinct bacterial groups (Table 1, Fig. 1). These insects have obligatory sexual reproduction with the young hatching from eggs (Borror et al., 1989). The endosymbionts are housed within bacteriocytes, and at least during some stage of the insect's life cycle, the bacteriocytes are associated with the ovarioles resulting in the transmission of endosymbionts to the eggs (Buchner, 1965).

Psyllid Endosymbionts

Fukatsu and Nikoh (1998) sequenced the 16S rDNA of endosymbionts from *Anomoneura mori*, while Spaulding and von Dohlen (1998) performed a similar study of the endosymbionts of *Blastopsylla occidentalis*, *Pachypsylla venusta*, and *Trioza magnoliae*. These authors came to the same conclusion, namely that the P-endosymbionts of psyllids constitute a distinct lineage within the γ-subdivision of the Proteobacteria (Fig. 1). These endosymbionts have an unusual property, namely, the lowest known G+C content of any 16S rDNA (36.4 mol%). In addition, this lineage appeared to have a substantial acceleration of the rate of evolutionary change within the 16S sequence. *A. mori*, *B. occidentalis*, and *T. magnoliae* all had different S-endosymbionts, which belong in the γ-subdivision. Only the P-endosymbiont was found in *P. venusta* (Spaulding and von Dohlen, 1998). The studies were limited to few taxa but were consistent with cospeciation of the P-endosymbiont and the psyllid host and multiple acquisitions of S-endosymbionts (Fukatsu and Nikoh, 1998; Spaulding and von Dohlen, 1998).

Psyllids contain bilobed bacteriomes made up of round uninucleate bacteriocytes and a multinucleate syncytial region (Buchner, 1965). Many psyllids have endosymbionts in both the bacteriocytes and the syncytium; some have endosymbionts only within the bacteriocytes (Buchner, 1965). Using electron microscopy, Chang and Musgrave (1969) and Waku and Endo (1987) found that two psyllid species have endosymbionts in the bacteriocytes which are distinguishable from those found in the syncytium and that both endosymbiont types have a Gram-negative cell wall. In general, the bacteriocyte-associated endosymbionts are more numerous than the syncytium-associated endosymbionts (Buchner, 1965). Fukatsu and Nikoh (1998), using an oligonucleotide probe specific for the P- or S-endosymbiont, showed by means of *in situ* hybridization that the former was localized in the bacteriocytes while the latter was in the syncytium (Fig. 10). There is currently no information on the requirement of the endosymbiont(s) by the psyllid host or on their function. The similarity in diet between psyllids and aphids raises the possibility that psyllid endosymbionts may provide nutrients as do *Buchnera*.

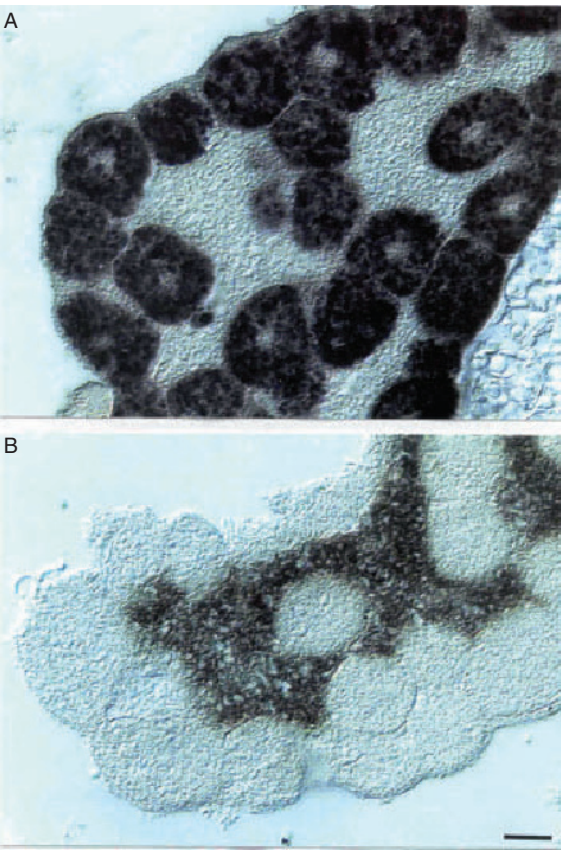

Fig. 10. Light micrographs of a bacteriome of the psyllid Anomoneura mori. (a) In situ hybridization using an oligonucleotide probe specific for the P-endosymbiont 16S rRNA, which reacts with bacteriocytes containing these endosymbionts. (b) In situ hybridization using an oligonucleotide probe specific for the 16S rRNA of the S-endosymbiont of this psyllid, which reacts with the endosymbiont located in the syncytium. Bar = 20 μm. From Fukatsu and Nikoh (1998) with permission from the authors and ASM Press.

Whitefly Endosymbionts

16S rDNA sequences have been obtained for endosymbionts of *Bemisia tabaci*, *B. argentifolii* (previously *B. tabaci* B biotype), *Siphonius phillyreae*, and *Trialeurodes vaporariorum* (Clark et al., 1992). The P-endosymbionts of these insects are a lineage within the γ-subdivision of the Proteobacteria (Fig. 1). *B. tabaci* and *B. argentifolii* have an S-endosymbiont which is

a member of the Enterobacteriaceae. The P-endosymbionts and the S-endosymbionts from these two species have identical 16S rDNA sequences, consistent with their close relationship (Brown et al., 1995; Clark et al., 1992).

The ultrastructure of the endosymbionts of *B. tabaci*, *B. argentifolii*, and *T. vaporariorum* has been studied by electron microcopy (Costa et al., 1993b, 1995). There is evidence for at least two morphological types. Whiteflies are unusual in that they transmit an entire bacteriocyte containing endosymbionts to the egg (Buchner, 1965; Costa et al., 1996). Some prokaryote-specific antibiotics affect the growth and development of whiteflies, indicating a requirement for the endosymbiont(s) by the host (Costa et al., 1993a, 1997).

Mealybug Endosymbionts

16S rDNA sequences have been obtained for endosymbionts of *Pseudococcus longispinus*, *P. maritimus*, and *Dysmicoccus neobrevipes* (Munson et al., 1992), and these organisms were found to be a distinct lineage within the β-subdivision of the Proteobacteria (Fig. 1). The morphology of endosymbionts from several mealybug species has been studied by means of electron microscopy (Tremblay, 1989). Within the bacteriocytes the endosymbionts appear to be embedded in mucous spherules of unknown composition. There is no information on the function or the requirement for the endosymbionts by the host.

Tsetse Fly Endosymbionts

Tsetse flies (genus *Glossina*) are important vectors of trypanosomes, which are causative agents of African sleeping sickness and various diseases of animals (Harwood and James, 1979). They have a somewhat unusual reproductive cycle in that the female gives birth to fully grown larvae. Only one larva is carried at a time within the uterus. During this stage the larva is fed nutritive fluids from special glands, commonly known as "milk glands." The female requires several blood meals to complete the development period of each larva, and it is these blood meals which result in the transmission of trypanosomes (Harwood and James, 1979).

Tsetse may be associated with three prokaryotes: 1) *Wigglesworthia* (P-endosymbionts), 2) *Sodalis* (S-endosymbionts), and 3) *Wolbachia*. The last are parasites found in reproductive tissue and causing reproductive disorders (O'Neill et al., 1997) and will not be considered here.

Table 8. Species of tsetse (*Glossina*) for which the 16S rDNA sequence of *Wigglesworthia* has been determined.[a]

G. austeni
G. brevipalpis
G. fuscipes
G. morsitans centralis
G. m. morsitans
G. palpalis gambiensis
G. p. palpalis
G. tachinoides

[a]Chen et al. (1999).

Wigglesworthia—the Primary Endosymbiont of Tsetse Flies

PHYLOGENY Based on 16S rDNA, *Wigglesworthia* was found to constitute a distinct lineage within the γ-3 subgroup of the Proteobacteria (Fig. 1; Aksoy et al., 1995; Chen et al., 1999). These organisms are related to but distinct from *Buchnera* of aphids and the P-endosymbionts of carpenter ants (Fig. 1). Using the host rDNA transcribed spacer-2, it was found that the phylogeny of the host was the same as that of *Wigglesworthia*, indicating cospeciation of the host and the endosymbiont (Chen et al., 1999). These results suggest a single infection of a tsetse ancestor with a bacterium followed by long-term vertical transmission of the endosymbiont, that is, a lack of exchange of *Wigglesworthia* between different tsetse fly species. The age of this association has been estimated to be at least 50 million years (Aksoy et al., 1997). A list of the species of tsetse flies for which the 16S rDNA of *Wigglesworthia* has been sequenced is presented in Table 8.

TAXONOMY The genus *Wigglesworthia* contains one species, *W. glossinidia*, which designates the lineage consisting of the P-endosymbionts of tsetse flies (Aksoy, 1995b). The type strain of this species is the P-endosymbiont of *G. morsitans morsitans*.

HABITAT Tsetse flies contain a U-shaped bacteriome located in the anterior region of the gut, which is made up of bacteriocytes containing *Wigglesworthia* (Aksoy, 1995b; Aksoy et al., 1995). These endosymbionts have a Gram-negative cell wall and are somewhat pleomorphic, occurring mostly as 4–5 m long rods. They are found free (not enclosed within host-derived vesicles) in the bacteriocyte cytoplasm. *Wigglesworthia* is maternally transmitted. Since neither the milk gland nor the developing eggs contain *Wigglesworthia*, the mechanism of their transmission is not known (Aksoy et al., 1997).

PHYSIOLOGY The feeding of tsetse flies on animals immunized with *Wigglesworthia* results in

elimination of the P-endosymbiont and sterility of the flies (Nogge, 1976). A similar effect is observed upon treatment of tsetse with prokaryote-specific antibiotics (Aksoy et al., 1995; Nogge, 1976, 1982). These results indicate that the P-endosymbiont is essential for reproduction. There is evidence that one of the functions of the *Wigglesworthia* is the production of B-complex vitamins (Nogge, 1982). *Wigglesworthia* produces a high level of GroEL (Aksoy, 1995a). In this respect it is similar to a number of other endosymbionts as well as other intracellular organisms (Hogenhout et al., 1998).

GENETICS The *Wigglesworthia* genome has one copy of the 16S rRNA gene (Aksoy, 1995a). In this organism 16S-rRNA gene is directly upstream of 23S-rRNA gene, suggesting that, as in the case of many other bacteria, these genes are a part of a single transcription unit. The presence of one copy of the rRNA operon is characteristic of slow-growing bacteria and also is found in several other endosymbionts (Baumann et al., 1995).

Sodalis—The Secondary Endosymbiont of Tsetse Flies

Tsetse flies may also contain S-endosymbionts. These are primarily found within midgut cells but also have been detected in hemolymph and in a variety of other tissues excluding ovaries (Aksoy et al., 1997; Beard et al., 1993b; Cheng and Aksoy, 1999). Their numbers are age-dependent, being higher in older insects (Cheng and Aksoy, 1999). The 16S rDNA has been sequenced from the S-endosymbionts of five different tsetse fly species, and it was found that they are members of the Enterobacteriaceae (Aksoy et al., 1997; Beard et al., 1993b). The sequences were found to be virtually identical, indicating either multiple recent infections with the same organism or horizontal transmission of the S-endosymbiont. The S-endosymbiont is maternally transmitted via the "milk gland" secretions to developing larvae (Aksoy et al., 1997).

The S-endosymbionts have been cultivated in cell-free liquid media (Beard et al., 1993b) and recently on solid media (Dale and Maudlin, 1999). The latter allowed a phenotypic characterization of this organism and led to its assignment into a new genus and species, *Sodalis glossinidius* (Dale and Maudlin, 1999). This species consists of Gram-negative rods 1–1.5 m in diameter and 2–12 m in length. It is microaerophilic, lacking catalase, and has a relatively limited capacity for carbohydrate utilization.

The S-endosymbiont has seven copies of 16S rDNA, a number which is similar to that found in rapidly growing free-living organisms (Aksoy, 1995a). Plasmids of 80 kb and about 130 kb have been detected in these organisms (Beard et al., 1993b). The S-endosymbiont has been transformed with pSUP204, and plasmid-encoded resistance to ampicillin, tetracycline and chloramphenicol was expressed (Beard et al., 1993b). Similarly the S-endosymbiont has been transformed with a pSUP204 derivative, which expressed the green fluorescent protein, allowing ready visualization of this organism in insect tissues (Cheng and Aksoy, 1999).

In one case, the S-endosymbiont from one tsetse fly species when microinjected into another species became pathogenic, killing the flies within 48 h (Cheng and Aksoy, 1999). This result is similar to the observations made with the S-endosymbiont of aphids (Chen and Purcell, 1997). The variation in the number of S-endosymbionts and their possible absence from some insects suggest that they do not perform a function essential for the survival of tsetse flies.

Sitophilus (Weevils) Endosymbionts

Weevils of the genus *Sitophilus* are major pests of stored grain (Borror et al., 1989). The female bores a hole in kernels and deposits an egg. The larva develops inside the grain from which the young adults emerge. Three related species have been studied with respect to their endosymbionts, *S. oryzae*, *S. granarius*, and *S. zeamais* (Dasch et al., 1984). Of these three species, the most extensive studies deal with *S. oryzae* (Nardon and Grenier, 1988). In addition, weevils may harbor the pathogen *Wolbachia* (O'Neill et al., 1997).

PHYLOGENY Early studies of *Sitophilus* endosymbiont morphology and the G+C content of its DNA suggested that weevils have different endosymbionts (Dasch, 1975; Dasch et al., 1984; Grinyer and Musgrave, 1966; Musgrave and Grinyer, 1968). *S. oryzae* has one endosymbiont with a G+C content of about 54 mol%, *S. granarius* has one with a G+C content of 50 mol%, while *S. zeamays* appears to have both endosymbionts (Dasch, 1984; Heddi et al., 1998). One type of endosymbiont 16S-rDNA sequence was detected in *S. oryzae*, and two types were detected in *S. zeamais* (Campbell et al., 1992; Heddi et al., 1998). Phylogenetic analysis indicated that all of these endosymbionts

are members of the family Enterobacteriaceae (Fig. 1).

Habitat

In larvae, endosymbionts are present in bacteriocytes which make up a bacteriome located at the junction of the foregut and the midgut as well as in the rudimentary ovaries (Charles et al., 1995; Nardon and Grenier, 1988). The endosymbionts are transmitted via the eggs. *S. oryzae* endosymbionts are rod-shaped, 5–15 m long, and free (not within host-derived vesicles) in the cytoplasm (Dasch et al., 1984; Nardon and Grenier, 1988).

Physiology

S. oryzae may be cured of endosymbionts by treatment with heat or antibiotics (Baker and Lum, 1973; Nardon and Grenier, 1988). Such aposymbiotic weevils are softer and paler, have an increased development time, and the fertility of their eggs is reduced (Nardon and Gernier, 1988). On some nutrient-rich grains, they can grow indefinitely. Aposymbiotic weevils lack bacteriomes, indicating that the endosymbiont triggers their development. Naturally occurring aposymbiotic weevils also may be found. There is evidence that one of the functions of the endosymbiont is the synthesis of vitamins as well as possibly phenylalanine or tyrosine (Baker, 1975, 1979; Wicker and Nardon, 1982). An additional function is the conversion of excess methionine in the diet to methionine sulfoxide (Gasnier-Fauchet and Nardon, 1986, 1986). Aposymbiotic weevils also have mitochondria with reduced levels of enzymes involved in respiration (Heddi et al., 1991). Isolated endosymbionts of *S. oryzae* do not consume oxygen and lack a number of enzymes of respiratory metabolism (Heddi et al., 1991, 1993). These results suggest that they have an anaerobic metabolism. As in the case of *Buchnera* and *Wigglesworthia*, the endosymbionts of *S. oryzae* overproduce GroEL (Charles et al., 1997b). Unlike *Buchnera*, the endosymbionts of *S. oryzae* have a heat shock response, as is indicated by an increase of *groEL* mRNA (Charles et al., 1997b).

Genetics

The genome of the endosymbiont of *S. oryzae* is 3.0 Mb and has two copies of the rRNA operon (Charles et al., 1997a). In addition, the endosymbiont contains a plasmid of about 138 kb. This genome size puts the endosymbiont within the range of many free-living bacteria (Heddi et al., 1998).

Comparisons with Other Associations

Although the information is somewhat limited, comparison of the *Sitophilus*-endosymbiont association with other insect-endosymbiont associations suggests that the former has some unique features. In several insect endosymbiotic associations, the S-endosymbionts are members of the Enterobacteriaceae, suggesting that organisms within this lineage have an enhanced capacity to enter into such associations. An interesting feature of the *Sitophilus* association is that these organisms are the sole endosymbionts. The morphological diversity of the endosymbionts, the large endosymbiont genome size, and the fact that aposymbiotic weevils are viable suggest that the associations arose through multiple recent infections and that major adaptations resulting in obligatory mutual interdependence of both partners have not evolved as yet. This association may consequently be an example of an endosymbiosis at an early stage of its development.

Carpenter Ant Endosymbionts

Ants feed on complex diets, and the presence of endosymbionts has been reported in only two groups. These two groups are the genus *Formica* and the genus *Camponotus*, commonly known as the carpenter ants (Borror et al., 1989; Buchner, 1965; Dasch et al., 1984). Both groups can use a broad range of food types but typically utilize plant nectar and honeydew (the liquid feces of sap-feeding Homoptera) as major components of their diet. Only in the carpenter ants have symbionts been studied using modern methods, and we focus on these. The contribution of ant endosymbionts to host nutrition is not clear (Dasch et al., 1984).

Phylogeny

The G+C content of the DNA of carpenter ants is 30–32 mol% (Dasch, 1975; Dasch et al., 1984). The sequence of the 16S rDNA has been determined for endosymbionts of the species *Camponotus floridanus*, *C. rufipes*, *C. ligniperdus* and *C. herculeanus* (Schröder et al., 1996). A phylogenetic analysis indicated that the endosymbionts constitute a distinct, monophyletic group related to, but different from, the endosymbionts of aphids, tsetse flies, and the members of the Enterobacteriaceae (Fig. 1). The order of branching reflects the relationships between the carpenter ant species and is consistent with a single infection and subsequent vertical evolution of the endosymbionts. The age of the association is estimated at over 100 million years

(Schröder et al., 1996). In endosymbionts of carpenter ants, the 16S-rRNA gene is not directly upstream of the 23S-rRNA gene, suggesting that these genes are organized in two transcription units, as is the case in *Buchnera* (C. Sauer and R. Gross, personal communication).

Habitat

In both workers and queens of *Camponotus*, the endosymbionts are located in bacteriocytes, which are intercalated between epithelial cells of the midgut (Fig. 11a) (Buchner, 1965; Schröder et al., 1996). The endosymbionts are rods of 1 µm in width to 5–15 µm in length (Fig. 11b). They have a Gram-negative type cell wall and are free (not enclosed in host-derived vesicles) in the cytoplasm (Schröder et al., 1996). Transmission is via infection of the ovaries and incorporation into the eggs.

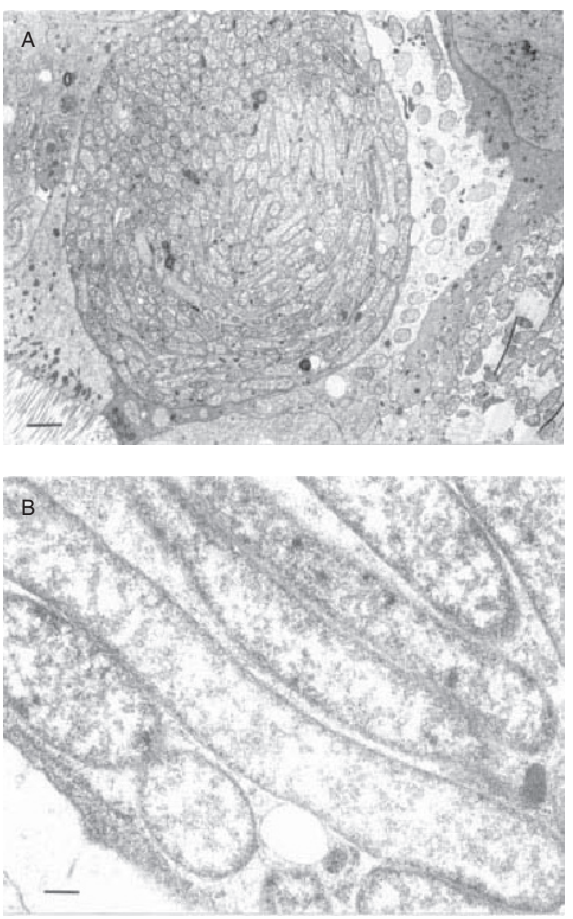

Fig. 11. Electron micrographs of the endosymbionts of the carpenter ant *Camponotus floridanus*. (a) Bacteriocyte containing the endosymbionts, bar= 3.0 µm; (b) ultrastructure of the endosymbionts showing the Gram-negative cell wall and the absence of a vesicular membrane, bar= 0.3 µm. Photos courtesy of C. Sauer and R. Gross.

Blattabacterium-Endosymbionts of Cockroaches and Termites

Cockroaches (order Blattaria) utilize a complex diet and harbor prokaryotic endosymbionts (Dasch et al., 1984). It has been hypothesized that cockroaches and termites (order Isoptera) are phylogenetically related (Kambhampati, 1995). Common ancestry is suggested from the fact that the wood-eating cockroach, *Cryptocercus punctulatus*, has a cellulose-digesting protozoal gut flora which is similar to that of termites (reviewed in Bandi and Sacchi, 1999). In addition, *Mastotermes darwiniensis*, a primitive termite, lays eggs in rows resembling those made by cockroaches (Borror et al., 1989; Sacchi et al., 1998b).

Phylogeny

The G+C content of the DNA of *Blattabacterium* is 26–28 mol% (Dasch, 1975; Dasch et al., 1984). Phylogenetic analysis of the 16S rDNA from *Blattabacterium* of cockroaches and the termite *M. darwiniensis* indicates that the endosymbionts form a distinct lineage within the *Flavobacter-Bacteroides* group of bacteria (Bandi et al., 1994, 1995; Fig. 1). The phylogenetic tree obtained on the basis of endosymbiont 16S rDNA is the same as that derived from host taxonomy. This result is consistent with a single infection in an ancestor of cockroaches and termites and vertical evolution of the endosymbionts, that is, a lack of endosymbiont exchange among different species. *M. darwiniensis* is the only termite known to harbor *Blattabacterium*, suggesting that, with the exception of this termite, the endosymbionts were eliminated in the lineage leading to the present termite species (Bandi and Sacchi, 1999; Bandi et al., 1997). The 16S-rDNA sequence has been determined for endosymbionts of the cockroach species *Periplaneta australasiae*, *P. americana*, *Blatella germanica*, *Pynoscelus surinamensis*, *Nauphoeta cinerea* and *C. punctulatus*, as well as the termite species *M. darwiniensis* (Bandi et al., 1994, 1995). The association between *Blattabacterium* and termites is estimated to be 135–300 million years old (Bandi et al., 1995).

Taxonomy

The genus *Blattabacterium* contains one species, *B. cuenoti*, and currently designates the lineage consisting of the bacteriocyte-associated endosymbionts of cockroaches and one termite (Bandi et al., 1995; Dasch et al., 1984). The type strain is the endosymbiont of *Blatta orientalis* (Dasch et al., 1984).

Habitat

Blattabacterium is found in bacteriocytes, polyploid cells which are found within the abdominal fat bodies of cockroaches and the termite *M. darwiniensis* (Fig. 12a) (Bigliardi et al., 1995; Sacchi et al., 1996, 1998a, b). The endosymbionts are rods of 1 μm in width and 1.6–9 μm in length (Fig. 12b; Dasch et al., 1984). They have a Gram-negative cell wall and are located within vesicles derived from the host cell. The bacteriocytes originate from plasmatocytes, which are phagocytic cells present in the hemolymph. There are extensive electron microscopic studies that document the infection and differentiation of plasmatocytes into bacteriocytes and the transmission of the endosymbionts to oocytes and eggs (Bigliardi et al., 1995; Sacchi et al., 1996, 1998a, b).

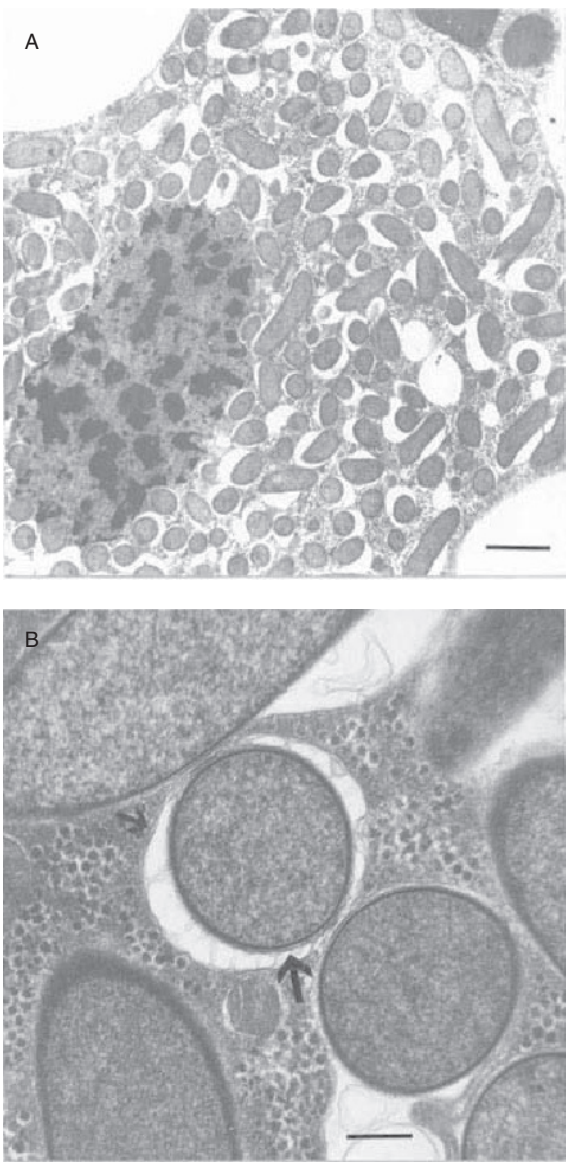

Fig. 12. Electron micrographs of *Blattabacterium cuenoti*, the endosymbiont of cockroaches and termites. (a) Endosymbiont within bacteriocytes of the cockroach *Periplaneta americana*, bar= 3.0 μm (photo courtesy of L. Sacchi); (b) ultrastructure of the endosymbiont of *Cryptocercus punctulatus* showing the Gram-negative cell wall (large arrow) and the vesicular membrane (small arrow), bar= 0.3 μm. From Sacchi et al. (1998) with permission of the authors and Balaban Publishers.

Physiology

Aposymbiotic cockroaches can be obtained by rearing the insects on antibiotic-containing foods (Bandi and Sacchi, 1999; Dasch et al., 1984). The resulting insects can be propagated on enriched diets. The aposymbiotic insects are smaller in size, light in color, and have a reduced fecundity and an increased development time. They also have an increased level of uric acid in fat bodies, suggesting that one function of *Blattabacterium* is nitrogen recycling (Cochoran, 1985). The proximity of bacteriocytes and urate cells (which store uric acid) in the fat body and the presence of adhesion sites between their plasma membranes suggest direct metabolic interactions between these cells (Sacchi et al., 1998a). There is also evidence that the bacteria provide essential amino acids for the host (Henry, 1962).

Isolation

P-endosymbionts have been isolated from aphids and also from *Sitophilus*. In the case of aphids, the starting material is usually whole insects; in the case of *Sitophilus*, the starting material is dissected bacteriomes. Since the two methods are similar, only the method for the isolation of *Buchnera* from aphids will be considered. Details of the isolation of the endosymbionts from *Sitophilus* are described by Heddi et al. (1991).

The methods for the isolation of *Buchnera* have been developed by Ishikawa (1982), Sasaki and Ishikawa (1995), and Charles and Ishikawa (1999). The resulting preparations are suitable for isolation of high molecular weight DNA for genome analysis as well as for physiological studies. The best criteria of purity have involved examination of the preparations by electron microscopy, which also allows determination of whether the endosymbionts are still within host-derived vesicles. Both the aphids and the endosymbionts have a similar mol% G+C in their DNA (Ishikawa, 1987; Unterman and Baumann, 1990). Consequently, endosymbiont DNA

cannot be separated from host DNA by CsCl density gradient centrifugation.

As starting material, it is best to use an aphid strain that has only *Buchnera* and lacks S-endosymbionts. All of the reagents and equipment are kept on ice, and the procedures are performed as rapidly as possible. Approximately 2 to 3 g (wet weight) aphids are transferred to a 1.5 cm diameter tissue grinder. Ten ml of buffer A of Ishikawa (1982) is added, and the aphids are ground with a lose fitting plunger for 5 min. [Buffer A contains 0.25 M sucrose, 35 mM Tris-HCl (pH 7.6), 25 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol.] The preparation is then passed through a double layer of a nylon mesh to remove large particulate material. The filtrate is brought to a volume of about 100 ml with buffer A and then quickly passed through a 100-µm nylon filter followed by filtration through 20-µm and 10-µm nylon filters (in some cases filtration through 5- and 3-µm filters is also performed). Only slight vacuum pressure is applied during the last two filtration steps. The suspension is centrifuged in a swinging bucket rotor for 6–10 min at 1,500 g. The pellets are gently resuspended in 1–2 ml buffer A and centrifuged through a Percoll gradient (12,000 g, 15 min). The gradient consists of 27–70% Percoll in buffer A, 5% PEG 6000, 1% Ficoll, and 1% bovine serum albumin (Pharmacia Biotech, Uppsala, Sweden; Charles and Ishikawa, 1999). Mitochondria are in the upper phase, host nuclei are in the pellet, and *Buchnera* appears as a green band.

Identification

Currently none of the P-endosymbionts has been cultured and consequently identification is based primarily on sequence analysis of their 16S rDNAs. Since these studies are just beginning, we will briefly discuss the methods used for both their characterization and identification. The success of the approach used in most of the studies has been dependent on the use of fresh or frozen insect samples which meet the following criteria: 1) the predominant bacterial flora of the insect consists of one or a few endosymbiont types, 2) there is no significant gut flora, and 3) the samples are relatively clean.

An ideal study would utilize the full-cycle rRNA analysis formulated by Amann et al. (1995). In this approach the 16S rDNA is amplified by PCR and sequenced. Based on comparisons with 16S rDNA(s) in databases, the organism(s) are identified. Specific oligonucleotide probes are designed and used in *in situ* hybridization to identify the endosymbiont associated with the sequence. This approach is extremely useful when more than one endosymbiont is present and, in the case of insects, has been applied to the identification of two types of psyllid endosymbionts (Fukatsu and Nikoh, 1998).

In studies in which the primary goal was a phylogenetic characterization of the P-endosymbiont, one approach used is an initial thorough study of one or more representative insects that can be cultivated or are readily available. Then characterization is extended to taxa obtained as field collections and preserved in dry ice or absolute ethanol. For example, in the initial study of aphid endosymbionts, the insect specimen chosen (Ap) was known from electron microscopy to harbor two morphologically distinct endosymbionts (Unterman et al., 1989). Using total aphid DNA, a probe to *E. coli* 16S rDNA, and restriction enzyme and Southern blot analysis, only two restriction maps could be constructed corresponding to the two endosymbionts. There were differences in the intensities of the bands, indicating that one of the endosymbionts was present in larger numbers than the other. In addition, the results were consistent with the presence of only one copy of the 16S-rRNA gene per endosymbiont genome. Based on the restriction maps, DNA fragments containing 16S-rRNA genes of both endosymbionts were cloned and sequenced. In addition, DNA was isolated from dissected bacteriomes, and restriction enzyme and Southern blot analysis indicated the association of both endosymbionts with the bacteriome. Subsequently, restriction enzyme and Southern blot analysis using DNA from other aphid species indicated the presence of only one copy of the 16S-rRNA gene, a finding consistent with only one or a single predominant endosymbiont (Munson et al., 1991b). Upon amplification of the 16S rDNA by PCR, the sequences detected were all related to *Buchnera*. This approach is suitable for the study of insects in which there is one predominant P-endosymbiont and does not exclude the possibility of not detecting S-endosymbionts that are present in lower numbers.

Instead of initial studies involving restriction enzyme and Southern blot analysis, it is much more convenient to use oligonucleotides complementary to the front and back of 16S rDNA and PCR to amplify DNA fragments for cloning, sequencing, or both. Localization of the endosymbiont may be performed by dissection of different tissues, purification of the DNA, and amplification of the 16S rDNA by PCR (Aksoy et al., 1995; Bandi et al., 1994, 1995; Schröder et al., 1996). Differences in the types of rDNA amplified may be established by restriction fragment polymorphism (Clark et al., 1992; Fukatsu and Nikoh, 1998). Once a pattern of relationship is established this method may be applied using

whole insect DNA. It should be noted that there are potential problems associated with PCR such as selective amplification of some sequences and hybrid formation, which are reviewed by Wintzingerode et al. (1997).

One additional approach to the detection of different kinds of organisms in the DNA samples is to use oligonucleotide primers that are complementary to the front of the 16S-rDNA and the middle of the 23S-rDNA. In bacteria in which the order of genes is 16S-23S (most species), there is a spacer region between the genes the length of which is labile and usually differs among species. If, after PCR, several bands are observed, they probably correspond to different bacteria. This approach has been applied to the cloning of P-endosymbionts of psyllids and to the putative S-endosymbionts which differ in the size of their 16S-23S spacer (Thao, M. L. and Baumann, P., unpublished observations).

Specific oligonucleotide primers also can be made complementary to unique sequences of the endosymbiont 16S and 23S rDNA. This has been done for *Buchnera* 16S rDNA (Rouhbakhsh et al., 1994). Another approach is to take advantage of unique linkage relationships. *Buchnera*-specific probes have been made which span the region *argS-16SrDNA* and *aroE-23SrDNA* (Munson et al., 1993; Rouhbakhsh and Baumann, 1995; Rouhbakhsh et al., 1994). The latter is especially useful, since most bacteria have 16S rDNA directly upstream of 23S rDNA.

Application

Aphids, psyllids, whiteflies, and mealybugs, which utilize plant sap as food, are of major economic importance in that they may cause plant debilitation and the transmission of a variety of plant pathogens (Gray and Banerjee, 1999; Sylvester, 1985). Tsetse flies, which suck blood, are important in the transmission of human and animal disease, especially in tropical regions of Africa (Harwood and James, 1979). Since these organisms are dependent on their P-endosymbionts for survival, an understanding of the genetics and physiology of the endosymbionts may be of use in devising strategies, which are based on interference with their endosymbionts, for the control of these insect pests. An additional approach, which is currently being developed, is the potential use of endosymbionts to produce compounds that would result in modification of the vector competence of an insect. Such an insect would be unable to harbor and/or transmit the disease agent. This approach has been discussed by Beard et al. (1993a) and Durvasula et al. (1997).

Literature Cited

Akhtar, S., and H. F. van Emden. 1994. Ultrastructure of the symbionts and mycetocytes of bird cherry aphid (Rhopalosiphum padi). Tissue Cell 26:513–522.

Aksoy, S. 1995a. Molecular analysis of the endosymbionts of tsetse flies: 16S rDNA locus and over-expression of a chaperonin. Insect Mol. Biol. 4:23–29.

Aksoy, S. 1995b. Wigglesworthia gen. nov. and Wigglesworthia glossinidia sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. Int. J. Syst. Bacteriol. 45:848–851.

Aksoy, S., X. Chen, and V. Hypsa. 1997. Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera: Glossinidae). Insect Mol. Biol. 6:183–190.

Aksoy, S., A. A. Pourhosseini, and A. Chow. 1995. Mycetome endosymbionts of tsetse flies constitute a distinct lineage related to the Enterobacteriaceae. Insect Mol. Biol. 4:15–22.

Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.

Andersson, S. G. E., A. Zomorodipour, J. O. Andersson, T. Sicheritz-Ponten, U. C. M. Alsmark, R. M. Podowski, A. K. Naslund, A. S. Eriksson, H. H. Winkler, and C. G. Kurland. 1998. The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature 396:133–140.

Baker, J. E. 1975. Vitamin requirements of larvae of Sitophilus oryzae. J. Insect Physiol. 21:1337–1342.

Baker, J. E. 1979. Requirements for the essential dietary amino acids of larvae of the rice weevil. Environ. Entomol. 8:451–453.

Baker, J. E., and P. T. M. Lum. 1973. Development of aposymbiosis in larvae of Sitophilus oryzae by dietary treatment with antibiotics. J. Stored Prod. Res. 9:241–245.

Bandi, C., and L. Sacchi. In Press. Intracellular Symbiosis. *In:* T. Abe, M. Higashi, and D. Bignel (eds.) Termites: their symbiosis, behavior, and global diversification. Kluwer. Dordrecht, The Netherlands.

Bandi, C., T. J. C. Anderson, C. Genchi, and M. L. Blaxter. 1998. Phylogeny of Wolbachia in filarial nematodes. Proc. Roy. Soc. Lond. B265:2407–2414.

Bandi, C., M. Sironi, C. A. Nalepa, S. Corona, and L. Sacchi. 1997. Phylogenetically distant intracellular symbionts in termites. Parassitologia 39:71–75.

Bandi, C., G. Damiani, L. Magrassi, A. Grigolo, R. Fani, and L. Sacchi. 1994. Flavobacteria as intracellular symbionts in cockroaches. Proc. Roy. Soc. Lond. B257:43–48.

Bandi, C., J. W. McCall, C. Genchi, S. Corona, L. Venco, and L. Sacchi. 1999. Effects of tetracycline on the filarial worms Brugia pahangi and Dirofilaria immitis and their bacterial endosymbionts Wolbachia. Int. J. Parasitol. 29:357–364.

Bandi, C., M. Sironi, G. Damiani, L. Magrassi, C. A. Nalepa, U. Laudani, and L. Sacchi. 1995. The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. Proc. Roy. Soc. Lond. B259:293–299.

Barancin, C. E., J. C. Smoot, R. H. Findlay, and L. A. Actis. 1998. Plasmid-mediated histamine biosynthesis in the bacterial fish pathogen Vibrio anguillarum. Plasmid 39:235–244.

Baumann, L., and P. Baumann. 1994. Growth kinetics of the endosymbiont Buchnera aphidicola in the aphid Schizaphis graminum. Appl. Environ. Microbiol. 60:3440–3443.

Baumann, L., and P. Baumann. 1998. Characterization of ftsZ, the cell division gene of Buchnera aphidicola (endosymbiont of aphids) and detection of the product. Curr. Microbiol. 36:85–89.

Baumann, P., and N. A. Moran. 1997. Non-cultivable microorganisms from symbiotic associations of insects and other hosts. Antonie van Leeuwenhoek 72:39–48.

Baumann, P., L. Baumann, and M. A. Clark. 1996. Levels of Buchnera aphidicola chaperonin GroEL during growth of the aphid Schizaphis graminum. Curr. Microbiol. 32:279–285.

Baumann, L., P. Baumann, and N. A. Moran. 1998a. The endosymbiont (Buchnera) of the aphid Diuraphis noxia contains all the genes of the tryptophan biosynthetic pathway. Curr. Microbiol. 37:58–59.

Baumann, P., L. Baumann, M. A. Clark, and M. L. Thao. 1998b. Buchnera aphidicola: the endosymbiont of aphids. ASM News 64:203–209.

Baumann, L., P. Baumann, and M. L. Thao. 1999a. Detection of messenger RNA transcribed from genes encoding enzymes of amino acid biosynthesis in Buchnera aphidicola (endosymbiont of aphids). Curr. Microbiol. 38:135–136.

Baumann, L., P. Baumann, M. A. Moran, J. Sandström, and M. L. Thao. 1999b. Genetic characterization of plasmids containing genes encoding enzymes of leucine biosynthesis in endosymbionts (Buchnera) of aphids. J. Mol. Evol. 48:77–85.

Baumann, P., N. A. Moran, and L. Baumann. 1997a. The evolution and genetics of aphid endosymbionts. Bioscience 47:12–20.

Baumann, L., M. A. Clark, D. Rouhbakhsh, P. Baumann, N. A. Moran, and D. J. Voegtlin. 1997b. Endosymbionts (Buchnera) of the aphid Uroleucon sonchi contain plasmids with trpEG and remnants of trpE pseudogenes. Curr. Microbiol. 35:18–21.

Baumann, P., L. Baumann, C. Y. Lai, D. Roubakhsh, N. A. Moran, and M. A. Clark. 1995. Genetics, physiology, and evolutionary relationships of the genus Buchnera: Intracellular symbionts of aphids. Annu. Rev. Microbiol. 49:55–94.

Beard, C. B., S. L. O'Neill, R. B. Tesh, F. F. Richards, and S. Aksoy. 1993a. Modification of arthropod vector competence via symbiotic bacteria. Parasitol. Today 9:179–183.

Beard, C. B., S. K. O'Neill, P. Mason, L. Mandelco, C. R. Woese, R. B. Tesh, F. F. Richards, and S. Aksoy. 1993b. Genetic transformation and phylogeny of bacterial symbionts from tsetse. Insect Mol. Biol. 1:123–131.

Bensaadi-Merchermek, N., J. C. Salvado, C. Cagnon, S. Karama, and C. Mouches. 1995. Characterization of the unlinked 16S rDNA and 23S-5S rRNA operon of Wolbachia pipientis, a prokaryotic parasite of insect gonads. Gene 165:81–86.

Berlyn, M. K. B. 1998. Linkage map of Escherichia coli K-12, edition 10: the traditional map. Microbiol. Mol. Biol. Rev. 62:814–984.

Bigliardi, E., M. G. Selmi, S. Corona, C. A. Bandi, and L. Sacchi. 1995. Membrane systems in endocytobiosis. III. Ultrastructural features of symbionts and vacuolar membrane in bacteriocytes of the wood-eating cockroach Cryptocercus punctulatus (Dictyoptera, Cryptocercidae). Boll. Zool. 62:235–238.

Blackman, R. L. 1984. Reproduction, cytogenetics and development. A. K. Minks and P. HarrewijnAphids: their biology, natural enemies and control. Elsevier Biomedical Press, Amsterdam 2A:163–195.

Blackman, R. L., and V. F. Eastop. 1984. Aphids on the world's crops. Wiley. Chichester, UK.

Blattner, F. R., G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. ColladoVides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of Escherichia coli K-12. Science 277:1453–1462.

Blua, M. J., T. M. Perring, and M. A. Madore. 1994. Plant virus-induced changes in aphid population development and temporal fluctuations in plant nutrients. J. Chem. Ecol. 20:691–707.

Boman, H. G., and D. Hultmark. 1987. Cell-free immunity in insects. Annu. Rev. Microbiol. 41:103–126.

Borror, D. J., C. A. Triplehorn, and N. F. Johnson. 1989. An Introduction to the Study of Insects. Harcourt Brace College Publishers. Fort Worth, TX.

Bracho, A. M., D. Martínez-Torres, A. Moya, and A. Latorre. 1995. Discovery and molecular characterization of a plasmid localized in Buchnera sp., bacterial endosymbiont of the aphid Rhopalosiphum padi. J. Mol. Evol. 41:67–73.

Brenner, D. J. 1984. Enterobacteriaceae. N. R. Krieg and J. G. Holt. Bergey's manual of systematic bacteriology. Williams and Wilkins Co.. Baltimore, MD. 1:408–506.

Brough, C. N., and A. F. G. Dixon. 1990. Ultrastructual features of egg development in oviparae of vetch aphid, Megura viciae. Tissue Cell 22:51–63.

Brown, J. K., D. R. Frohlich, and R. C. Rosell. 1995. The sweetpotato or silverleaf whiteflies - biotypes of Bemisia tabaci or a species complex. Annu. Rev. Entomol. 40:511–534.

Brynnel, E. U., C. G. Kurland, N. A. Moran, and S. G. E. Andersson. 1998. Evolutionary rates for tuf genes in endosymbionts of aphids. Mol. Biol. Evol. 15:574–582.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers, Inc. New York.

Campbell, B. C., T. S. Bragg, and C. E. Turner. 1992. Phylogeny of symbiotic bacteria of four weevil species (Coleoptera, Curculionidae) based on analysis of 16S ribosomal DNA. Insect Biochem. Mol. Biol. 22:415–421.

Campbell, B. C., J. D. Stefen-Campbell, and R. J. Gill. 1994. Evolutionary origin of whiteflies (Hemiptera: Sternorrhyncha: Aleyrodidae) inferred from 18S rDNA sequences. Insect Mol. Biol. 3:73–88.

Chang, K. P., and A. J. Musgrave. 1969. Histochemistry and ultrastructure of the mycetome and its "symbiotes" in the pear psylla, Psylla pyricola Foerster (Homoptera). Tissue Cell 1:597–606.

Charles, H., and H. Ishikawa. 1999. Physical and genetic map of the genome of Buchnera, the primary endosymbiont of the pea aphid Acyrthosiphon pisum. J. Mol. Evol. 48:142–150.

Charles, H., H. Ishikawa, and P. Nardon. 1995. Presence of a protein specific of endocytobiosis (symbionin) in the weevil Sitophilus. C. R. Acad. Sci. Paris (ser. III) 318:35–41.

Charles, H., G. Condemine, C. Nardon, and P. Nardon. 1997a. Genome size characterization of the principal endocellular symbiotic bacteria of the weevil Sitophilus oryzae, using pulsed field gel electrophoresis. Insect Biochem. Mol. Biol. 27:345–350.

Charles, H., A. Heddi, J. Guillaud, C. Nardon, and P. Nardon. 1997b. A molecular aspect of symbiotic interactions between the weevil Sitophilus oryzae and its endosym-

biotic bacteria: over-expression of a chaperonin. Biochem. Biophys. Res. Com. 239:769–774.

Chen, D.-Q., and A. H. Purcell. 1997. Occurence and transmission of facultative endosymbionts in aphids. Curr. Microbiol. 34:220–225.

Chen, D. Q., B. C. Campbell, and A. H. Purcell. 1996. A new rickettsia from a herbivorous insect, the pea aphid Acyrthosiphon pisum (Harris). Curr. Microbiol. 33:123–128.

Chen, X. A., S. Li, and S. Aksoy. 1999. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus Glossina and its bacteriome-associated endosymbiont, Wigglesworthia glossinidia. J. Mol. Evol. 48:49–58.

Cheng, Q., and S. Aksoy. 1999. Tissue tropism, transmission and expression of foreign genes in vivo in midgut symbionts of tsetse flies. Insect Mol. Biol. 8:125–132.

Clark, M. A., L. Baumann, and P. Baumann. 1998a. Sequence analysis of a 34.7-kb DNA segment from the genome of Buchnera aphidicola (endosymbiont of aphids) containing groEL, dnaA, the atp operon, gidA, and rho. Curr. Microbiol. 36:158–163.

Clark, M. A., L. Baumann, and P. Baumann. 1998b. Buchnera aphidicola (aphid endosymbiont) contains genes encoding enzymes of histidine biosynthesis. Curr. Microbiol. 37:356–358.

Clark, M. A., P. Baumann, and M. A. Moran. 1999a. Buchnera plasmid-associated trpEG probably originated from a chromosomal location between hslU and fpr. Curr. Microbiol. 38:309–311.

Clark, M. A., N. A. Moran, and P. Baumann. 1999b. Sequence evolution in bacterial endosymbionts having extreme base compositions. Mol. Biol. Evol. 16:1586–1598.

Clark, M. A., N. A. Moran, and P. Baumann. 2000. Cospeciation between bacterial endosymbionts (Buchnera) and a recent radiation of aphids (Uroleucon). Evolution In Press: In Press–In Press.

Clark, M. A., L. Baumann, P. Baumann, and D. Rouhbakhsh. 1996. Ribosomal protein S1(RpsA) of Buchnera aphidicola, the endosymbiont of aphids: characterization of the gene and detection of the product. Curr. Microbiol. 32:89–94.

Clark, M. A., L. Baumann, M. A. Munson, P. Baumann, B. C. Campbell, J. E. Duffus, L. S. Osborne, and N. A. Moran. 1992. The eubacterial endosymbionts of whiteflies (Homoptera, Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. Curr. Microbiol. 25:119–123.

Cochoran, D. G. 1985. Nitrogen excretion in cockroaches. Annu. Rev. Entomol. 30:29–49.

Costa, H. S., T. J. Henneberry, and N. C. Toscano. 1997. Effects of antibacterial materials on Bemisia argentifolii (Homoptera: Aleyrodidae) oviposition, growth, survival, and sex ratio. J. Econ. Entomol. 90:333–339.

Costa, H. S., N. C. Toscano, and T. J. Henneberry. 1996. Mycetocyte inclusion in the oocytes of Bemisia argentifolii (Homoptera, Aleyrodidae). Ann. Entomol. Soc. Amer. 89:694–699.

Costa, H. S., D. E. Ullman, M. W. Johnson, and B. E. Tabashnik. 1993a. Antibiotic oxytetracycline interferes with Bemisia tabaci (Homoptera, Aleyrodidae) oviposition, development, and ability to induce squash silverleaf. Ann. Entomol. Soc. Amer. 86:740–748.

Costa, H. S., D. M. Westcot, D. E. Ullman, and M. W. Johnson. 1993b. Ultrastructure of the endosymbionts of the whitefly, Bemisia tabaci and Trialeurodes vaporariorum. Protoplasma 176:106–115.

Costa, H. S., D. M. Westcot, D. E. Ullman, R. Rosell, J. K. Brown, and M. W. Johnson. 1995. Morphological variation in Bemisia endosymbionts. Protoplasma 189:194–202.

Crawford, I. P. 1989. Evolution of a biosynthetic pathway—the tryptophan paradigm. Annu. Rev. Microbiol. 43: 567–600.

Dadd, R. H. 1985. Nutrition: organisms. G. A. Kerkut and L. I. Gilbert (eds.) Comprehensive insect physiology, biochemistry, and pharmacology. Pergamon Press, Inc.. Elmsford, NY. 4:315–319.

Dale, C., and I. Maudlin. 1999. Sodalis gen. nov. and Sodalis glossinidius sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly Glossina morsitans morsitans. Int. J. Syst. Bacteriol. 49:267–275.

Dasch, G. A. 1975. Morphological and molecular studies on intracellular bacterial symbiotes of insects.. Yale University. New Haven.

Dasch, G. A., E. Weiss, and K.-P. Chang. 1984. Endosymbionts of insects. N. R. Krieg and J. G. Holt (eds.) Bergey's manual of systematic bacteriology. Williams and Wilkins Co. Baltimore, 1:811–833.

Denk, D., and A. Böck. 1987. L-Cysteine biosynthesis in Escherichia coli: nucleotide sequence and expression of the serine acetyltransferase (cysE) gene from wild-type and a cysteine-excreting mutant. J. Gen. Microbiol. 133:515–525.

Dixon, A. F. G. 1973. Biology of aphids. Edward Arnold, Ltd. London.

Dixon, A. F. G. 1992. Constraints on the rate of parthenogenetic reproduction and pest status of aphid. Invertebr. Rep. Devel. 22:159–163.

Douglas, A. E. 1988. Sulfate utilization in an aphid symbiosis. Insect Biochem. 18:159–163.

Douglas, A. E. 1989. Mycetocyte symbiosis in insects. Biol. Rev. Camb. 64:409–434.

Douglas, A. E. 1990. Nutritional interactions between Myzus persicae and its symbionts. R. K. Campbell and R. D. Eikenbary (eds.) Aphid-plant genotype interactions. Elsevier Biomedical Press. Amsterdam, 319–327.

Douglas, A. E. 1997. Parallels and contrasts between symbiotic bacteria and bacterial-derived organelles: evidence from Buchnera, the bacterial symbiont of aphids. FEMS Microbiol. Ecol. 24:1–9.

Douglas, A. E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. Annu. Rev. Entomol. 43:17–37.

Douglas, A. E., and A. F. G. Dixon. 1987. The mycetocyte symbiosis of aphids: variation with age and morph in virginoparae of Megoura viciae and Acyrthosiphon pisum. J. Insect Physiol. 33:109–113.

Douglas, A. E., and W. A. Prosser. 1992. Synthesis of the essential amino acid tryptophan in the pea aphid (Acyrthosiphon pisum) symbiosis. J. Insect Physiol. 38:565–568.

Douglas, A. E., and D. C. Smith. 1989. Are endosymbioses mutualistic? Trends Ecol. Evol. 4:350–352.

Durvasula, R. V., A. Gumbs, A. Panackal, O. Kruglov, S. Aksoy, R. B. Merrifield, F. F. Richards, and C. B. Beard. 1997. Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc. Natl. Acad. Sci. USA 94:3274–3278.

Eisen, J. A. 1995. The RecA Protein as a model molecule for molecular systematic studies of bacteria—comparison of

trees of RecAs and 16S rRNAs from the same species. J. Mol. Evol. 41:1105–1123.

Eremeeva, M. E., W. M. Ching, Y. L. Wu, D. J. Silverman, and G. A. Dasch. 1998. Western blotting analysis of heat shock proteins of Rickettsiales and other eubacteria. FEMS Microbiol. Lett. 167:229–237.

Faye, I. 1978. Insect immunity: early fate of bacteria injected in a saturniid pupae. J. Invertebr. Pathol. 31:19–26.

Filichkin, S. A., S. Brumfield, T. P. Filichkin, and M. J. Young. 1997. In vitro interactions of the aphid endosymbiotic SymL chaperonin with barley yellow dwarf virus. J. Virol. 71:569–577.

Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. Fitzhugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter. 1995. Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science 269:469–512.

Forrest, J. M. S. 1987. Galling aphids. A. K. Minks and P. Harrewijn Aphids: their biology, natural enemies and control. Elsevier. Amsterdam, 2A:341–353.

Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, J. L. Fritchman, J. F. Weidman, K. V. Small, M. Sandusky, J. Fuhrmann, D. Nguyen, T. R. Utterback, D. M. Saudek, C. A. Phillips, J. M. Merrick, J. F. Tomb, B. A. Dougherty, K. F. Bott, P. C. Hu, T. S. Lucier, S. N. Peterson, H. O. Smith, C. A. Hutchison, and J. C. Venter. 1995. The minimal gene complement of Mycoplasma genitalium. Science 270:397–403.

Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. vanVugt, N. Palmer, M. D. Adams, J. Gocayne, J. Weidman, T. Utterback, L. Watthey, L. McDonald, P. Artiach, C. Bowman, S. Garland, C. Fujii, M. D. Cotton, K. Horst, K. Roberts, B. Hatch, H. O. Smith, and J. C. Venter. 1997. Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. Nature 390:580–586.

Fukatsu, T., and H. Ishikawa. 1992a. A novel eukaryotic extracellular symbiont in an aphid, Astegopteryx styraci (Homoptera, Aphididae, Hormaphidinae). J. Insect Physiol. 38:765–773.

Fukatsu, T., and H. Ishikawa. 1992b. Soldier and male of an eusocial aphid Colophina arma lack endosymbiont: implications for physiological and evolutionary interaction between host and symbiont. J. Insect Physiol. 38:1033–1042.

Fukatsu, T., and H. Ishikawa. 1992c. Synthesis and localization of symbionin, an aphid endosymbiont protein. Insect Biochem. Mol. Biol. 22:167–174.

Fukatsu, T., and H. Ishikawa. 1993. Occurrence of chaperonin-60 and chaperonin-10 in primary and secondary bacterial symbionts of aphids—implications for the evolution of an endosymbiotic system in aphids. J. Mol. Evol. 36:568–577.

Fukatsu, T., and H. Ishikawa. 1996. Phylogenetic position of yeast-like symbiont of Hamiltonaphis styraci (Homoptera, Aphididae) based on 18S rDNA sequence. Insect Biochem. Mol. Biol. 26:383–388.

Fukatsu, T., and H. Ishikawa. 1998. Differential immunohistochemical visualization of the primary and secondary intracellular symbiotic bacteria of aphids. Appl. Entomol. Zool. 33:321–326.

Fukatsu, T., and N. Nikoh. 1998. Two intracellular symbiotic bacteria from the mulberry psyllid Anomoneura mori (Insecta, Homoptera). Appl. Environ. Microbiol. 64:3599–3606.

Fukatsu, T., K. Watanabe, and Y. Sekiguchi. 1998. Specific detection of intracellular symbiotic bacteria of aphids by oligonucleotide-probed in situ hybridization. Appl. Entomol. Zool. 33:461–472.

Fukatsu, T., S. Aoki, U. Kurosu, and H. Ishikawa. 1994. Phylogeny of Cerataphidini aphids revealed by their symbiotic microorganisms and basic structure of their galls: implications for host-symbiont coevolution and evolution of sterile soldier castes. Zool. Sci. 11:613–623.

Gasnier-Fauchet, F., and P. Nardon. 1986. Comparison of methionine metabolism in symbiotic and aposymbiotic larvae of Sitophilus oryzae L. (Coleopetera: curculionidae) II. Involvement of the symbiotic bacteria in the oxidation of methionine. Comp. Biochem. Physiol. 85B:251–254.

Gasnier-Fauchet, F., A. Gharib, and P. Nardon. 1986. Comparison of methionine metabolism in symbiotic and aposymbiotic larvae of Sitophilus oryzae L. (Coleopetera: Curculionidae) I. Evidence for a glycine N-methyltransferase-like activity in the aposymbiotic larvae. Comp. Biochem. Physiol. 85B:245–250.

Gray, S. M., and N. Banerjee. 1999. Mechanisms of arthropod transmission of plant and animal viruses. Microbiol. Mol. Biol. Rev. 63:128–148.

Grenier, A. M., C. Nardon, and Y. Rahbe. 1994. Observations on the micro-organisms occurring in the gut of the pea aphid Acyrthosiphon pisum. Entomol. Exp. Appli. 70:91–96.

Griffiths, G. W., and S. D. Beck. 1973. Intracellular symbiotes of the pea aphid, Acyrthosiphon pisum. J. Insect Physiol. 19:75–84.

Griffiths, G. W., and S. D. Beck. 1974. Effect of antibiotics on intracellular symbiotes in the pea aphid, Acyrthosiphon pisum. Cell Tissue Res. 148:287–300.

Grinyer, I., and A. J. Musgrave. 1966. Ultrastructure and peripheral membranes of the mycetomal microorganism of Sitophilus granarius (L. coleoptera). J. Cell Sci. 1:181–186.

Gross, C. A. 1996. Function and regulation of heat shock proteins. F. C. Neidhard (ed.) Escherichia coli and Salmonella. ASM Press. Washington, DC, 1:1382–1399.

Gross, R., and R. Rappuoli. 1988. Positive regulation of pertussis toxin expression. Proc. Nat. Acad. Sci. USA 85:3913–3917.

Hara, E., and H. Ishikawa. 1990. Purification and partial characterization of symbionin, an aphid endosymbiont-specific protein. Insect Biochem. 20:421–427.

Harada, H., and H. Ishikawa. 1993. Gut microbe of aphid closely related to its intracellular symbiont. Biosystems 31:185–191.

Harada, H., H. Oyaizu, and H. Ishikawa. 1996. A consideration about the origin of aphid intracellular symbiont in connection with gut bacterial flora. J. Gen. Appl. Microbiol. 42:17–26.

Harrison, C. P., A. E. Douglas, and A. F. G. Dixon. 1989. A rapid method to isolate symbiotic bacteria from aphids. J. Invertebr. Pathol. 53:427–428.

Harwood, R. F., and M. T. James. 1979. Entomology in human and animal health. Macmillan Publishing Co. New York.

Hassan, A. K. M., S. Moriya, P. Baumann, H. Yoshikawa, and N. Ogasawara. 1996. Structure of the dnaA region of the endosymbiont, Buchnera aphidicola, of the aphid Schizaphis graminum. DNA Res. 3:415–419.

Heddi, A., F. Lefebvre, and P. Nardon. 1991. The influence of symbiosis on the respiratory control ratio (RCR) and the ADP/O Ratio in the adult weevil-Sitophilus oryzae (Coleoptera, Curculionidae). Endocytobiosis Cell Res. 8:61–73.

Heddi, A., F. Lefebvre, and P. Nardon. 1993. Effect of endocytobiotic bacteria on mitochondrial enzymatic activities in the weevil Sitophilus oryzae (Coleoptera, Curculionidae). Insect Biochem. Mol. Biol. 23:403–411.

Heddi, A., H. Charles, C. Khatchadourian, G. Bonnot, and P. Nardon. 1998. Molecular characterization of the principal symbiotic bacteria of the weevil Sitophilus oryzae: a peculiar G—C content of an endocytobiotic DNA. J. Mol. Evol. 47:52–61.

Henry, S. M. 1962. The significance of microorganisms in the nutrition of insects. Trans. N. Y. Acad. Sci. 24:676–683.

Hinde, R. 1971a. The control of mycetome symbiotes of the aphids Brevicoryne brassicae, Myzus persicae, and Macrosiphon rosae. J. Insect Physiol. 17:1971–1800.

Hinde, R. 1971b. The fine structure of mycetome symbiotes of the aphids Brevicoryne brassicae, Myzus persicae, and Macrosiphum rosae. J. Insect Physiol. 17:2035–2050.

Hogenhout, S. A., F. van derWilk, M. Verbeek, R. W. Goldbach, and J. F. J. M. van den Heuvel. 1998. Potato leafroll virus binds to the equatorial domain of the aphid endosymbiotic GroEL homolog. J. Virol. 72:358–365.

Houk, E. J., and G. W. Griffiths. 1980. Intracellular symbiotes of the Homoptera. Annu. Rev. Entomol. 25:161–187.

Houk, E. J., G. W. Griffiths, N. E. Hadjokas, and S. D. Beck. 1977. Peptidoglycan in the cell wall of the primary intracellular symbiote of the pea aphid. Science 198:401–403.

Humphreys, N. J., and A. E. Douglas. 1997. Partitioning of symbiotic bacteria between generations of insect: a quantitative study of a Buchnera sp. in the pea aphid (Acyrthosiphon pisum) reared at different temperatures. Appl. Environ. Microbiol. 63:3294–3296.

Iaccarino, F. M., and E. Tremblay. 1973. Comparazione ultrastrutturale della disimbiosi di Macrosiphum rosae (L.) e Dactynotus jaceae (L.) (Homoptera, Aphididae). Boll. Lab. Entomol. Ag. Filipo Silvestri 30:319–335.

Ishikawa, H. 1982. Isolation of the intracellular symbionts and partial characterizations of their RNA species of the elder aphid, Acyrthosiphon magnoliae. Comp. Biochem. Physiol. 72B:239–247.

Ishikawa, H. 1987. Nucleotide composition and kinetic complexity of the genomic DNA of an intracellular symbiont in the pea aphid Acyrthosiphon pisum. J. Mol. Evol. 24:205–211.

Kakeda, K., and H. Ishikawa. 1991. Molecular chaperon produced by an intracellular symbiont. J. Biochem. 110:583–587.

Kambhampati, S. 1995. A phylogeny of cockroaches and related insects based on DNA sequence of mitochondrial ribosomal RNA genes. Proc. Natl. Acad. Sci. USA 92:2017–2020.

Katsumata, R., and M. Ikeda. 1993. Hyperproduction of tryptophan in Corynebacterium glutamicum by pathway engineering. Biotechnology 11:921–925.

Klotz, M. G., and J. M. Norton. 1998. Multiple copies of ammonia monooxygenase (amo) operons have evolved under biased AT/GC mutational pressure in ammonia-oxidizing autotrophic bacteria. FEMS Microbiol. Lett. 168:303–311.

Komaki, K., and H. Ishikawa. 1999. Intracellular symbionts of aphids are bacteria with numerous genomic copies. J. Mol. Evol. 48:717–722.

Kreditch, N. M. 1996. Biosynthesis of cysteine. F. C. Neidhardt (ed.) Escherichia coli and Salmonella. ASM Press. Washington, DC, 1:514–527.

Kusano, T., T. Takeshima, C. Inoue, and K. Sugawara. 1991. Evidence for two sets of structural genes coding for ribulose bisphosphate carboxylase in Thiobacillus ferrooxidans. J. Bacteriol. 173:7313–7323.

Lai, C. Y., L. Baumann, and P. Baumann. 1994. Amplification of trpEG: adaptation of Buchnera aphidicola to an endosymbiotic association with aphids. Proc. Natl. Acad. Sci. USA 91:3819–3823.

Lai, C.-Y., P. Baumann, and N. A. Moran. 1995. Genetics of the tryptophan biosynthetic pathway of the prokaryotic endosymbiont (Buchnera) of the aphid Schlechtendalia chinensis. Insect Mol. Biol. 4:47–59.

Lai, C. Y., and P. Baumann. 1992a. Genetic analysis of an aphid endosymbiont DNA fragment homologous to the rnpA-rpmH-dnaA-dnaN-gyrB region of eubacteria. Gene 113:175–181.

Lai, C. Y., and P. Baumann. 1992b. Sequence analysis of a DNA fragment from Buchnera aphidicola (an endosymbiont of aphids) containing genes homologous to dnaG, rpoD, cysE andsecB. Gene 119:113–118.

Lai, C. Y., P. Baumann, and N. Moran. 1996. The endosymbiont (Buchnera sp.) of the aphid Diuraphis noxia contains plasmids consisting of trpEG and tandem repeats of trpEG pseudogenes. Appl. Environ. Microbiol. 62:332–339.

Lambert, J. D., and N. A. Moran. 1998. Deleterious mutations destabilize ribosomal RNA in endosymbiotic bacteria. Proc. Natl. Acad. Sci. USA 95:4458–4462.

Liadouze, I., G. Febvay, J. Guillaud, and G. Bonnot. 1996. Metabolic fate of energetic amino acids in the aposymbiotic pea aphid Acyrthosiphon pisum (Harris) (Homoptera, Aphididae). Symbiosis 21:115–127.

Lipsitch, M., M. A. Nowak, D. Ebert, and R. M. May. 1995. The population dynamics of vertically and horizontally transmitted parasites. Proc. Roy. Soc. Lond. B 260:321–327.

Llanes, C., P. Gabant, M. Couturier, L. Bayer, and P. Plesiat. 1996. Molecular analysis of the replication elements of the broad-host-range RepA/C replicon. Plasmid 36:26–35.

Margolis, N., D. Hogan, K. Tilly, and P. A. Rosa. 1994. Plasmid location of Borrelia purine biosynthesis gene homologs. J. Bacteriol. 176:6427–6432.

Maynard Smith, J., and E. Szathmáry. 1995. The major transitions in evolution. W. H. Freeman Spektrum. Oxford, New York.

McLean, D. L., and E. J. Houk. 1973. Phase contrast and electron microscopy pf the mycetocytes and symbiotes of the pea aphid, Acyrthosiphon pisum. J. Insect Physiol. 19:625–633.

McMillan, D. J., M. Mau, and M. J. Walker. 1998. Characterisation of the urease gene cluster in Bordetella bronchiseptica. Gene 208:243–251.

Messer, W., and C. Weigl. 1996. Initiation of chromosome replication. F. C. Neidhardt (ed.) Escherichia coli and Salmonella. ASM Press. Washington, DC, 2:1579–1601.

Moran, N. A. 1996. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc. Natl. Acad. Sci. USA 93:2873–2878.

Moran, N., and P. Baumann. 1994. Phylogenetics of cytoplasmically inherited microorganisms of arthropods. Trends Ecol. Evol. 9:15–20.

Moran, N. A., M. E. Kaplan, M. J. Gelsey, T. G. Murphy, and E. A. Scholes. 1999. Phylogeny and evolution of the aphid genus Uroleucon based on nuclear and mitochondrial DNA sequences. System. Entomol. 24:85–93.

Moran, N. A., M. A. Munson, P. Baumann, and H. Ishikawa. 1993. A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc. Roy. Soc. Lond. B253:167–171.

Moran, N. A., and A. Telang. 1998. Bacteriocyte-associated symbionts of insects: a variety of insect groups harbor ancient prokaryotic endosymbionts. Bioscience 48:295–304.

Moran, N. A., C. D. von Dohlen, and P. Baumann. 1995. Faster evolutionary rates in endosymbiotic bacteria than in cospeciating insect hosts. J. Mol. Evol. 41:727–731.

Munson, M. A., L. Baumann, and P. Baumann. 1993. Buchnera aphidicola (a prokaryotic endosymbiont of aphids) contains a putative 16S rRNA operon unlinked to the 23S rRNA-encoding gene: sequence determination, and promoter and terminator analysis. Gene 137:171–178.

Munson, M. A., P. Baumann, and M. G. Kinsey. 1991a. Buchnera gen. nov. and Buchnera aphidicola sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. Int. J. Syst. Bacteriol. 41:566–568.

Munson, M. A., P. Baumann, M. A. Clark, L. Baumann, N. A. Moran, D. J. Voegtlin, and B. C. Campbell. 1991b. Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. J. Bacteriol. 173:6321–6324.

Munson, M. A., P. Baumannn, and M. A. Moran. 1992. Phylogenetic relationships of the endosymbionts of mealybugs (Homoptera:Pseudococcidae) based on 16S rDNA sequences. Mol. Phylogen. Evol. 1:26–30.

Musgrave, A. J., and I. Grinyer. 1968. Membranes associated with the disintegration of mycetomal micro-organisms in Sitophilus zea-mais (Mots. Coleoptera). J. Cell Sci. 3:65–70.

Nakabachi, A., and H. Ishikawa. 1997. Differential display of mRNAs related to amino acid metabolism in the endosymbiotic system of aphids. Insect Biochem. Mol. Biol. 27:1057–1062.

Nakabachi, A., and H. Ishikawa. 1999. Provision of riboflavin to the host aphid, Acyrthosiphon pisum, by endosymbiotic bacteria, Buchnera. J. Insect Physiol. 45:1–6.

Nardon, P., and A. M. Grenier. 1988. Genetical and biochemical interactions between the host and its endocytobiotes in the weevil Sitophilus (Coleoptere, Curculionidae) and other related species. S. ScanneriniCell to cell signals in plant, animal and microbial symbiosis. Springer Verlag. Heidelberg, 255–270.

Nicholson, M. L., M. Gaasenbeek, and D. E. Laudenbach. 1995. Two enzymes together capable of cysteine biosynthesis are encoded on a cyanobacterial plasmid. Mol. Gen. Genet. 247:623–632.

Nogge, G. 1976. Aposymbiotic tsetse flies, Glossina morsitans morsitans obtained by feeding adults on rabbits immunized specificaly with symbionts. J. Insect Physiol. 24:299–304.

Nogge, G. 1982. Significance of symbionts for the maintenance of an optimal nutritional state of successful reproduction in hematophagous arthropods. Parasitology 82:299–304.

Norton, J. M., J. M. Low, and G. Martin. 1996. The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in Nitrosospira sp. NpAV. FEMS Microbiol. Lett. 139:181–188.

O'Neill, A., A. Hoffman, and J. H. Werren. 1997. Influential passengers; inherited microorganisms and arthropod reproduction. Oxford University Press. Oxford.

Ohtaka, C., H. Nakamura, and H. Ishikawa. 1992. Structures of chaperonins from an intracellular symbiont and their functional expression in Escherichia coli groE mutants. J. Bacteriol. 174:1869–1874.

Patten, C. L., and B. R. Glick. 1996. Bacterial biosynthesis of indole-3-acetic acid. Can. J. Microbiol. 42:207–220.

Pennisi, E. 1998. Evolution—heat shock protein mutes genetic changes. Science 282:1796–1796.

Remaudié, G., and M. Remaudi 1997. Catalogue des aphididae du monde. Institut National de la Recherche Agronomique. Paris.

Riley, M., and B. Labedan. 1996. Escherichia coli gene products: physiological functions and common ancestries. F. C. Neidhardt (ed.) Escherichia coli and Salmonella. ASM Press. Washington, DC, 2:2118–2202.

Romero, D., and R. Palacios. 1997. Gene amplification and genomic plasticity in prokaryotes. Annu. Rev. Gen. 31:91–111.

Roth, J. R., N. Benson, T. Galitski, K. Haack, J. G. Lawrence, and L. Miesel. 1996. Rearrangements of the bacterial chromosome: formation and applications. F. C. Neidhardt (ed.) Escherichia coli and Salmonella. ASM Press. Washington, DC, 2:2256–2276.

Rouhbakhsh, D., and P. Baumann. 1995. Characterization of a putative 23S-5S rRNA operon of Buchnera aphidicola (endosymbiont of aphids) unlinked to the 16S rRNA-encoding gene. Gene 155:107–112.

Rouhbakhsh, D., M. A. Clark, L. Baumann, N. A. Moran, and P. Baumann. 1997. Evolution of the tryptophan biosynthetic pathway in Buchnera (aphid endosymbionts): studies of plasmid-associated trpEG within the genus Uroleucon. Mol. Phylogen. Evol. 8:167–176.

Rouhbakhsh, D., N. A. Moran, L. Baumann, D. J. Voegtlin, and P. Baumann. 1994. Detection of Buchnera, the primary prokaryotic endosymbiont of aphids, using the polymerase chain reaction. Insect Mol. Biol. 3:213–217.

Rouhbakhsh, D., C. Y. Lai, C. D. von Dohlen, M. A. Clark, L. Baumann, P. Baumann, N. A. Moran, and D. J. Voegtlin. 1996. The tryptophan biosynthetic pathway of aphid endosymbionts (Buchnera): genetics and evolution of plasmid-associated anthranilate synthase (trpEG) within the Aphididae. J. Mol. Evol. 42:414–421.

Rutherford, S. L., and S. Lindquist. 1998. Hsp90 as a capacitor for morphological evolution. Nature 396:336–342.

Sacchi, L., S. Corona, A. Grigolo, U. Laudani, M. G. Selmi, and E. Bigliardi. 1996. The fate of the endocytobionts of Blattella germanica (Blattaria, Blattellidae) and Periplaneta americana (Blattaria, Blattidae) during embryo development. Ital. J. Zool. 63:1–11.

Sacchi, L., C. A. Nalepa, E. Bigliardi, S. Corona, A. Grigolo, U. Laudani, and C. Bandi. 1998a. Ultrastructural studies of the fat body and bacterial endosymbionts of Cryptocercus punctulatus Scudder (Blattaria: Cryptocercidae). Symbiosis 25:251–269.

Sacchi, L., C. A. Nalepa, E. Bigliardi, M. Lenz, C. Bandi, S. Corona, A. Grigolo, S. Lambiase, and U. Laudani. 1998b.

Some aspecys of intracellular symbiosis during embryo development of Mastotermes darwiniensis (Isoptera: Mastotermididae). Parassitologia 40:309–316.

Sandström, J., and N. Moran. 1999. How nutritionally imbalanced is phloem sap for aphids? Entomol. Exp. Appl. 91:203–210.

Sandström, J., and J. Pettersson. 1994. Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (Acyrthosiphon pisum) performance. J. Insect Physiol. 40:947–955.

Sandström, J., A. Telang, and N. A. Moran. 2000. Nuritional enhancement of host plants by aphids- a comparison of three aphid species on grasses. J. Insect Physiol. 46:33–40.

Sasaki, T., and H. Ishikawa. 1995. Production of essential amino acids from glutamate by mycetocyte symbionts of the pea aphid, Acyrthosiphon pisum. J. Insect Physiol. 41:41–46.

Sasaki, T., T. Aoki, H. Hayashi, and H. Ishikawa. 1990. Amino acid composition of the honeydew of symbiotic and aposymbiotic pea aphids Acyrthosiphon pisum. J. Insect Physiol. 36:35–40.

Sato, S., and H. Ishikawa. 1997a. Expression and control of an operon from an intracellular symbiont which is homologous to the groE operon. J. Bacteriol. 179:2300–2304.

Sato, S., and H. Ishikawa. 1997b. Structure and expression of the dnaKJ operon of Buchnera, an intracellular symbiotic bacteria of aphid. J. Biochem. 122:41–48.

Schröder, D., H. Deppisch, M. Obermayer, G. Krohne, E. Stackebrandt, B. Holldobler, W. Goebel, and R. Gross. 1996. Intracellular endosymbiotic bacteria of Camponotus species (carpenter ants): systematics, evolution and ultrastructural characterization. Mol. Microbiol. 21:479–489.

Silva, F. J., R. C. H. J. van Ham, B. Sabater, and A. Latorre. 1998. Structure and evolution of the leucine plasmids carried by the endosymbiont (Buchnera aphidicola) from aphids of the family Aphididae. FEMS Microbiol. Lett. 168:43–49.

Smith, D. C., and A. E. Douglas. 1987. The biology of symbiosis. Edward Arnold London.

Smith, O. H., and C. Yanofsky. 1962. Enzymes involved in the biosynthesis of tryptophan. Meth. Enzymol. 5:794–806.

Spaulding, A. W., and C. D. vonDohlen. 1998. Phylogenetic characterization and molecular evolution of bacterial endosymbionts in psyllids (Hemiptera: Sternorrhyncha). Mol. Biol. Evol. 15:1506–1513.

Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. X. Zhao, E. V. Koonin, and R. W. Davis. 1998. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science 282:754–759.

Sylvester, E. S. 1985. Multiple acquisition of viruses and vector-dependent prokaryotes: consequences on transmission. Annu. Rev. Entomol. 30:71–88.

Telang, A., J. Sandström, E. Dyreson, and N. A. Moran. 1999. Feeding damage by Diuraphis noxia results in nutritionally enhanced phloem diet. Entomol. Exp. Appl. 91:403–412.

Thao, M. L., and P. Baumann. 1998. Sequence analysis of a DNA fragment from Buchnera aphidicola (aphid endosymbiont) containing the genes dapD-htrA-ilvI-ilvH-ftsL-ftsI-murE. Curr. Microbiol. 37:214–216.

Thao, M. L., L. Baumann, P. Baumann, and N. A. Moran. 1998. Endosymbionts (Buchnera) from the aphids Schizaphis graminum and Diuraphis noxia have different copy numbers of the plasmid containing the leucine biosynthetic genes. Curr. Microbiol. 36:238–240.

Tremblay, E. 1989. Coccoidea endosymbiosis. W. Schwemmler and G. GassnerInsect endocytobiosis: morphology, physiology, genetics, evolution. CRC Press, Inc.. Boca Raton, 145–173.

Unterman, B. M., and P. Baumann. 1990. Partial characterization of ribosomal RNA operons of the pea-aphid endosymbionts: evolutionary and physiological implications. R. K. Campbell and R. D. Eikenbary (eds.) Aphid-plant genotype interactions. Elsevier Biomedical Press. Amsterdam, 329–350.

Unterman, B. M., P. Baumann, and D. L. McLean. 1989. Pea aphid symbiont relationships established by analysis of 16S rRNAs. J. Bacteriol. 171:2970–2974.

van den Heuvel, J. F. J. M., A. Bruyere, A. Hogenhout, V. ZieglerGraff, V. Brault, M. Verbeek, F. van derWilk, and K. Richards. 1997. The N-terminal region of the luteovirus readthrough domain determines virus binding to Buchnera GroEL and is essential for virus persistence in the aphid. J. Virol. 71:7258–7265.

van den Heuvel, J. F. J. M., M. Verbeek, and F. van der Wilk. 1994. Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by Myzus persicae. J. Gen. Virol. 75:2559–2565.

van Ham, R. C. H. J., D. Martínez-Torres, A. Moya, and A. Latorre. 1999. Plasmid-encoded anthranilate synthase (TrpEG) in Buchnera aphidicola from the family Pemphigidae. Appl. Environ. Microbiol. 65:117–125.

van Ham, R. C. H. J., A. Moya, and A. Latorre. 1997. Putative evolutionary origin of plasmids carrying the genes involved in leucine biosynthesis in Buchnera aphidicola (endosymbiont of aphids). J. Bacteriol. 179:4768–4777.

von Dohlen, C. D., and N. A. Moran. 1995. Molecular phylogeny of the Homoptera—a paraphyletic taxon. J. Mol. Evol. 41:211–223.

von Wintzingerode, F., U. B. Gobel, and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21:213–229.

Waku, Y., and Y. Endo. 1987. Ultrastructure and life cycle of the symbionts in a Homopteran insect, Anomoneura mori Schwartz (Psyllidae). Appl. Entomol. Zool. 22:630–637.

Wernegreen, J. J., and N. A. Moran. 1999. Evidence for genetic drift in endosymbionts (Buchnera): analyses of protein-coding genes. Mol. Biol. Evol. 16:83–97.

Werren, J. H., and S. L. O'Neill. 1997. The evolution of heritable symbionts. S. L. O'Neill, A. A. Hoffman, and J. H. WerrenInfluential passengers; inherited microorganisms and arthropod reproduction. Oxford University Press. Oxford, 1–41.

Whitehead, L. F., and A. E. Douglas. 1993. A metabolic study of Buchnera, the intracellular bacterial symbionts of the pea aphid Acyrthosiphon pisum. J. Gen. Microbiol. 139:821–826.

Wicker, C., and P. Nardon. 1982. Development responses of symbiotic and aposymbiotic weevils Sitophilus oryzae L. (Coleoptera, Curculonidae) to a diet supplemented with aromatic amino-acids. J. Insect. Physiol. 28:1021–1024.

Wilkinson, T. L. 1998. The elimination of intracellular microorganisms from insects: an analysis of antibiotic-treatment in the pea aphid (Acyrthosiphon pisum). Comp. Biochem. Physiol. A119:871–881.

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CHAPTER 2.4

Symbiotic Associations Between Termites and Prokaryotes

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Introduction

The symbiotic associations of termites with microorganisms comprise different levels of interaction, ranging from the extracorporal cultivation of fungus gardens to the most intimate associations, where bacteria reside intracellularly in dedicated bacteriocytes. However, the majority of prokaryotic symbionts of termites are located in the intestinal tract, where they are free-swimming, attached to the gut epithelium, or associated with the intestinal protozoa (Fig. 1). Although it is suggestive that the gut microbiota of termites is directly or indirectly involved in the digestion of lignocellulose or has other nutritional implications, the exact nature of the associations and possible benefits for the partners of each particular symbiosis are often far from clear. Therefore, this chapter will use the term "symbiosis" in its broader sense, as originally defined by Anton de Bary (de Bary, 1878). A definitive classification of the associations into the different categories of symbiosis, such as mutualism, parasitism, or commensalism, would require a level of understanding that is yet to be reached.

In view of the enormous body of literature on the intestinal microbiota of termites and its role in lignocellulose digestion, the subject cannot be covered exhaustively. This chapter will attempt to summarize the current state of knowledge on the prokaryotic communities within the intestinal tracts of termites, the major populations and their metabolic activities, and their interactions. In addition, it will focus on the gut as a microbial habitat. The chapter will touch only briefly on the intestinal flagellates, which are most important in the phylogenetically lower termites, the exosymbiotic fungi in fungus-cultivating Macrotermitinae, and the intracellular bacteria in termite tissues. For details on these subjects and for many other aspects of the termite gut symbiosis, the reader will be referred to the pertinent review articles.

Symbiotic Digestion

Termites, like other insects thriving on a lignocellulosic diet, possess a pronounced gut microbiota housed in specially adapted regions of the alimentary tract (Fig. 2). The symbionts convert a substantial portion of the dietary components to microbial fermentation products, which are then eventually resorbed by the intestinal epithelia. It is generally assumed that the intestinal symbioses provide metabolic capacities that are otherwise not available to the host. For reviews, see Breznak and Brune (1994b), Kane (1997), Brune (1998), Brune (2003), Bignell (2000), Breznak (2000), Brune and Friedrich (2000a), and Ohkuma (2003).

The symbiotic digestion of lignocellulose by termites is a complex series of events involving both the host and its gut microbiota (Fig. 3). While the events in the foregut and midgut seem to be mainly due to host activities, the digestive processes in the hindgut are largely controlled by the symbionts. Many aspects of lignocellulose digestion are common to all termites, but there are also several noteworthy differences between the phylogenetically lower and higher taxa.

Fiber Degradation

The degradation of plant cell walls requires the synergistic action of many different enzymes and, in the case of lignified substrates, also a mechanism to break up the lignocellulose complex (Breznak and Brune, 1994b). Microorganisms, i.e., bacteria, protozoa, and fungi, are the most efficient cellulose and hemicellulose degraders in nature, and fungi and certain actinomycetes are also the only organisms that have developed a strategy for the chemical breakdown of lignin (Béguin and Aubert, 1994; Jeffries, 1994). Not surprisingly, termites and other animals have made use of these capacities by employing microbial symbionts in the digestion of lignocellulosic food (Martin, 1983).

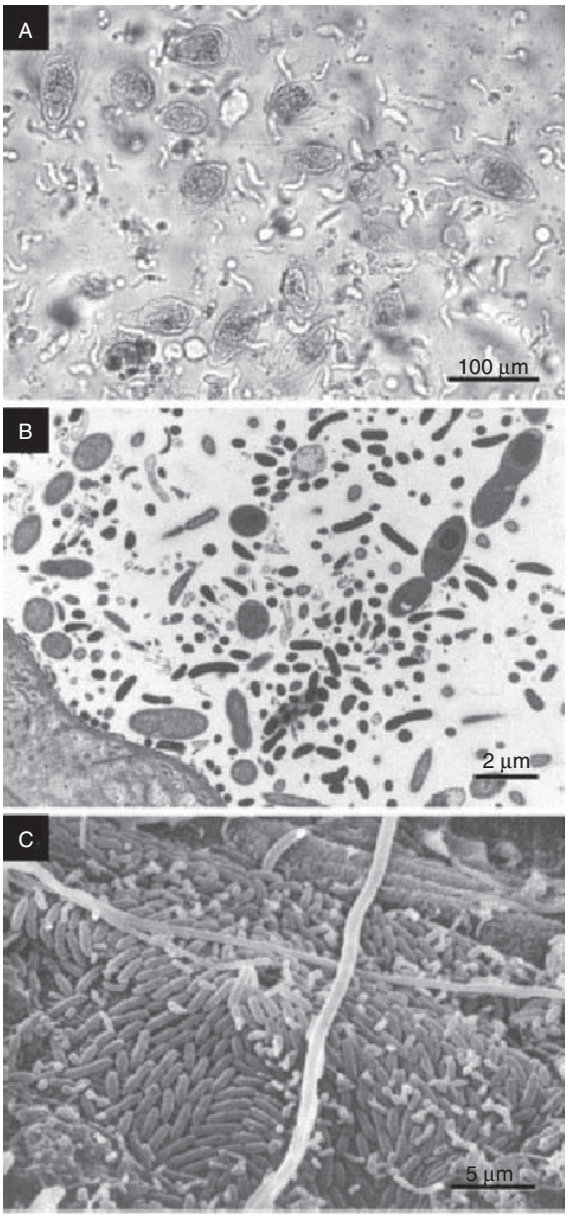

Fig. 1. Examples of microbial symbionts in the hindgut of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae), a wood-feeding lower termite. A) Preparation of anaerobic protozoa from the hindgut of a worker larva, showing the large hypermastigote flagellate *Trichonympha agilis*, filled with wood particles, and numerous smaller flagellates (mainly oxymonads, *Dinenympha* spp.). Differential interference contrast photomicrograph taken by U. Stingl. B) Transverse section through the peripheral hindgut, showing the diverse bacterial microbiota associated with the thin cuticle of the hindgut wall (bottom left). Transmission electron micrograph provided by J. A. Breznak. C) Preparation of the hindgut wall, showing the dense colonization of the cuticle by numerous rod-shaped and filamentous bacterial morphotypes. Scanning electron micrograph provided by J. A. Breznak. Reproduced from Brune (2003).

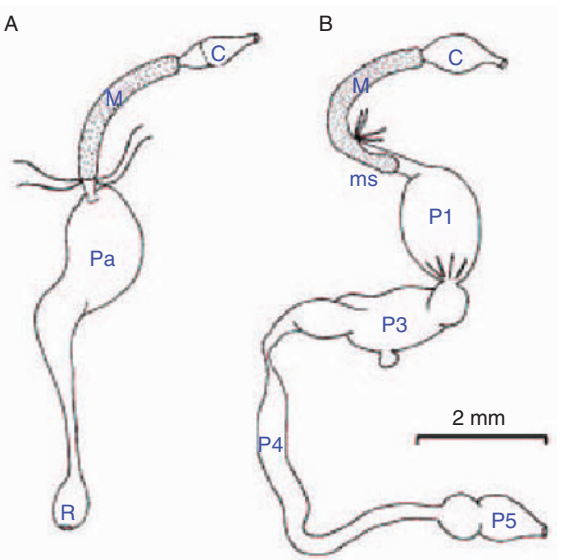

Fig. 2. Structure of the intestinal tract of *Reticulitermes* species (A) and *Cubitermes* species (B). All lower termites harbor the gut microbiota in a single, strongly dilated hindgut paunch (Pa) that tapers out via the colon into the rectum (R). In most higher termites, especially the soil-feeding species, the hindgut is more elongated and has additional dilatations. Abbreviations: crop (C), midgut (M), mixed segment (ms), and proctodeal segments (P1–P5).

Role of Intestinal Protozoa The presence of protozoa in termite guts was recognized very early, although they were initially considered to be parasites (e.g., Leidy, 1881; Koidzumi, 1921). The American protozoologist L. R. Cleveland recognized that the wood-feeding lifestyle in the evolutionarily "lower" termites is based on a mutualistic association with their intestinal protozoa (Cleveland, 1925a; Cleveland, 1926). In a series of elegant experiments, he established that the ability of lower termites to live on a diet of wood or cellulose depends on the digestive capacities of their intestinal flagellates. His pioneering work, published in 1923–1928, paved the way for many later studies (see reviews by Honigberg [1970], Inoue et al. [2000], and Brune and Stingl [2005]).

About 15 years later, Hungate elucidated the biochemical basis for this symbiosis (Hungate, 1939; Hungate, 1943). He showed that the gut flagellates depolymerize and ferment lignocellulose to short-chain fatty acids, which are resorbed and oxidized by the host (reviewed by Hungate [1955] and Breznak and Brune [1994b]). The importance of protozoa for the metabolic processes in the hindgut of lower termites is most impressively evidenced by their enormous numbers, which may constitute more than one-third of the body mass in *Zootermopsis* species (Katzin and Kirby, 1939).

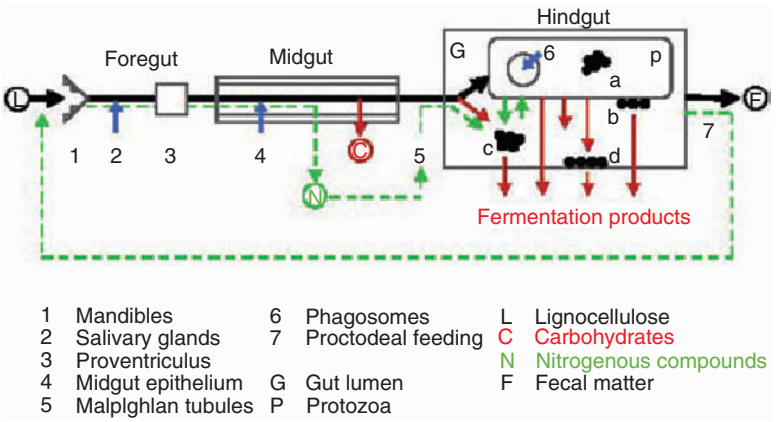

Fig. 3. Major events in the symbiotic digestion of lignocellulose by wood-feeding lower termites. The black lines show the path of the insoluble material whose lignin-rich residues are released as feces, whereas the red lines represent soluble degradation products, which are eventually resorbed by the host. The green lines indicate the cycling of nitrogenous compounds. Blue arrows mark the sites where cellulolytic enzymes are secreted. Lower-case letters refer to the different groups of bacteria, which are either endobionts (a) or epibionts (b) of the protozoa, suspended in the gut lumen (c) or attached to the gut wall (d). The scheme has been simplified for the sake of clarity; not all possible interactions are shown. Adapted from Brune (2003).

There is a large body of literature on the decomposition of wood and cellulose by termite gut flagellates (for references, see O'Brien and Slaytor [1982], Breznak and Brune [1994b], and Inoue et al. [2000]). Apparently, the different flagellate species are nutritionally specialized, and each species might fill a specific niche in lignocellulose digestion (Yoshimura et al., 1996; Inoue et al., 2000). Most of the endoxylanase and β-xylosidase activity in the lower termite *Reticulitermes speratus* is located in the anterior hindgut and is lost upon defaunation (removal of protozoa by ultraviolet irradiation; Inoue et al., 1997), and the effects of artificial diets on the composition of the protozoan community corroborate that different gut flagellates are involved in xylan and cellulose degradation (Inoue et al., 1997; Cook and Gold, 2000).

The protozoa possess their own cellulase genes, which fall into different glycosyl hydrolase families (Ohtoko et al., 2000; Nakashima et al., 2002a; Watanabe et al., 2002; Li et al., 2003; Inoue et al., 2005) and may even exploit host cellulases that are secreted in the anterior gut regions (Li et al., 2003). There is no evidence that the prokaryotic symbionts of the gut flagellates (see the section Interactions Between Prokaryotes and Protozoa in this Chapter) confer cellulolytic activity to their hosts.

The Role of Fungi The role of fungi in the digestion of lignocellulose by termites is less clear. There are termites that can thrive on sound wood, but many species show a strong preference for decaying wood colonized by saprophytic fungi, which may either precondition the wood for digestion or provide metabolic products important for termite nutrition (Sands, 1969; Rouland, 2000; Cornelius et al., 2002). Schäfer et al. suggested that the yeasts and other fungi present in the guts of the lower termites *Zootermopsis angusticollis* and *Neotermes castaneus* are involved in hemicellulolytic degradation (Schäfer et al., 1996).

Higher termites of the subfamily Macrotermitinae have established a unique exosymbiosis with basidiomycetes of the genus *Termitomyces*, which are maintained on predigested plant litter in so-called "fungus gardens" within the nests. The symbiosis has rendered fungus-cultivating termites independent of the intestinal protozoa, which probably allowed for the obvious diversification in their diet (Sands, 1969; Rouland, 2000). The specificity of this symbiosis, whose enormous evolutionary success is impressively evidenced by the huge nests of fungus-cultivating termites populating the grasslands of Africa, is documented by several instances of coevolution between the termites and their fungal partners, indicating both horizontal and vertical transmission of the fungal symbionts (Aanen et al., 2002; Katoh, 2002; Rouland-Lefevre et al., 2002; Taprab et al., 2002).

The association with the lignin-degrading fungus enables the fungus-cultivating termites to utilize lignocellulose nearly completely, as reflected in the small volume of their final feces (Darlington, 1994). The key activities attributed to the fungal partner in this mutualistic symbiosis are extensive delignification of the substrate (Hyodo et al., 1999, 2000; Johjima et al., 2003) and the conversion of plant fiber to fungal biom-

ass, as proposed earlier in the *lignin degradation hypothesis* of Grassé and Noirot (1958). Evidence for an activity within the gut of fungal cellulases ingested by the termites together with the fungus comb material (Abo-Khatwa, 1978; Martin and Martin, 1978; Martin and Martin, 1979) gave rise to the *acquired enzyme hypothesis* of Martin (1983). However, claims that the fungal cellulases are essential for cellulose digestion in the termite gut remain controversial (Slaytor, 1992; Bignell et al., 1994b; Crosland et al., 1996), especially in view of the recently discovered ability of termites to produce their own cellulases (see the section The Role of Host Enzymes in this Chapter).

The Role of Host Enzymes Since phylogenetically higher termites (family: Termitidae) lost their gut flagellates in the course of termite evolution, it was initially assumed that either ingested fungal enzymes (see the section The Role of Fungi in this Chapter) or prokaryotic symbionts took over the function of the cellulolytic protozoa. However, there is still no clear evidence that bacteria play a major role in cellulose degradation in any of the termites investigated (see the section Cellulolytic Bacteria in this Chapter), which may find its explanation in the recently discovered ability of termites to produce their own cellulases.

In all insects, the digesta are exposed to a variety of digestive enzymes secreted by the salivary glands and the midgut epithelium (Terra, 1990). Cook (1943) had already demonstrated that termites are able to absorb sugars directly, and evidence is accumulating that termites secrete a full complement of enzymes necessary for the digestion of plant structural polysaccharides, including cellulose, into the midgut (e.g., Rouland et al., 1989; Slaytor, 1992; Rouland, 2000). The presence of protease and lysozyme activities has been documented for several termites, which indicates that also microbial cells can be digested (Rohrmann and Rossman, 1980; Fujita et al., 2001; Fujita and Abe, 2002a; Fujita et al., 2002b). Although experimental evidence is scarce, one can safely assume that—as in other insects—most of the easily digestible material has been mobilized and resorbed by the time the digesta reach the end of the midgut (Fig. 3).

The persisting dogma that higher animals do not possess their own cellulases has been unequivocally refuted by the demonstration of endoglucanase genes in the termite genome and their expression in the cells of the midgut epithelium and in the salivary glands (reviewed by Watanabe and Tokuda, 2001). Even in lower termites, host cellulases secreted by the salivary glands and complement (and surpass) the cellulolytic activities of the intestinal protozoa in the hindgut (Nakashima et al., 2002b; Tokuda et al. 2004); There is evidence that glycosyl hydrolase family 9 (GHF9) cellulases present in the genomes of termites are ancient and widespread in Metazoa (Lo et al., 2003b; Davison and Blaxter, 2005).

Soil-Feeding Termites The majority of termite species are humivorous, yet little is known about the exact nature of the dietary components exploited by these ecologically important soil macroinvertebrates (Brauman et al., 2000). Besides fragments of plant tissue, fungal hyphae, and numerous microorganisms, their diet consists largely of undefined humic material intimately associated with the mineral soil matrix (Donovan et al., 2001). While the aromatic component of humus was initially assumed to be the principal substrate of soil-feeding termites (Noirot, 1992; Bignell, 1994a), feeding trials with soil-feeding *Cubitermes* spp. have shown that peptidic soil components—free or polymerized into humic model compounds—are preferentially digested and mineralized (Ji et al., 2000; Ji and Brune, 2001; Ji and Brune, 2005).

The anterior hindgut of soil-feeding termites is extremely alkaline (Bignell and Eggleton, 1995; Brune and Kühl, 1996), which favors the extraction of organic matter from the soil (Brune, 1998; Kappler and Brune, 1999). Microbial biomass and its structural components are assimilated more efficiently than cellulose, which supports the hypothesis that soil microorganisms and the nitrogenous components of humus are an important dietary resource for humivorous soil macroinvertebrates (Ji et al., 2000; Ji and Brune, 2001).

Host Nutrition

Irrespective of their contribution to polymer degradation, the majority of prokaryotes in termite guts are probably involved in the fermentation of the soluble products released into the gut (see the section Microbial Fermentations in this Chapter), which are derived either directly from the food by the digestive enzymes (see the section The Role of Host Enzymes in this Chapter) or by the fermentative activity of the intestinal protozoa (see the section Role of Intestinal Protozoa in this Chapter). The major products of the hindgut metabolism are acetate and, to a smaller extent, other short-chain fatty acids (mainly propionate and butyrate), which accumulate in the hindgut fluid and are eventually resorbed by the hindgut epithelium (Fig. 3). Termites—like other insects—cannot use acetate as a substrate for gluconeogenesis, but as long as the digestive processes in the midgut release sufficient amounts of soluble sugars and amino acids, this is not a problem. Breznak calculated

that acetate produced by the hindgut microbiota of *Reticulitermes flavipes* would suffice to support the respiratory activity of the host (Breznak, 2000).

Besides being difficult to degrade, lignocellulose is also an extremely nutrient-poor substrate. While non-lignified plant cells are usually rich in protein and other nitrogenous compounds, the C-to-N ratio of sound wood is up to 100-fold higher than that of the insect body (La Fage and Nutting, 1978). Moreover, a lignocellulosic diet lacks most of the essential nutrients required by animals, such as amino acids, vitamins, and sterols. Many microorganisms are capable of fixing dinitrogen, assimilating nitrate and ammonia, or synthesizing those amino acids and vitamins essential for the host. Animals, including termites, have developed means of exploiting these biosynthetic capacities, which include—in the simplest case—the digestion of the intestinal symbionts.

Wood-feeding termites, especially those feeding on sound wood, have an extreme shortage of nutrients in their diet, and the digestion of microbial biomass acquired in the course of anal trophallaxis supplies them with high-quality nutrients (Machida et al., 2001). The gut microbiota supplies essential precursors for the biosynthesis, e.g., of methyl-branched hydrocarbons (Guo et al., 1991), and might play a role in nestmate recognition (Matsuura, 2001). To date, the lack of knowledge on the individual components of the prokaryotic microbiota and their metabolic capacities and activities in situ still makes it difficult to define the essential functions and understand the complex interactions.

The Gut Microenvironment

The intestinal tract of insects is organized into three major gut regions: a short foregut, a midgut (which is the main site of digestion), and a usually short hindgut (proctodeum). The hindguts of all termites, however, have immensely increased in length and volume over the course of evolution (Fig. 2). In the more primitive, lower termites, the hindgut is still relatively simple, consisting of a dilated "hindgut paunch" that tapers out into the colon and ends in the rectal compartment (Noirot, 1995). While this organization has been retained in the fungus-cultivating termites (Termitidae: Macrotermitinae), all other lineages of higher termites show a trend towards a further elongation and additional compartmentalization of the hindgut (Noirot, 2001), which is most pronounced in the soil-feeding representatives. The gut morphologies of lower and higher termites and the significance of the adaptations for the digestive process have been reviewed exhaustively (Noirot, 1995; Noirot, 2001).

The proctodeal dilatations increase the residence time of the digesta, thereby prolonging the exposure to the activities of the intestinal microbiota. Moreover, host factors and microbial activities give rise to physicochemical gradients that create distinct microenvironmental conditions in each gut compartment. This has been shown for oxygen, hydrogen, redox potential, and intestinal pH and has to be expected also for any other metabolite when source and sink are spatially separated (Brune and Friedrich, 2000a), especially since the microbiota are not randomly distributed within the gut (see the section Spatial Organization in this Chapter).

Physicochemical Gradients

Redox Conditions and Oxygen Status The general concept of termite guts as anoxic habitats had been based on several pieces of circumstantial evidence (outlined by Veivers et al., 1980): 1) the oxygen sensitivity of the intestinal protozoa, already recognized by Cleveland (Cleveland, 1925a; Cleveland, 1925b); 2) the demonstration of a fermentative metabolism of cellulose by these flagellates (Hungate, 1939; Hungate, 1943) and the high concentrations of microbial fermentation products in the hindgut of all termites investigated; and 3) the presence of oxygen-sensitive or strictly anaerobic processes, such as nitrogen fixation and methanogenesis (Breznak et al., 1973; Breznak, 1975). Also, the subsequent isolation of anaerobic bacteria from termite guts (see the section Isolates and Major Metabolic Activities in this Chapter) supported the general assumption that the principle of symbiotic digestion in termite guts was analogous to that in the rumen.

Slaytor and coworkers were the first to question the anoxic status of termite guts. Following the color reaction in the hindgut of redox indicator dyes fed to *Nasutitermes exitiosus* and *Coptotermes lacteus*, they initially claimed that the hindgut paunch was "aerobic," since methylene blue remained oxidized (Eutick et al., 1976). In a later study, however, using a more refined technique, they obtained E_h values between –230 mV and –270 mV in the hindgut paunch of these and seven other termite species (Veivers et al., 1980). The initial error had been caused by the color of the reduced dye within the gut being obscured by that of the oxidized dye, which had also impregnated the oxic gut epithelium.

Moreover, Slaytor and coworkers demonstrated that the vitality of *Nasutitermes exitiosus* and *Coptotermes lacteus* depended on the presence of their prokaryotic gut microbiota (Eutick et al., 1978b) and that bacteria play an important

role in maintaining the low redox potential of the hindgut paunch (Veivers et al., 1982), which led to the postulation that they maintain anoxic conditions by removing oxygen from the hindgut.

Bignell (1984) pointed out that arthropods are relatively small animals with surface-to-volume ratios higher than those in practically all vertebrates, and that they are likely to reach equilibrium with their environment unless efficient permeability barriers for oxygen are established or oxygen is sequestered by the animal or by intestinal microorganisms. In a series of studies employing oxygen microsensors, Brune and coworkers clarified the situation (Brune et al., 1995a; Ebert and Brune, 1997; Schmitt-Wagner and Brune, 1999) by demonstrating that the steep gradient in oxygen partial pressure between the oxic gut epithelium and the anoxic gut contents drives a continuous influx of oxygen into the hindgut (Fig. 4). In all termites investigated, oxygen penetrated 50–200 µm into the periphery of the hindgut lumen, leaving only the central portion of the dilated compartments anoxic (Ebert and Brune, 1997; Brune, 1998; Kappler and Brune, 1999; Schmitt-Wagner and Brune, 1999).

The maintenance of anoxia in the termite hindgut is not a trivial issue. Since the removal of oxygen in the gut periphery is fueled by the fermentative processes in the hindgut lumen, there must be a lower size limit for arthropods with a symbiotic digestion. However, even the smallest of all termites investigated to date (*Anoplotermes pacificus*, Termitidae: Apicotermitinae) seems to possess a symbiotic gut microbiota, although the spectrum of fermentation products in the hindgut differs from that of other termites (Bauer et al., 2000).

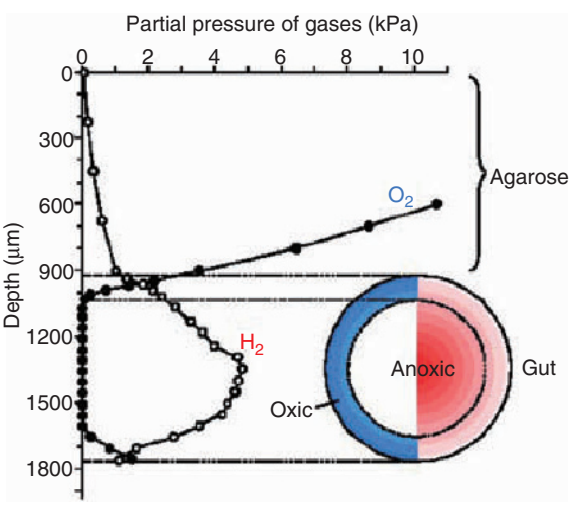

Fig. 4. Radial gradients of oxygen (●) and hydrogen (○) in an agarose-embedded hindgut (paunch region) of *Reticulitermes flavipes* worker larva. A schematic cross-section through the paunch illustrates the relative sizes of the oxic and anoxic zones. Modified after Brune (1998).

Fine-scaled redox measurements with platinum microelectrodes in wood- and soil-feeding termites (Ebert and Brune, 1997; Kappler and Brune, 2002) have shown that the redox potential in the gut mirrors the oxygen gradients (Fig. 5), and also the absolute values are in good agreement with the previously published data based on feeding experiments with redox dyes (Veivers et al., 1980). The most negative redox potentials are found in the regions of high hydrogen partial pressure, although in soil-feeding termites of the genus *Cubitermes*, parameters other than oxygen or hydrogen partial pressure seem to control the redox status of the intestinal contents (Kappler and Brune, 2002). There is evidence for ferric iron reduction in the gut of soil-feeding and wood-feeding species, which may be a microbial process, possibly mediated by the presence of humic acids or other phenolic polymers (Kappler and Brune, 2002; Vu et al., 2004).

Hydrogen Partial Pressure Despite the massive hydrogen production by the intestinal protozoa (Odelson and Breznak, 1985b), the hydrogen emission rates of termites are relatively low (Odelson and Breznak, 1983; Ebert and Brune, 1997; Sugimoto et al., 1998; Schmitt-Wagner and Brune, 1999). Originally, it had been assumed that the situation in the intestinal tract of termites was similar to that in other methanogenic habitats, where low hydrogen partial pressures result from a tight coupling between hydrogen-producing and hydrogen-consuming processes (Breznak, 1994a; Breznak and Brune, 1994b). However, hydrogen microsensor measurements revealed that the situation in termites is quite different, giving rise to steep radial gradients of hydrogen towards the gut epithelium and enormous differences in hydrogen partial pressure along the gut axis (Ebert and Brune, 1997; Schmitt-Wagner and Brune, 1999; Kappler and Brune, 2002), which gave rise to the hypothesis that the spatial organization of the hydrogen-producing and hydrogen-consuming populations controls the hydrogen partial pressure in different gut regions.

Intestinal pH The intestinal pH in the hindgut of most phylogenetically lower termites seems to be around neutral (Eutick et al., 1976; Bignell and Anderson, 1980a; Veivers et al., 1980; Brune et al., 1995a). Kovoor (1967) was the first to report the alkaline region in the anterior hindgut of wood-feeding termites; this observation was later extended also to soil-feeding species (Bignell and Anderson, 1980a). Since then, a large body of data has accumulated (Bignell, 1994a;

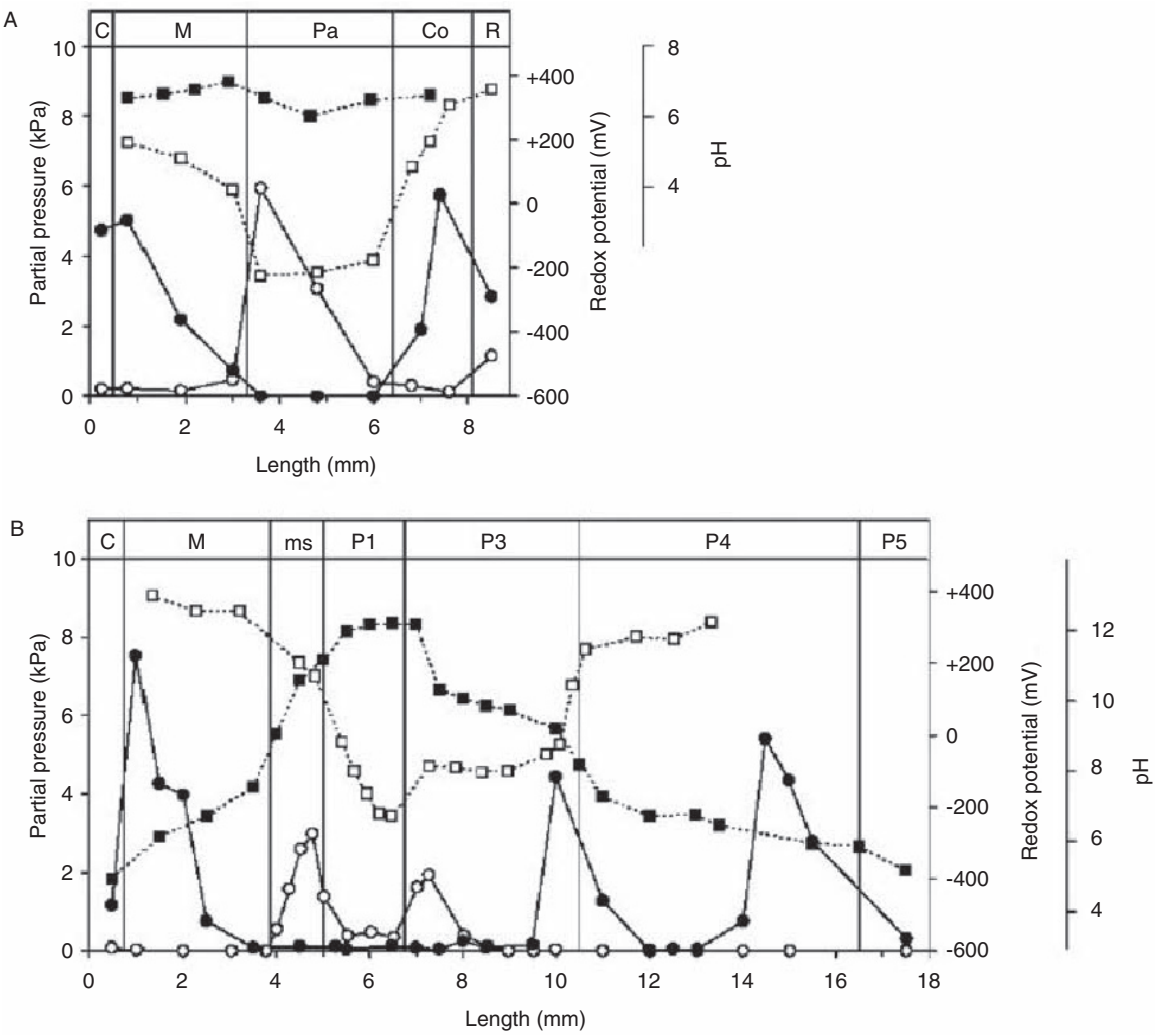

Fig. 5. Profiles of physicochemical conditions along the gut axis of *Reticulitermes flavipes* (A) and *Cubitermes orthognathus* (B). Oxygen (●) and hydrogen (○) partial pressures, intestinal pH (■), and apparent redox potential (□) (against a standard hydrogen reference electrode [SHE]) were measured with the respective microsensors. Guts were embedded in agarose-solidified Ringer's solution. The borders between the different gut regions (see legend to Fig. 2) are indicated by the vertical lines. Data from Brune et al. (1995a), Brune and Kühl (1996), Ebert and Brune (1997), Schmitt-Wagner and Brune (1999), and Kappler and Brune (2002).

Bignell and Eggleton, 1995), documenting a tendency towards strong alkalinity in the anterior hindgut of all higher termites except the Macrotermitinae (Anklin-Mühlemann et al., 1995).

While the initial measurements (performed mostly by spotting pooled, disrupted samples of individual gut regions on pH indicator paper) still lacked accuracy and resolution, studies with pH microsensors allowed alkaline regions to be precisely located (Brune et al., 1995a; Brune and Kühl, 1996). The latter measurements are not biased by homogenization and documented that guts of soil-feeding termites are even more alkaline than reported previously. The most alkaline values (pH 11–12.5) were found among soil-feeding Termitinae and represent the highest values ever encountered in biological systems (Brune and Kühl, 1996). In all species tested, the pH of the gut contents increases sharply from circumneutral in the midgut to highly alkaline between the midgut–hindgut junction and the first proctodeal dilation (P1), which coincides exactly with the location of the mixed segment (Fig. 5), a morphologically unique gut region present in all higher termites except the Macrotermitinae (Noirot, 2001).

Gut Compartmentation and Microhabitats

Each gut compartment provides various microhabitats differing in many environmental parameters (see the section Physicochemical Gradients

in this Chapter). The small size of the guts results in large surface-to-volume ratios (Brune, 1998), and the epithelial surfaces provide ample attachment sites for gut microorganisms, which are thus protected from washout (Bignell, 1984). Additional compartmentalization is created by the protozoa inhabiting the hindgut lumen of lower termites.

Midgut Epithelium, Ectoperitrophic Space As in other insects, the midgut epithelium is not protected by a cuticle, but a peritrophic membrane separates the epithelial surface from the digesta (Terra, 1990). The ectoperitrophic space harbors a distinct bacterial microbiota, which can be intimately associated with the microvilli of the brush border (Breznak and Pankratz, 1977). In addition, the so-called "mixed segment" in many Termitidae, a region where midgut and hindgut epithelia overlap (Noirot, 2001), is a microhabitat that harbors a specific bacterial microbiota. Kovoor (1968) described a "pure culture of spore-forming fusiform bacteria" in the mixed segment of *Microcerotermes edentatus*, located outside of the peritrophic membrane in a posterior pocket formed by the mesenteric side. Also Potts and Hewitt (1973) observed a prominent flora of "thin long filaments with terminal spores" in the mixed segment of the harvester termite, *Trinervitermes trinervoides* (Nasutitermitidae), located in the ectoperitrophic space posterior to the Malphigian tubules. Other authors described dense populations of different but also relatively uniform microorganisms in the mixed segment of *Nasutitermes exitiosus* (Czolij et al., 1985) and of soil-feeding Termitinae (*Procubitermes aburiensis* and *Cubitermes severus*; Bignell et al., 1980b; Bignell et al., 1983). Recently, Tokuda et al. (2000) demonstrated that the bacteria populating the mixed segment of *Nasutitermes takasagoensis* are phylogenetically within the radiation of the Clostridiales (see the section Clostridiales in this Chapter). Electron microscopy confirmed their close association with the mesenteric epithelium, suggesting that there is some kind of interaction with the gut tissue (Tokuda et al., 2001).

Hindgut Cuticle Electron-microscopy studies revealed intimate associations of microorganisms with the cuticle of the hindgut epithelium in all termites investigated (Breznak and Pankratz, 1977; To et al., 1980; Czolij et al., 1985; Yara et al., 1989; Fig. 1). Bacteria are associated with cup-like indentations on the epithelial surface of the hindgut in *Reticulitermes flavipes* (Breznak and Pankratz, 1977). Although *Mastotermes darwiniensis* and *Nasutitermes exitiosus* possess similar structures, they seem not to be associated with microorganisms (Czolij et al., 1984). In certain soil-feeding termites, cuticular spines protrude from the hindgut wall into the lumen of the P4 compartment and form additional attachment sites for the gut microbiota (*Procubitermes aburiensis*; Bignell et al., 1980c).

Hindgut Protozoa The protozoa in the hindgut of lower termites occupy the bulk of the hindgut volume (Katzin and Kirby, 1939) and represent an enormous surface area in the hindgut (Berchtold et al., 1999). Pierantoni (1936) was first to point out the association of gut flagellates with bacteria; since then, ectobiotic and endocytobiotic bacteria have been found on and in almost every flagellate investigated. For example, the hypermastigote flagellate *Joenia annectens*, a symbiont in the hindgut of *Kalotermes flavicollis*, is densely colonized by prokaryotic microorganisms (Hollande and Valentin, 1969). The body is covered with rod-shaped bacteria, and the nucleus and the cytoplasm contain various types of endocytobiotic bacteria (Radek et al., 1992; Patricolo et al., 2001). Also the oxymonadid flagellate *Streblomastix strix*, a hindgut symbiont *Zootermopsis* species, is associated with several, morphologically distinct types of bacteria that are orderly arranged end-to-end on six or seven longitudinal vanes, lending *S. strix* a stellate appearance in transverse section (Leander and Keeling, 2004). Adhesion of bacteria to the flagellate surfaces is based on different mechanisms and facilitated by special surface structures (e.g., Radek et al., 1996; Radek and Tischendorf, 1999b; Rother et al., 1999; Patricolo et al., 2001). The literature has been recently reviewed by Radek (1999). Possible significance of these associations of prokaryotes with hindgut flagellates is discussed in a different section (see the section Interactions Between Prokaryotes and Protozoa in this Chapter).

Prokaryotic Gut Symbionts

In view of the variety of microhabitats and microenvironmental conditions in the intestinal tracts of termites (see the section The Gut Microenvironment in this Chapter), it is not astonishing to find an equally large diversity among the microorganisms colonizing the gut. The amount of diversity indicated already by the morphological and ultrastructural features of the microbiota is greatly exceeded by that encountered at the phylogenetic level.

Morphological Diversity

Already the observation of gut preparations with a phase-contrast light microscope reveals a wide

variety of prokaryotic life forms. Several comprehensive studies of the bacterial gut microbiota of termites using transmission electron microscopy have provided detailed accounts of the morphological diversity of gut microorganisms for several termite species from different families. In addition to the abundant protozoan fauna in all so-called "lower termites" (Yamin, 1979), at least 20–30 different bacterial morphotypes have been distinguished among the microorganisms colonizing the intestinal tract of *Reticulitermes flavipes* and *Coptotermes formosanus* (Rhinotermitidae; Breznak and Pankratz, 1977) and *Pterotermes occidentis* (Kalotermitidae; To et al., 1980).

The so-called "higher termites" (Termitidae) lack intestinal flagellates, but the morphology of their prokaryotic microbiota appears to be equally diverse. The hindgut of the wood-feeding *Nasutitermes exitiosus* (Termitidae: Nasutitermitinae) contains almost 30 different bacterial morphotypes (Czolij et al., 1985), and also the hindgut microbiota of the fungus-growing termite *Odontotermes formosanus* (Termitidae: Macrotermitinae) comprises at least 20 different morphotypes (Yara et al., 1989). Numerous bacterial morphotypes, including many filamentous forms, colonize the intestinal epithelia and the ectoperitrophic space of the soil-feeding termite *Procubitermes aburiensis* (Termitidae: Termitinae; Bignell et al., 1980b).

Although the morphological features usually do not allow the affiliation of a bacterium to a specific taxon, members of the termite gut microbiota have conspicuous forms or other morphological features that are of (albeit limited) taxonomic value, or conspicuous forms that seem to occur in different species of termites. One example is the thin, spore-forming filaments described by Leidy (1881) as "Arthromitus" species, which occur in many invertebrates, including termites (Leidy, 1881; Margulis et al., 1990). Also, many of the spirochetal forms are so large and conspicuous that they can be morphologically distinguished (Breznak, 1984a; see also Termite Gut Spirochetes in Volume 7). On the basis of the detailed morphological features visualized by transmission electron microscopy, Margulis and coworkers (Bermudes et al., 1988; Wier et al., 2000; see also Large Symbiotic Spirochetes: *Clevelandia*, *Cristispira*, *Diplocalyx*, *Hollandina*, and *Pillotina* in Volume 7) proposed a number of new species for the larger spirochetes.

Phylogenetic Diversity and Community Structure

In the recent years, the microbiota in the intestinal tracts of termites has been investigated also with molecular tools (see Ohkuma [2002a] for a review). Most studies employed the 16S rRNA gene as a molecular marker. As in most other environments, phylogenetic diversity of the intestinal microbiota is enormous, and there is still little overlap between the phylotypes recovered with cultivation-independent techniques and the isolates obtained by cultivation (see the section Isolates and Major Metabolic Activities in this Chapter). Molecular fingerprinting has been used to compare the structure of gut communities and to follow temporal changes. The bias inherent in all polymerase chain reaction (PCR)-based approaches has been addressed by backing the results with independent methods, such as fluorescence in situ hybridization (FISH).

Bacterial Diversity

König and coworkers were among the first to use the 16S rRNA-based approach to identify the phylogenetic position of uncultivated spirochetes in the gut of *Mastotermes darwiniensis* (Berchtold et al., 1994; Berchtold and König, 1996); a parallel study of Paster et al. (1996) was aimed at characterizing the spirochetes in the gut of *Nasutitermes lujae*. At about the same time, Ohkuma and coworkers attempted to characterize the full diversity of archaea and bacteria in the intestinal tract of *Reticulitermes speratus* and *Cryptotermes domesticus* using a similar strategy (Ohkuma et al., 1995; Ohkuma and Kudo, 1996a; Ohkuma and Kudo, 1998).

Although the cultivation-independent approach documented the presence of many new, hitherto uncultivated phylotypes in the intestinal tracts of termites (for a review, see Kudo et al., 1998), these early studies lacked resolution since only small numbers of clones were investigated. Later studies documented that diversity coverage of the clone libraries was far from exhaustive, even if larger numbers of clones were used. The most comprehensive assessment of molecular diversity and bacterial community structure in termite guts to date involved the gut microbiota of *Reticulitermes* species. Hongoh et al. analyzed 14 clone libraries (96 clones each) of the bacterial 16S rRNA genes in the hindgut of the Japanese termite species *Reticulitermes speratus* to characterize phylogenetic diversity and to address the bias introduced by different primer combinations and PCR conditions (Hongoh et al., 2003a; Hongoh et al., 2003b). Yang et al. (2005) performed a similar analysis with more than 500 clones from the European termite species *Reticulitermes santonensis*, focusing on the differences between the bacterial communities in the four major intestinal habitats: the midgut, the wall of the hindgut paunch, the hindgut fluid, and the intestinal protozoa (see the section Gut

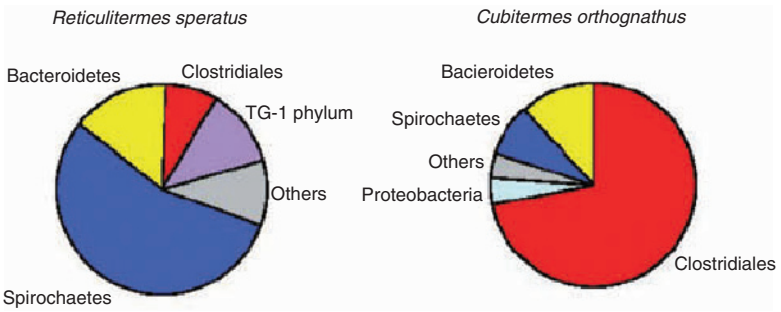

Fig. 6. Relative abundance of the major bacterial phyla in clone libraries of 16S rRNA genes from the hindgut of the wood-feeding, lower termite *Reticulitermes speratus* and the soil-feeding, higher termite *Cubitermes orthognathus*. Data from Hongoh et al. (2003a) and Schmitt-Wagner et al. (2003b).

Compartmentation and Microhabitats in this Chapter).

The intestinal community of the two *Reticulitermes* species is quite similar, comprising representatives of several bacterial phyla (Fig. 6). Both termite species harbor Gram-positive bacteria (mainly clostridia, streptococci, and Mycoplasmatales-related clones), members of the Bacteroidetes, spirochetes, and a number of Proteobacteria, albeit at slightly different ratios. A large number of clones fall into the so-called "Termite group 1" (TG-1) phylum, which were most abundant in *Reticulitermes santonensis* (Yang et al., 2005); spirochetal clones were less abundant in this termite but accounted for approximately half of the analyzed clones in *Reticulitermes speratus* (Hongoh et al., 2003b).

The situation in soil-feeding termites is quite different. In a study analyzing bacterial diversity in the highly compartmentalized intestinal tract of *Cubitermes orthognathus* (Schmitt-Wagner et al., 2003a; Schmitt-Wagner et al., 2003b), the authors combined clone analysis with FISH and a molecular fingerprinting analysis, which not only substantiated the data obtained by the individual approaches but also allowed differences in community structure between the different gut compartments to be investigated. In contrast to the situation in *Reticulitermes speratus*, the bacterial clone libraries contained no clones from the TG-1 phylum and only few spirochetal clones. In the anterior gut sections, most clones represented Firmicutes. In the posterior gut sections, clones belonging to the Bacteroidetes and different subgroups of the Proteobacteria gained some numerical significance. A study of the bacterial microbiota in the P1 compartment of several higher termites extended the presence of a compartment-specific microbiota and a predominance of Firmicutes in the highly alkaline gut regions also to representatives of other feeding guilds (Thongaram et al., 2005).

All PCR bias notwithstanding, these large datasets, together with the numerous clones obtained from other termite species (for references, see Ohkuma, 2003), allow a reasonably accurate picture of the dominant phylogenetic groups to be drawn. Most clones obtained in the different studies represent lineages of microorganisms that were exclusively recovered from the intestinal tract of termites. The termite specificity of these lineages was underscored by the finding that the closest relatives of the bacterial clones within each lineage were usually derived also from the most-closely related termites, supporting the concept of coevolution between gut microbiota and host (Yang et al., 2005).

Spirochaetes Not astonishingly, in view of the numerical predominance of this morphologically diverse and conspicuous group in most wood-feeding termites (Breznak, 2002), the majority of the clones obtained with bacteria-specific primers from the hindgut of *Reticulitermes speratus* represent spirochetes (Hongoh et al., 2003a). Already the first molecular studies had indicated that termite gut spirochetes represent a lineage phylogenetically distinct from other Spirochaetes (Berchtold and König, 1996; Ohkuma and Kudo, 1996a; Paster et al., 1996). Better diversity coverage was achieved by Lilburn et al. (1999), who targeted the intestinal spirochetes of *Reticulitermes flavipes* with spirochete-specific primers. They demonstrated that the 12–15 spirochete morphotypes in *Reticulitermes flavipes* (Breznak and Pankratz, 1977) were paralleled by 21 different spirochete phylotypes, which formed two major clusters of treponemes, one of them containing only clones of termite origin (Lilburn et al., 1999). *Treponema*-related clones have also been recovered from a variety of other termite species (Lilburn et al., 1999; Ohkuma et al., 1999a). In several cases, the ectosymbiotic association of certain phylotypes with flagellate protozoa has been documented using FISH with group-specific oligonucleotide probes (Berchtold and König, 1996; Iida et al., 2000; Noda et al., 2003).

"Endomicrobia" In their first survey of the bacterial diversity in *Reticulitermes speratus*, Ohkuma and Kudo (1996a) obtained a number

of clones whose sequences were only distantly related to other bacteria and which were subsequently recognized as a novel bacterial phylum (Hugenholtz et al., 1998). Also many clones in the comprehensive libraries subsequently obtained with *Reticulitermes speratus* (Hongoh et al., 2003a) and *Reticulitermes santonensis* (Yang et al., 2005) belong to this lineage, indicating that members of the TG-1 phylum represent a hitherto uncultivated but numerically dominant group of prokaryotes in the gut of *Reticulitermes* species.

Using a full-cycle molecular approach, combined with transmission electron microscopy, Stingl et al. (2005) showed that the TG-1 bacteria in *Reticulitermes* species are endosymbionts that colonize—exclusively and in high abundance—the cytoplasm of the larger flagellate species. The symbionts were specific for their respective host flagellate and were provisionally classified in the candidate genus "*Endomicrobium*." Members of the TG-1 phylum, for which the name "Endomicrobia" has been proposed, are phylogenetically quite diverse and seem to be present in and also restricted to the guts of those insects (lower termites and wood-feeding cockroaches of the genus *Cryptocercus*) that are in mutualistic association with such cellulose-fermenting flagellates (Stingl et al., 2005).

Firmicutes In clone libraries of lower termites, clones affiliated with the Clostridia are abundant and fall into apparently termite-specific lineages (Hongoh et al., 2003a; Yang et al., 2005). In the higher termite *Cubitermes orthognathus*, they dominated the clone library of the alkaline hindgut sections (Schmitt-Wagner et al., 2003b), which was confirmed using FISH with cluster-specific probes and supported by molecular fingerprints of the different gut compartments (Schmitt-Wagner et al., 2003a).

One of the clostridial clusters from the *Cubitermes orthognathus* clone libraries falls into the *Clostridium propionicum* group (Schmitt-Wagner et al., 2003b). Interestingly, this cluster comprises also clones from the termite *Nasutitermes takasagoensis*, which were localized in the mixed segment between the midgut epithelium and the peritrophic membrane using FISH (Tokuda et al., 2000). Other clones are affiliated with homoacetogenic isolates (see the section Homoacetogenic Bacteria in this Chapter).

Clone libraries and molecular fingerprints indicated that Clostridia dominate also the bacterial microbiota in the most alkaline hindgut compartment (P1) of *Termes comis*, *Pericapritermes lathignathus*, and a *Microcerotermes species* (all Termitinae), whereas Bacilli dominate the P1 of a *Speculitermes* species (Apicotermitinae) (Thongaram et al., 2005). Many of the clones derived from the P1 region form phylogenetic clusters that are unique to termites and are often related to clones obtained from the other insects with alkaline digestive tracts, which suggests that they represent lineages of alkaliphilic bacteria (Schmitt-Wanger et al., 2003; Thongaram et al. 2005).

Bacteroidetes Clones affiliated with the Bacteroidetes were recovered from the guts of numerous termite species (Ohkuma et al., 2002b; Schmitt-Wagner et al., 2003b). There is an enormous diversity of such phylotypes in *Reticulitermes* species (Hongoh et al., 2003a; Yang et al., 2005). Most clones are only distantly related to described taxa and often form monophyletic clusters with clones recovered from the gut of other termite species. While some of the phylotypes seem to be associated with the hindgut cuticle (Yang et al., 2005), others represent epibionts of protozoa (see the section Interactions Between Prokaryotes and Protozoa in this Chapter).

Other Groups Clone libraries of *Reticulitermes* species contained clones related to the Mycoplasmatales in a distinct and apparently termite-specific lineage (Hongoh et al., 2003a; Yang et al., 2005) that were abundant in the protozoan fraction of *Reticulitermes santonensis* (Yang et al., 2005) and comprised also a clone obtained from a symbiont of the termite gut flagellate *Koruga bonita* from *Mastotermes darwiniensis* by single-cell PCR (Fröhlich and König, 1999a). Among the Lactobacillales, most clones were affiliated with the genus *Streptococcus* and were mainly from the midgut clone library. Most clones affiliated with the Proteobacteria formed distinct, termite-specific lineages in the α-subgroup (only distantly related to other lineages of the Rickettsiales) or in the β-subgroup (most closely related to *Dechlorimonas agitatus* or to fermenting bacteria of the genus *Propionivibrio*; Brune et al., 2002). Clones belonging to the δ-subgroup were rare but virtually identical to the sequences of *Desulfovibrio desulfuricans* and of a sulfate-reducing isolate from *Reticulitermes santonensis* (Kuhnigk et al., 1996).

Only a single clone among the >100 clones retrieved from the hindgut of the soil-feeding termite *Cubitermes orthognathus* was affiliated with the Planctomycetales (Schmitt-Wagner et al., 2003b). However, a large fraction of the cells in the posterior hindgut of the closely related *Cubitermes ugandensis* hybridized with a mixture of FISH probes targeting this phylum. This severe underestimation of this phylum in the clone libraries is probably caused by the inadequacy of the commonly used Bacteria-specific

PCR primers to amplify the 16S rRNA genes of planctomycetes (Derakshani et al., 2001) and underlines the importance of backing the results of PCR-based analyses with an independent method. Although FISH analysis indicates that more than one-third of the bacteria in the second hindgut compartment (P3 segment) of *Cubitermes ugandensis* may be planctomycetes (Schmitt-Wagner et al., 2003b), their metabolic function remains obscure.

Archaeal Diversity Molecular phylogenetic profiling of the microbial communities by dot-blot hybridization with domain-specific probes has indicated that archaea represent 0.1–2.6% of small subunit (SSU) rRNA extracted from the guts of 24 nutritionally and taxonomically diverse termite species (Brauman et al., 2001). Interestingly, the relative abundance of archaea seems to be related to the host diet. The percentage of archaeal 16S rRNA among prokaryotic 16S rRNA in the gut of soil-feeding termite species (1.4–3.1%) was significantly higher than in wood-feeding and litter-feeding termite species (0.1–1.7%). This is in agreement with the methane emission rates, which are generally higher among soil-feeding termite species (Brauman et al., 1992), and it has been speculated that the majority of the archaea in termite guts are methanogens (Brauman et al., 2001).

Methanogenic Archaea Partial sequences of the genes encoding for 16S rRNA and for subunit A of the methyl coenzyme M reductase (*mcrA*) of methanogens indicated that the methanogens in *Reticulitermes speratus* belong to the order Methanobacteriales (Ohkuma et al., 1995) and are closely related but not identical to the *Methanobrevibacter* species isolated from *Reticulitermes flavipes* (Leadbetter and Breznak, 1996; Leadbetter et al., 1998; also, see the subsection Methanogenic Archaea in section "Hydrogen Metabolism"). Later studies concentrated on the 16S rRNA genes and confirmed the presence of Methanobacteriales in *Reticulitermes speratus* (Shinzato et al., 1999), *Cryptotermes domesticus* (Ohkuma and Kudo, 1998; Shinzato et al., 2001), *Hodotermopsis sjöstedti* (Ohkuma et al., 1999c), *Neotermes koshunensis*, *Reticulitermes kanmonensis*, *Coptotermes formosanus*, and *Mastotermes darwiniensis* (Shinzato et al., 2001). All sequences cluster within the radiation of the genus *Methanobrevibacter*, but the sequences from termites differ from those of known methanogens, forming unique lineages in the phylogenetic trees. A single clone related to Methanomicrobiales was recovered from *Reticulitermes speratus* (Shinzato et al., 1999).

Dot-blot hybridization indicated that Methanobacteriales constitute one-third to more than one-half of the archaea in the guts of almost all termite species studied (Brauman et al., 2001). By contrast, Methanosarcinales seem to be present only in the guts of about half of the termite species, apparently forming the dominant group of methanogens in 4 of the 24 species studied and accounting for the total archaeal signal in the fungus-growing species *Macrotermes subhyalinus* (Brauman et al., 2001). Additionally, the clones retrieved from the guts of the phylogenetically higher termites *Nasutitermes takasagoensis*, *Odontotermes formosanus* and *Pericapritermes nitobei* clustered mostly among the Methanomicrobiales and Methanosarcinales (Ohkuma et al., 1999c). In a detailed study of archaeal diversity in the gut of the soil-feeding higher termite, *Cubitermes orthognathus*, most archaeal clones were affiliated with Methanobacteriales, Methanomicrobiales and Methanosarcinales, and a few clones had their closest relatives among the Methanococcales (Friedrich et al., 2001). Similar results were obtained in a study with *Cubitermes* fungifaber, which also corroborated that there is little overlap between the communities of methanoarchaea present in the gut and in the food soil (Donovan et al., 2004). In contrast, the similarities between the methanoarchaeal communities of congeneric termites are substantial, and many clones obtained from the intestinal tract of termites cluster with clones retrieved from other insects. However, a purely vertical transmission of the methanogenic gut microbiota is not supported (Donovan et al., 2004).

Methanobrevibacter spp. in *Reticulitermes* are associated with the hindgut wall (Leadbetter and Breznak, 1996; Leadbetter et al., 1998) and, in the gut of *Reticulitermes speratus* and *Hodotermopsis sjoestedti*, attached to the flagellated protist species *Dinenympha* and *Microjoenia* (Tokura et al., 2000): there are indications that the lineages attached to the flagellates are phylogenetically different from those associated with the gut epithelium. Fröhlich and König (1999a) retrieved single cells of endosymbiotic methanogens from the anaerobic flagellate *Pentatrichomonoides scroa* occurring in the hindgut of *Mastotermes darwiniensis* that were affiliated with the genus *Methanobrevibacter*.

Methanogens are among the few groups of organisms for which one can infer metabolic information from the 16S rRNA gene sequence. FISH with an archaea-specific probe revealed that archaea are largely restricted to the gut sections P3 and P4 in *Cubitermes ugandensis*, which is in agreement with the distribution of F_{420}-fluorescent cells and methane-emission rates along the gut axis of *Cubitermes* species (Schmitt-Wagner and Brune, 1999). Cells hybridizing with the archaea-specific probe presented

1.6% and 3.8% of the DAPI-stained cells in the P3 and P4 section, respectively (Schmitt-Wagner et al., 2003b), but since many of the abundant and morphologically diverse F_{420}-fluorescent microorganisms in these gut sections were filamentous forms and appeared to be fragmented or destroyed during homogenization, they were likely underestimated by the FISH analysis.

Non-methanogenic Archaea In a dot-blot analyses of many termite species, the total combined value of the subgroup-specific probes was much lower than that of the Archaea domain probe, indicating that termite guts may contain (possibly non-methanogenic) archaeal populations whose 16S-like rRNAs do not hybridize with probes for methanoarchaeal subgroups employed in this study (Brauman et al., 2001).

Shinzato et al. (1999) provided the first evidence for the presence of Thermoplasmales in the intestinal tract of *Reticulitermes speratus*. Friedrich et al. (2001) obtained numerous clones of Thermoplasmales and a few clones related to the Thermococcales from the gut of the soil-feeding termite *Cubitermes orthognathus* and also documented for the first time the presence of crenarchaeota in an intestinal tract. Donovan et al. (2004) retrieved several clones related to the haloalkaliphile genus *Natronococcus* from *Cubitermes fungifaber*, which is quite intriguing in view of the extreme alkalinity of the anterior hindgut of *Cubitermes* species (Brune and Kühl, 1996).

Spatial Organization As mentioned above, termite guts are axially and radially structured, providing numerous microhabitats with different physicochemical microenvironments (see the section The Gut Microenvironment in this Chapter). Not astonishingly, therefore, the distribution of gut microbiota is not random but seems to be spatially organized. Detailed descriptions of the spatial arrangement of the intestinal prokaryotes in situ (Breznak and Pankratz, 1977; To et al., 1978; To et al., 1980; Czolij et al., 1985; Yara et al., 1989), together with numerous other observations of certain morphotypes in particular regions of the gut (see the section Gut Compartmentation and Microhabitats in this Chapter), indicate that many microhabitats harbor characteristic microbial populations.

Until recently, most of such evidence was based purely on morphological data, and the line of evidence is far from complete. With the advent of molecular tools, however, it became possible to address not only the diversity of the termite gut microbiota but also the spatial distribution of individual phylotypes or phylogenetic groups. A study employing whole-cell hybridization in homogenates of different gut regions and in situ hybridization of gut cryosections with group-specific oligonucleotide probes provided the first results documenting differences in microbial community structure between different regions of the hindgut of *Mastotermes darwiniensis* at the level of phylogenetically defined microbial groups (Berchtold et al., 1999). Other studies used FISH to document the association of gut protozoa with certain phylotypes of hitherto uncultivated spirochetes (Berchtold and König, 1996; Iida et al., 2000; Noda et al., 2003), Bacteroidetes (Wenzel et al., 2003; Stingl et al., 2004), members of the TG-1 phylum (Stingl et al., 2005), or methanogenic archaea (Tokura et al., 2000).

Also, PCR-based approaches allow one to resolve differences in the community structure of different gut regions or gut compartments (Friedrich et al., 2001; Schmitt-Wagner et al., 2003b; Yang et al., 2005). Costs and effort involved in sequencing and phylogenetic analysis limit investigations based on clone analysis, but molecular fingerprinting allows expansion of the investigation of diversity and—observing the necessary cautions inherent to all PCR-based techniques—community structure to include a larger number of samples. Terminal-restriction-fragment-length polymorphism (T-RFLP) analysis in the higher, soil-feeding termite *Cubitermes orthognathus* documented that the different archaeal and bacterial populations are not randomly distributed along the gut and that the prokaryotic communities in the individual gut segments differ considerably with respect to diversity and abundance (Friedrich et al., 2001; Schmitt-Wagner et al., 2003a). By contrast, the bacterial community structure in homologous compartments in three different species of *Cubitermes* was quite similar, indicating the existence of gut-segment-specific communities (Schmitt-Wagner et al., 2003a).

Isolates and Major Metabolic Activities

The major metabolic activities of the gut microbiota have been outlined (Breznak, 2000; Slaytor, 2000; Tholen and Brune, 2000; Fig. 7), but there are still considerable gaps in our knowledge that underline the need for a refined concept (Tholen and Brune, 2000). Numerous attempts have been made to characterize the prokaryotic gut microbiota of termites by isolating microorganisms that either were numerically abundant or possessed a metabolic potential considered important in the metabolism of the hindgut. Many of these efforts have yielded results of uncertain significance, either because the methods were not fully described or because no quantitation of bacteria was made relative to

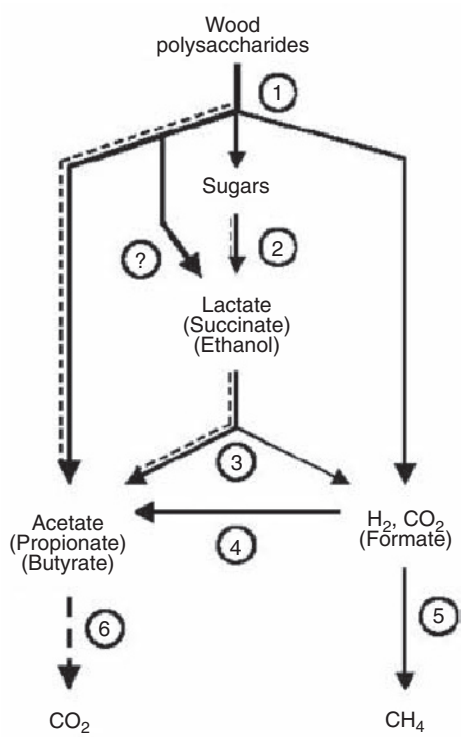

Fig. 7. Schematic presentation of the metabolic processes involved in the fermentative degradation of polysaccharides in the hindgut of *Reticulitermes flavipes*. The dashed lines indicate metabolic fluxes which seem to be strongly influenced by the continuous influx of oxygen into the gut periphery. Line thickness indicates the relative importance of the process. The major metabolic groups are gut flagellates (1), primary (2) and secondary (3) fermenting bacteria, homoacetogens (4), and methanogens (5); it remains to be clarified whether the flagellates are also a major source of lactate (?). Short-chain fatty acids are oxidized by the host (6). Adapted from Brune (2003).

the total number of microorganisms present. As there is little overlap between the existing isolates and the 16S rRNA genes obtained in the molecular studies (see the section Phylogenetic Diversity and Community Structure in this Chapter), it is apparent that many of the microorganisms responsible for the major metabolic activities remain to be cultivated.

Numerically Predominant Isolates Most cultivable heterotrophic bacteria in the hindgut of *Reticulitermes flavipes* are *Streptococcus* and *Enterococcus* species, followed by *Bacteroides* species and representatives of the Enterobacteriaceae, mostly *Citrobacter* species and *Enterobacter cloacae* (Schultz and Breznak, 1978; Tholen et al., 1997). Coccoid lactic acid bacteria also dominated among the isolates obtained from the hindguts of the lower termites *Mastotermes darwiniensis* and *Cryptotermes primus* (Eutick et al., 1978a) and the higher termites *Nasutitermes arborum*, *Thoracotermes macrothorax* and *Anoplotermes pacificus* (Bauer et al., 2000). While *Enterobacter* species were found to dominate among the isolates from the rhinotermitid species *Heterotermes ferox*, *Coptotermes acinaciformes*, *Coptotermes lacteus* and *Schedorhinotermes intermedius* (Eutick et al., 1978a), most isolates from termitid species *Nasutitermes exitiosus*, *Nasutitermes graveolus* and *Nasutitermes walkeri* were staphylococci. Isolates from these genera have been recovered also in earlier studies (e.g., Mannesmann and Piechowski, 1989).

Although some of these studies were at best semiquantitative and many employed only aerobic techniques, the pattern of bacterial species cultivated from each host species is remarkably constant. Most importantly, the majority of the isolates obtained from termite guts are either aerobes or aerotolerant anaerobes. The absence of obligate anaerobes among the isolates in those studies that did not attempt to apply a methodology appropriate for the successful cultivation of such bacteria is not astonishing. However, even in the studies that explicitly used the Hungate technique and employed reduced media for cultivation, the fraction of obligate anaerobes was always smaller than that of aerotolerant anaerobes and aerobes (Schultz and Breznak, 1978; Tholen et al., 1997). It is not clear whether this phenomenon is caused by the oxygen status of the termite gut (see the section Redox Conditions and Oxygen Status in this Chapter) or the strong cultivation bias against certain groups of bacteria (see the section Cultivation Bias in this Chapter).

Cultivation Bias A comparison of the viable counts of heterotrophic bacteria to the direct microscopic counts of the microorganisms in the hindgut of *Reticulitermes flavipes* indicates that about 90% of the microbial cells have escaped cultivation (Schultz and Breznak, 1978; Tholen et al., 1997). Viable counts obtained in a similar study attempting to characterize the major gut bacteria of nine species of termites indicate an even larger cultivation bias (Eutick et al., 1978a).

When the isolates obtained in these studies are compared to the results of the cultivation-independent characterization (see the section Phylogenetic Diversity and Community Structure in this Chapter), there are enormous discrepancies between the frequencies of the phylogenetic groups dominating the clone libraries (Fig. 6) and the species recovered by cultivation. Nevertheless, many isolates are unique to the termite gut habitat, and their characterization has provided valuable information on metabolic properties and other physiological

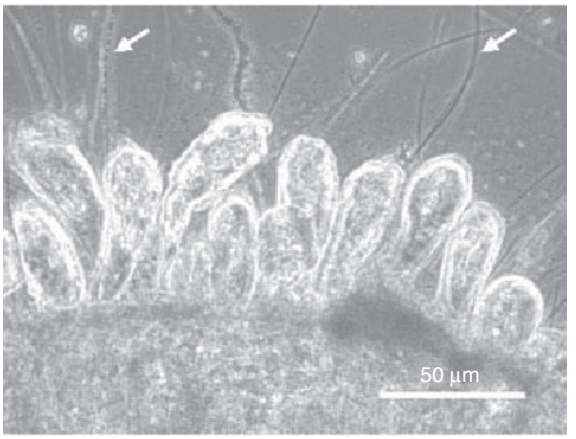

Fig. 8. Phase contrast photomicrograph of the hindgut wall of *Reticulitermes santonensis*, colonized by *Pyrsonympha flagellates* and "Arthromitus"-like filaments (arrows). Reprinted from Yang et al. (2005).

features relevant for the colonization of this particular habitat (Table 1). To increase cultivation efficiency, it will be necessary to develop new cultivation strategies that take into account the special environmental conditions within the gut, in particular the steep physicochemical gradients (see the section The Gut Microenvironment in this Chapter) and the metabolic interactions among the microbiota (see the section Microbe-Microbe Interactions in this Chapter).

Like many arthopods, termites harbor filamentous bacterial morphotypes with refractile inclusions resembling endospores. These bacteria are usually attached to the hindgut wall (Fig. 8) and were first described in 1849 as "Arthromitus" by Leidy (1849, 1881). The filaments have not been cultivated, but Margulis et al. (1998) have proposed that they represent a different life stage of aerobic, rod-shaped bacteria closely related to *Bacillus cereus*, a species group that occurs ubiquitously in soil. However, their conclusions were based merely on the isolation of such bacteria from the boiled intestines of ten species of soil arthropods containing "Arthromitus"-like filaments and on earlier reports on the isolation of *B. cereus* from arthropod guts. A phylogenetic identity of the filaments with the isolates has not been confirmed with molecular methods. In this context, it should be noted that other authors had previously identified the segmented filamentous bacteria in the gut of mice, rats, and chickens as a new lineage of Clostridiales, based on a 16S rRNA gene sequence analysis (Snel et al., 1994). The same group has proposed "Candidatus Arthromitus" as a provisional generic name for the segmented filamentous bacteria falling into this lineage (Snel et al., 1995)—unfortunately without verifying whether they are indeed related to the morphologically similar filaments in arthropods originally described by Leidy.

Lignocellulose Degradation There are numerous reports on the presence in termite guts of enzyme activities acting on different cellulose and hemicellulose preparations. The enzyme activities in foregut and midgut are most likely of host origin or are ingested fungal cellulases that remain active within the gut (see the section Fiber Degradation in this Chapter), whereas most activities in the hindgut are probably due to the microbiota. In the lower termites, it is also important to differentiate between protozoan and bacterial origin.

Cellulolytic Bacteria Cellulolytic prokaryotes have been isolated from the guts of lower and higher termites on numerous occasions (for references, see Breznak [1975], Breznak and Brune [1994b], and Wenzel et al. [2002]). However, many of these efforts either were unsuccessful or have yielded positive results of uncertain significance, either because the methods were not fully described or because the population size had not been established (Schultz and Breznak, 1978). Therefore, the contribution of bacteria to cellulose degradation in the termite gut has always been a matter of debate (see the section Fiber Degradation in this Chapter).

Interestingly, successful attempts to isolate cellulolytic bacteria usually employed oxic cultivation conditions; only a few anaerobic strains have been reported. Hungate isolated an anaerobic actinomycete, "*Micromonospora propionici*," from *Amitermes minimus* (Hungate, 1946). *Clostridium termitidis* was isolated from the gut of the wood-feeding termite *Nasutitermes lujae* (Hethener et al., 1992). All attempts to isolate anaerobic cellulolytic bacteria from the gut of *Reticulitermes flavipes* were negative (Schultz and Breznak, 1978). No cellulose-degrading bacteria were present among the numerically predominant isolates recovered from nine species of termites representing all major families, using both aerobic and anaerobic techniques (Eutick et al., 1978a). No anaerobic cellulolytic bacteria were isolated from the soil-feeding termite *Cubitermes speciosus* using serial dilutions of gut homogenates and the Hungate technique (Brauman et al., 1990b).

By contrast, numerous studies have yielded aerobic bacteria, albeit of often dubious numerical significance. Frequent isolates, found in numerous studies, were assigned to *Serratia marcescens* and *Bacillus cereus* (e.g., Thayer, 1976). Strains of both species, isolated from *Reticulitermes hesperus*, formed soluble, and in the case of *Bacillus cereus* also cell-bound, car-

Table 1. Described species of prokaryotes unique to the intestinal tract of termites.

Group species	Termite species[a]	Unusual feature	References
Firmicutes			
Acetonema longum	*Pterotermes occidentis* (K)	Homoacetogenic	Kane and Breznak, 1991
Bacillus oleronius	*Reticulitermes santonensis* (R)	Degrades aromatic compounds	Kuhnigk et al., 1995
Clostridium mayombei	*Cubitermes speciosus* (T)	Homoacetogenic	Kane et al., 1991
Clostridium termitidis	*Nasutitermes lujae* (T)	Cellulolytic	Hethener et al., 1992
Isoptericola variabilis (formerly *Cellulosimicrobium variabile*)	*Mastotermes darwiniensis* (M)	Cellulolytic	Bakalidou et al., 2002 Stackebrandt et al., 2004
Sporobacter termitidis	*Nasutitermes lujae* (T)	Homoacetogenic	Grech-Mora et al., 1996
Sporomusa aerivorans	*Thoracotermes macrothorax* (T)	Homoacetogenic	Boga and Brune, 2003
Sporomusa termitida	*Nasutitermes nigriceps* (T)	Homoacetogenic	Breznak et al., 1988
Sporotomaculum hydroxybenzoicum	*Cubitermes speciosus* (T)	Degrades aromatic compounds	Brauman et al., 1998
Proteobacteria			
Desulfovibrio intestinalis	*Mastotermes darwiniensis* (M)	Sulfate-reducing	Fröhlich et al., 1999
Desulfovibrio termitidis	*Heterotermes indicola* (R)	Sulfate-reducing	Trinkerl et al., 1990
Fusobacteria			
Sebaldella termitidis (formerly *Bacteroides termitidis*)	*Reticulitermes lucifugus* (R)	Uricolytic	Collins and Shah, 1986
Bacteroidetes			
Candidatus Vestibaculum illigatum[b]	*Neotermes cubanus* (K)	Epibiont of *Staurojoenina* sp.	Stingl et al., 2004
Spirochaetes			
Treponema azotonutricium	*Zootermopsis angusticollis* (Z)	Nitrogen-fixing	Graber et al., 2004
Treponema primitia	*Zootermopsis angusticollis* (Z)	Homoacetogenic	Graber et al., 2004
"Endomicrobia"			
Candidatus Endomicrobium trichonymphae[b]	*Reticulitermes santonensis* (R)	Endobiont of *Trichonympha agilis*	Stingl et al., 2005
Candidatus Endomicrobium pyrsonymphae[b]	*Reticulitermes santonensis* (R)	Endobiont of *Pyrsonympha vertens*	Stingl et al., 2005
Methanobacteriales			
Methanobrevibacter curvatus	*Reticulitermes flavipes* (R)	Methanogenic	Leadbetter and Breznak, 1996
Methanobrevibacter cuticularis	*Reticulitermes flavipes* (R)	Methanogenic	Leadbetter and Breznak, 1996
Methanobrevibacter filiformis	*Reticulitermes flavipes* (R)	Methanogenic	Leadbetter et al., 1998

[a]Termites belong to the families Kalotermitidae (K), Mastotermitidae (M), Rhinotermitidae (R), Termitidae (T), and Termopsidae (Z).
[b]*Candidatus* taxon: not cultivated, but well-characterized with respect to morphology, ultrastructure, phylogeny, and specific location.

boxymethylcellulases (Thayer, 1978). *Serratia marcescens* seems to be a common but minor inhabitant of the intestinal tract of insects that on occasion can become pathogenic (see the section Pathogens in this Chapter).

On the basis of the few studies where the number or density of cellulolytic bacteria in the gut had been determined (e.g., Paul et al., 1993; Wenzel et al., 2002), it appears that bacteria are not very relevant for cellulose digestion. The most abundant among 23 groups of Cellulolytic isolates from *Zootermopsis angusticollis* were closely affiliated with *Bacillus megaterium*, *Bacillus cereus* or *Paenibacillus polymyxa* (Wenzel et al., 2002). Many of the cellulolytic isolates are species known to occur also in soil and other habitats and may represent transient permanent populations of microorganisms ingested with the food, while others are apparently specific for the intestinal tracts of insects (Table 1).

Isoptericola variabilis (formerly *Cellulosimicrobium variabile*; Stackebrandt et al., 2004) and other closely related bacteria were isolated from the hindgut of *Mastotermes darwiniensis* and various other termites (Bakalidou et al., 2002) and form significant populations also in the gut of other insects (Cazemier et al., 2003).

Cellulolytic activity has been detected also among numerous actinomycetes isolated from the gut of several soil-feeding Termitidae (Pasti and Belli, 1985); some of the isolates were also lignolytic (Pasti et al., 1990). Although an unusual association of soil-feeding termites (Termitidae, Termitinae) with actinomycete-like bacteria has been documented (Bignell et al., 1979; Bignell et al., 1980b) and facultatively aerobic actinomycetes have been isolated from the gut, parent soil, and mound materials of the termites *Procubitermes aburiensis* and *Cubitermes severus* (Bignell et al., 1991), their significance in the degradation of lignocellulose remains to be established.

Hemicellulolytic Bacteria Compared to the information on cellulose degradation, our understanding of the degradation of the hemicellulose component of lignocellulose in termite guts is rather meager. Xylanase activity has been observed in midgut and hindgut of several termites, including wood-feeding, soil-feeding, and fungus-cultivating species (Breznak and Brune, 1994b; Rouland, 2000). In the fungus-cultivating *Macrotermes bellicosus*, there is evidence that the source of xylanase activity may be a symbiotic fungus (Matoub and Rouland, 1995), but analogous to the host cellulases, a host origin of the midgut xylanases remains to be scrutinized.

In lower termites, the gut flagellates also seem to play a major role in the degradation of xylan (see the section Role of Intestinal Protozoa in this Chapter). However, also bacteria and yeasts might be involved in the metabolism of hemicelluloses. Aerobic and facultatively anaerobic hemicellulose-degrading bacteria and yeasts were isolated from the guts of several wood-feeding termites, with xylan-degrading bacteria (10^6–10^7 per ml) dominating in *Mastotermes darwiniensis* and xylan-degrading yeasts (10^7–5×10^8 cells per ml) in *Zootermopsis angusticollis* and *Neotermes castaneus* (Schäfer et al., 1996). Gram-positive isolates belonged to the genera *Bacillus* and *Paenibacillus* or to the Actinobacteria, while Gram-negative strains were affiliated with the genera *Pseudomonas*, *Acinetobacter*, and *Ochrobactrum* or with the enterobacteria.

Several strains of alkaliphilic bacteria, which were isolated from the extremely alkaline P1 compartment the soil-feeding *Sinocapritermes mushae* and *Amitermes longignathus* and represent a novel lineage of *Paenibacillus*, express alkalitolerant xylanase activity with a high PH optimum (Ohkuma et al., 2003; Thongaram et al., 2003). An unusual xylanase, distantly related to xylanases of bacteria and fungi colonizing the bovine rumen (GHF11), has been discovered in a genomic library to microbial DNA extracted from the intestinal tract of an unspecified higher termite belonging to the subfamily Nasutiermitinae (Brennan et al., 2004).

Lignin-Degrading Bacteria The degradation of lignin involves the "enzymatic combustion" of the highly recalcitrant polyphenolic resin by peroxidases. Despite a few claims to the contrary, there is currently no conclusive evidence that bacteria lacking extracellular peroxidative activity solubilize or degrade polymeric core lignin significantly, and lignin degradation by the gut microbiota has been questioned (Breznak and Brune, 1994b; Kuhnigk et al., 1994). A ^{13}C-nuclear magnetic resonance (NMR) analysis of the feces of the wood-feeding termites *Microcerotermes parvus* and *Coptotermes formosanus* indicated a preferential loss of polysaccharide during gut passage, whereas lignin accumulated and was modified only in its O-aromatic-C and O-methyl-C components were modified (Hopkins et al., 1998; Hyodo et al., 1999).

However, extensive delignification occurs in the fungal gardens of fungus-cultivating termites (Hyodo et al., 2000) and has to be expected also in any wood colonized by lignolytic fungi. Many lignin-derived aromatic compounds (representing the major subunits and lignin-carbohydrate linkages found in lignins and their depolymerization products) can be degraded by aerobic bacteria, and numerous bacteria degrading lignin

monomers or certain dimeric lignin model compounds have been isolated from various wood-feeding termites (Kuhnigk et al., 1994; Kuhnigk and König, 1997; Harazono et al., 2003). They comprised a wide range of Gram-positive bacteria and Proteobacteria, with strict aerobes dominating in *Nasutitermes nigriceps* and enterobacteria in the lower termites. An aerobic isolate able to degrade hydroxybenzoic acids was described as a new species, *Bacillus oleronius* (Kuhnigk et al., 1995).

All attempts to enumerate bacteria degrading aromatic compounds indicated that termite guts contain significant numbers of aerobic bacteria capable of degrading aromatic rings, whereas anaerobic degradation of the aromatic nucleus appears to be of little significance (Brune et al., 1995b; Kuhnigk and König, 1997). Under anoxic conditions, only ring and side-chain modification seem to be of importance (Kuhnigk et al., 1994; Brune et al., 1995b). Some isolates from termite guts involved in anaerobic modification or degradation of aromatic compounds were novel taxa (Table 1).

The digestibility of lignocellulose is improved if the phenylpropanoic acid residues esterified to the hemicellulose chains and the diferulic ester linkages between the hemicellulose chains in plant cell walls are hydrolyzed, since this increases the solubility of the macromolecules and reduces steric hindrance of hydrolytic enzymes (Jeffries, 1994). A strain of *Clostridium xylanolyticum* (producing hydroxycinnamoyl esterases) that hydrolyzes and then hydrogenates ferulic and *p*-coumaric acid residues has been isolated from the gut of the grass-feeding termite *Tumulitermes pastinator* (McSweeney et al., 1999). Several termites have been shown to possess also activities cleaving non-phenolic β-O-4 type-lignin model compounds representing the major linkage within the lignin macromolecule (Hirai et al., 2000).

Lignin-solubilizing actinomycetes have been isolated from the gut of soil-feeding termites (Pasti et al., 1990), and all isolates were *Streptomyces* strains. Moreover, in lower termites, the only isolate decolorizing Remazol Brilliant Blue and Azure B was a *Streptomyces* strain (Kuhnigk and König, 1997). Since isolation and phylogenetic characterization of actinomycetes from termite guts indicated that the actinomycete flora of termites depends largely on their geographical origin (Watanabe et al., 2003), it is not clear whether such isolates simply represent spores ingested with the food.

Oxygen Reduction The bacteria and protozoa colonizing the gut periphery, especially those directly associated with the gut epithelium (Fig. 8), are exposed to the continuous influx of oxygen (Brune et al., 2000b). Oxygen microprofiles indicate that the anoxic status of the termite hindguts must be attributed to the oxygen consumption of the microbiota located in the gut periphery (see the section Redox Conditions and Oxygen Status in this Chapter), which can represent a substantial fraction of the respiratory activity of the host (Brune et al., 1995a). This conclusion is supported by the predominance of facultatively or even obligately aerobic isolates in all cultivation-based studies.

However, there are several indications that not all oxygen-consuming activities in termite guts are of a respiratory nature. In the extremely alkaline gut regions, the high rates of oxygen consumption might be partly attributable also to chemical reactions, such as the autoxidation of phenolic residues in lignin or humic substances (see Kappler and Brune, 1999). On the other hand, radiotracer analysis of the in situ metabolism in the hindgut of *Reticulitermes flavipes* demonstrated that the high oxygen fluxes also significantly influence fermentation processes in the hindgut (Tholen and Brune, 2000). This is supported by the oxygen reduction potential found in obligate anaerobes such as lactic acid bacteria (see the section Lactic Acid Bacteria in this Chapter), homoacetogenic bacteria (see the section Homoacetogenic Bacteria in this Chapter), and sulfate-reducing bacteria (see the section Sulfate-Reducing Bacteria in this Chapter).

Microbial Fermentations In lower termites, the bulk of the fermentative activity should be caused by the intestinal protozoa, which immediately phagocytize the wood particles entering the hindgut. The identity of the substrates of the prokaryotic microbiota is far from clear. Since most of the prokaryotic symbionts probably do not participate in fiber degradation, presumably they ferment either soluble products entering the hindgut from the midgut or intermediates released by the protozoa (Fig. 7). The composition of short-chain fatty acids and other fermentation products in the hindgut fluid of termites indicates that a variety of microbial fermentations occur simultaneously (Schultz and Breznak, 1979; Odelson and Breznak, 1983; Anklin-Mühlemann et al., 1995; Tholen and Brune, 2000). However, the exact nature and amount of the monomeric substrates entering the hindgut are not known, and the spectrum of fermentation products formed by the protozoa might be much larger than initially assumed (Tholen and Brune, 2000).

The apparent absence of pyruvate dehydrogenase activity in termite tissues (O'Brien and Breznak, 1984) and several other observations

prompted the suggestion that the termite releases pyruvate into the hindgut to be converted to acetate and reabsorbed by the host (Slaytor et al., 1997; Slaytor, 2000). However, recent findings documented that termite mitochondria possess a pyruvate dehydrogenase complex, although the difference in activity suggests differences in pyruvate metabolism between lower and higher termites (Itakura et al., 1999; Itakura et al., 2003).

Lactobacillales Coccoid lactic acid bacteria predominate among the carbohydrate-utilizing bacteria isolated from the hindguts of termites of the families Mastotermitidae, Kalotermitidae, and Rhinotermitidae (Eutick et al., 1978a; Schultz and Breznak, 1978; Tholen et al., 1997; Bauer et al., 2000) and have been isolated also from several representatives of the Termitidae (Eutick et al., 1978a; Bauer et al., 2000). The most abundant isolates obtained from the hindguts of *Reticulitermes flavipes* and *Thoracotermes macrothorax* belong to the genera *Enterococcus* and *Lactococcus* (Bauer et al., 2000). Howerver, the low frequency of such clones in cultivation-independent studies indicates that lactic acid bacteria might be present only in moderate numbers (Hongoh et al., 2003a; Schmitt-Wagner et al., 2003b; Yang et al., 2005).

Physiological characterization of the isolates has revealed high potential rates of O_2 reduction in the presence of fermentable substrates (Tholen et al., 1997; Bauer et al., 2000), which might represent an adaptation to variable oxygen tensions and could explain why lactococci and enterococci are regularly encountered in the intestinal tracts of termites and other insects and possibly restricted to specific compartments of the intestinal tract (Yang et al., 2005).

Clostridiales Compared to their abundance in bacterial clone libraries derived from termite guts, clostridial isolates are underrepresented in cultivation-based studies. Several isolates have been described as new species and seem to be unique to termite guts (Table 1), but their numerical significance is either low or has not been established. Bacteria distantly related to *Clostridium oroticum*, detected in an alkaline enrichment culture derived from gut homogenates of *Pericapritermes latignathus*, may prove to be first exception, since they were represented also in a clone library obtained from the P1 compartment (Thongaram et al., 2005).

Bacteroidetes Besides the coccoid lactic acid bacteria, a relatively large proportion of the isolates from the hindgut of *Reticulitermes flavipes* have been identified as *Bacteroides* species (Schultz and Breznak, 1978). The strains fall into two groups: aeroduric anaerobes fermenting lactate to propionate and acetate (Schultz and Breznak, 1979) and anaerobic strains forming butyrate and isobutyrate as characteristic products from complex media (Schultz and Breznak, 1978). The only described *Bacteroides* species isolated from termite guts, *Bacteroides termitidis* (Sebald) from *Reticulitermes lucifugus*, was phylogenetically misplaced (Paster et al., 1985) and recently reclassified among the Fusobacteria in the genus *Sebaldella* (Collins and Shah, 1986). A recent isolate, obtained from the hindgut of *Reticulitermes speratus* by dilution plating of gut suspensions, has a 16S rRNA gene sequence identical to that of a group of clones recovered exclusively from termite guts, representing a lineage of bacteria within the radiation of the Bacteroides subgroup but only distantly related to the genus *Bacteroides* (Ohkuma et al., 2002b).

The uricolytic activity of *Bacteroides* isolates from termite guts (Potrikus and Breznak, 1980) suggests a potential role in nitrogen cycling (see the section Nitrogen Recycling in this Chapter). Many of the clones from termite guts clustering among the Bacteroidetes fall into a lineage comprising epibionts of protozoa (see the section Interactions Between Prokaryotes and Protozoa in this Chapter).

Hydrogen Metabolism The enormous accumulation of hydrogen at the gut center and the steep radial hydrogen gradients in the gut periphery of several termites (Ebert and Brune, 1997; Schmitt-Wagner and Brune, 1999) indicate that molecular hydrogen is a key intermediate in the microbial food chain. Hydrogen-dependent CO_2 reduction by methanogens and homoacetogens is probably the most important hydrogen sink in termite hindguts. To understand the metabolism in termite guts, it is important to identify the hydrogen-producing and hydrogen-consuming populations and their functional interactions (see the section Microbe-Microbe Interactions in this Chapter).

Hydrogen-Producing Microorganisms On the basis of the few pure culture studies available, it is generally assumed that the polysaccharides of the wood particles taken up by the gut flagellates are oxidized to acetate and CO_2, and the reducing equivalents are released as H_2 (see Breznak and Brune [1994b] and Brune and Stingl [2005]; Fig. 7). This is in agreement with the enormous accumulation of hydrogen in the hindgut of *Reticulitermes flavipes* (Ebert and Brune, 1997; Fig. 4). In view of the wide variety of clostridial clones retrieved from *Reticulitermes* species (Hongoh et al., 2003a; Yang et al., 2005), bacterial fermentations may also contribute to the intestinal H_2 production. Such assumptions are

substantiated by the considerable amounts of H_2 produced in certain gut regions of soil-feeding *Cubitermes* species (Schmitt-Wagner and Brune, 1999), a group of termites that lack gut flagellates but contain a similar assemblage of clostridial clones (Schmitt-Wagner et al., 2003b). The cellulolytic bacterium *Clostridium termitidis* isolated from *Nasutitermes lujae* (Hethener et al., 1992) is affiliated with a number of clones from *Cubitermes orthognathus* guts (Schmitt-Wagner et al., 2003b), but nothing is known about the physiology of the bacteria represented by these 16S rRNA genes.

Homoacetogenic Bacteria Reductive acetogenesis from H_2 and CO_2 in termite gut homogenates was first demonstrated by Breznak and Switzer (1986). In the following years, the presence of homoacetogenic activities was established for a large number of termite species from all major feeding guilds, including representatives of wood-feeding, fungus-cultivating, and soil-feeding termites (Breznak and Kane, 1990; Brauman et al., 1992; Williams et al., 1994). Although reductive acetogenesis in gut homogenates of soil-feeding termites was always outcompeted as a hydrogen sink by methanogenesis (Brauman et al., 1992; Breznak, 1994a), microinjection of $H^{14}CO_3^-$ into intact hindguts of soil-feeding *Cubitermes* spp. has identified a high potential for reductive acetogenesis (Tholen and Brune, 1999), which indicates that the contribution of reductive acetogenesis to the overall electron flow in the guts of soil-feeding termites might be larger than expected. Possibly, part of the explanation lies in the ability of homoacetogens to grow mixotrophically on H_2 and other substrates (Breznak and Switzer, 1991; Breznak, 1994a).

Although reductive acetogenesis has been demonstrated to occur in a large number of termite species from all major feeding guilds (Brauman et al., 1992), only seven species of homoacetogenic bacteria from termites have been described to date (Table 1). *Sporomusa termitida* (isolated from *Nasutitermes nigriceps*; Breznak et al., 1988), *Sporomusa aerivorans* (isolated from *Thoracotermes macrothorax*; Boga et al., 2003b), and *Acetonema longum* (isolated from *Pterotermes occidentis*; Kane and Breznak, 1991a) are three spore-forming representatives in the *Sporomusa* group of Gram-positive bacteria that are characterized by a Gram-negative cell wall. All isolates are capable of H_2-dependent reduction of CO_2 to acetate and possess a large potential for hydrogen-dependent oxygen reduction (Boga and Brune, 2003a).

Clostridium mayombei has been isolated from *Cubitermes speciosus* (Kane et al., 1991b) and belongs to the *Clostridium lituseburense* group. A very unusual homoacetogen (*Sporobacter termitidis*, isolated from *Nasutitermes lujae*) clusters in the *Clostridium leptum* group together with numerous clones of 16S rRNA genes retrieved from the anterior hindgut of *Cubitermes orthognathus* (Schmitt-Wagner et al., 2003b). It grows exclusively by the disproportionation of methyl groups derived from methoxylated aromatic compounds but not on $H_2 + CO_2$ or other typical substrates of homoacetogens, and it methylates sulfide and cysteine if these compounds are present in the medium (Grech-Mora et al., 1996).

Recently, numerous spirochetal strains have been isolated from the gut of *Zootermopsis angusticollis* (Leadbetter et al., 1999). They belong to the *Treponema* branch of Spirochaetes that contains mainly sequences obtained from termite guts (Lilburn et al., 1999), and many are capable of H_2-dependent acetogenesis from CO_2 (Leadbetter et al., 1999). One of the homoacetogenic isolates, which has been described as a new species, *Treponema primitia* (Graber et al., 2004b), utilizes a wide range of substrates and is capable of mixotrophic growth, i.e., the simultaneous utilization of H_2 and organic substrates (Graber and Breznak, 2004a). These represent important clues for the unknown metabolic function of the enormous populations of spirochetes colonizing the hindgut lumen of termites and the surfaces of many intestinal protozoa (Breznak, 2002).

Generally, there seems to be a strong cultivation bias against homoacetogens from termite guts, and their numerical abundance and contribution to reductive acetogenesis in situ are not clear. Recently, sequences clustering with the formyl tetrahydrofolate synthase (FTHFS) homologues of termite-gut spirochetes were found to dominate the diversity of genes in the hindgut of *Zootermopsis angusticollis* coding for FTHFS (Salmassi and Leadbetter, 2003). FTHFS is a key enzyme in reductive acetogenesis, which makes the FTHFS gene a good functional marker for homoacetogenic bacteria (Leaphart and Lovell, 2001; Leaphart et al., 2003). Quite likely therefore homoacetogenic spirochetes are responsible for the large potential activities of H_2-dependent acetogenesis encountered in most wood-feeding termites (Breznak, 1994a; Breznak, 2002).

All investigated homoacetogens isolated from termite guts catalyzed hydrogen-dependent oxygen reduction (Boga and Brune, 2003a); the isolate *Sporomusa aerivorans* has the highest known oxygen-reducing capacity among obligately anaerobic bacteria other than sulfate-reducing bacteria of the genus *Desulfovibrio* (see the section Sulfate-Reducing Bacteria in this Chapter), which possibly indicates an adaptation to the oxygen status of the gut environment.

Although the location of homoacetogens relative to the oxygen gradient remains to be established, it is rather unlikely that the homoacetogens in *Reticulitermes flavipes* contribute substantially to the removal of oxygen diffusing into the gut: in contrast to methanogenesis, the in situ rates of reductive acetogenesis in this termite species are not affected by oxygen (Tholen and Brune, 2000).

Methanogenic Archaea Methane formation by termites, first documented by Breznak (Breznak, 1975; Odelson and Breznak, 1983), has received considerable attention owing to its implication for the global methane budget (Sanderson, 1996). Methane formation is restricted to the archaea; therefore, the methane production by all termites investigated indicates that methanogenic archaea are present in virtually all termites (Brauman et al., 1992). Furthermore, the gas bubbles around termites entrapped in amber (Fig. 9), which have been attributed to continuing methanogenesis in the fresh resin, are palaeontologic evidence for the presence of methanogenic archaea in termite guts.

The autofluorescence of coenzyme F_{420} allows easy visualization of methanogens by epifluorescence microscopy. They are either located free in the hindgut lumen, attached to the hindgut cuticle, or associated with other microorganisms (Breznak and Brune, 1994b). In *Zootermopsis*, methanogens are almost exclusively associated with the gut flagellates (Lee et al., 1987). On the basis of their F_{420}-like autofluorescence, filamentous bacteria colonizing the cuticular spines in the fourth proctodeal segment of soil-feeding termites (Bignell et al., 1980b) also appear to be methanogens (Schmitt-Wagner et al., 2003b).

Fig. 9. Termites entrapped in a block of copal (young amber) from the Andean uplift region of Boyaca Province, Colombia (Pleistocene). The photograph shows gas bubbles around the termites, presumably methane that escaped from the body after the termites had been engulfed by the resin.

Despite their relative abundance and phylogenetic diversity in termite guts (see the section Archaeal Diversity in this Chapter), to date only a few methanogens from *Reticulitermes flavipes* have been isolated in pure culture. The isolates represent new species in the genus *Methanobrevibacter* (Table 1), grow best with H_2 and CO_2, and form large populations that are attached to the luminal side of the gut epithelium or adhere to other prokaryotes colonizing the hindgut wall (Leadbetter and Breznak, 1996; Leadbetter et al., 1998). Already the location in the microoxic gut periphery and the presence of catalase activity in *Methanobrevibacter cuticularis* indicated a considerable oxygen tolerance (Leadbetter and Breznak, 1996). This was substantiated by the finding that – despite a general inability among Methanobacteriales to synthesize heme—the wetwood isolate *Methanobrevibacter arboriphilus* expresses a catalase when the medium is supplemented with hemin (Shima et al., 2001). The documentation of a $F_{420}H_2$ oxidase in *M. arboriphilus* (Seedorf et al., 2004) finally provided also a biochemical basis for the high rates of H_2-dependent O_2 reduction exhibited by *Methanobrevibacter* species colonizing the periphery of the termite hindgut (A. Tholen and A. Brune, unpublished results) and their apparent ability to cope with the continuous influx of oxygen (Leadbetter and Breznak, 1996; Ebert and Brune 1997).

Sulfate-Reducing Bacteria Sulfate-reducing bacteria have been isolated from the intestinal tracts of many different termite species (Brauman et al., 1990a; Brauman et al., 1990b; Trinkerl et al., 1990; Kuhnigk et al., 1996; Fröhlich et al., 1999b). All isolates are members of the genus *Desulfovibrio* and seem to form substantial populations in the gut of certain termites. The relevance of sulfate reduction under in situ conditions is not clear, but the sulfate-reducing bacteria in termite guts might partake also in the removal of oxygen or play a role in syntrophic fermentations (Kuhnigk et al., 1996). Like other *Desulfovibrio* species isolated from sediments (for references, see Cypionka, 2000), all strains isolated from termite guts show extremely high rates of O_2 reduction in the presence of H_2 (Kuhnigk et al., 1996; Fröhlich et al., 1999b).

Nitrogen Transformations The diet of the termites ranges from sound wood to lignocellulosic plant materials in various stages of humification, including soil and animal dung. Owing to the high C-to-N ratio of sound wood, many xylophagous termites are strongly limited by nitrogen (Collins, 1983). They conserve nitrogen by recycling—a strategy that has been termed "carbon

elimination" (Higashi et al., 1992). Another way to improve the C/N balance of the colony is through nitrogen fixation.

This section will summarize the present knowledge on these processes and the microorganisms involved. For details, the reader is referred to the comprehensive review of Breznak (2000).

Nitrogen Recycling The best way to deal with a rare resource is conservation and recycling. Termites, like other insects, secrete uric acid and urea, the waste products of nucleic acid and protein metabolism, via the Malpighian tubules into the intestinal tract (Terra, 1990; Fig. 3). Potrikus and Breznak (1981) demonstrated that termites lack uricase and that uric acid is recycled by anaerobic bacteria in the hindgut of *Reticulitermes flavipes*, including *Streptococcus* and *Citrobacter* species and *Bacteroides* (now *Sebaldella*) *termitidis* (Potrikus and Breznak, 1980).

Despite the low nitrogen content of the diet, nitrogen recycling creates high ammonia concentrations in the hindgut of wood-feeding termites, which allows the maintenance of an active gut microbiota and thus ensures high rates of carbon mineralization. The concentration of ammonia in the paunch of *Nasutitermes walkeri* is in the range of 3 mM (Slaytor and Chappell, 1994). The efficient assimilation of ammonium into microbial biomass (and possibly also resorption of ammonium in the posterior hindgut) avoids the loss of nitrogen via the feces (Breznak, 2000). The transformation to high-quality microbial protein also leads to an upgrading of any low-quality nitrogen in the diet.

The nitrogen cycle is closed by the digestion of microbial cells. Since termites cannot access the microbes in the hindgut directly, worker larvae solicit hindgut contents from their nestmates. This behavior, termed "proctodeal trophallaxis," is unique to this group of social insects and increases in frequency with the level of nitrogen limitation (Machida et al., 2001). Digestion of the hindgut contents and resorption of the nitrogenous products probably take place in the foregut and midgut (Fig. 3), as indicated by lysozyme and protease activities in these gut regions (Fujita et al., 2001; Fujita and Abe, 2002; Fujita, 2004). The efficiency of nitrogen conservation within the colony is increased further by the consumption of exuviae and dead nestmates.

Nitrogen Fixation Though nitrogen cycling is efficient in termites, termite colony growth is limited by the net nitrogen taken in with food (Breznak, 2000). Therefore, many termites show a preference for lignocellulosic substrates that are colonized by fungi and therefore have a decreased C-to-N ratio (Amburgey et al., 1980; Cornelius et al., 2002). Termites living on sound wood, however, rely on the exclusive capacity of their prokaryotic gut microbiota to fix atmospheric nitrogen.

After the first convincing demonstrations of N_2 fixation in termites using the acetylene reduction assay (Benemann, 1973; Breznak et al., 1973) and the incorporation of ^{15}N into biomass by termites incubated under an $^{15}N_2$-enriched atmosphere (Bentley, 1984), a large body of data on nitrogen fixation in termite guts has accumulated. A few aspects will be mentioned; for more details, the reader is again referred to the excellent review of this subject by Breznak (2000).

Nitrogen fixation is widespread among termites, although the rates differ considerably. Nitrogenase activity in certain *Nasutitermes* spp. would be sufficient to double the nitrogen content of a colony within a few years (Breznak, 2000), and stable isotope analysis has revealed that 30–60% of the nitrogen in *Neotermes koshunensis* workers is derived via this pathway (Tayasu et al., 1994).

However, the nitrogen content of the diet increases with humification of the organic matter, and peptides, amino sugars, and microbial biomass are potential sources of nutrition for soil-feeding *Cubitermes* spp. (Ji et al., 2000; Ji and Brune, 2001). The natural abundance of the ^{15}N isotope indicates that dietary nitrogen and not atmospheric nitrogen fixation is an important nitrogen source in humivorous termite species (Tayasu et al., 1997; Tayasu, 1998). The enormous ammonium concentrations in feces and nests (constructed from feces) of soil-feeding termites (Ndiaye et al., 2004; R. Ji and A. Brune, unpublished results) suggest that termites in this feeding guild are not nitrogen-limited.

There are also many indications that the nitrogen fixation rates cannot easily be extrapolated to the colony level or ecosystem level. Apart from methodological aspects, there are interspecific differences, intraspecific variations, seasonal patterns, and effects of laboratory maintenance (Curtis and Waller, 1995; Curtis and Waller, 1998) to consider. Additionally, the reduced oxygen partial pressure in subterraneous nests and galleries has to be taken into account (Curtis and Waller, 1996). The problems are discussed in detail by Breznak (2000).

Dinitrogen-fixing bacteria isolated from termite guts include enterobacteria (French et al., 1976; Potrikus and Breznak, 1977; Eutick et al., 1978a), sulfate-reducing bacteria (Kuhnigk et al., 1996), and spirochetes (Leadbetter et al., 1999; Graber et al., 2004b). Molecular approaches, using the *nifH* gene as a functional marker, have revealed a much wider spectrum of potentially N_2-fixing microorganisms, including clostridia, Proteobacteria, and methanogenic archaea, comprising several sequence clusters

unique to termites (Ohkuma et al., 1996b; Ohkuma et al., 1999b). However, a gene-expression study at the community level has revealed that only a few *nifH* homologs, probably representing alternative nitrogenases (*anf* genes), were actually transcribed under in situ conditions in the gut of *Neotermes koshunensis* (Noda et al., 1999). The preferential expression of *anf* genes might be related to an insufficient molybdenum (Mo) supply with the lignocellulosic diet (Ohkuma, 2002a). The number of unrelated *nifH* homologs in a single spirochete species and the variety of apparently spirochete-related *nifH* homologs in hindgut clone libraries indicate that spirochetes are potentially important nitrogen-fixing microorganisms in termite guts (Lilburn et al., 2001).

Symbiotic Interactions

The enormous complexity of this subject emerges from the large number of species present within the gut, the different organizational levels of the potential partners, their metabolic capacities and topological orientation, and the numerous possibilities for metabolic interactions especially within anaerobic communities. In addition, symbiotic interactions can be extremely specific with respect to the partners involved or may simply consist of a cross-feeding of metabolites or nutrients between populations that are not even in direct contact with each other.

Microbe–Microbe Interactions

Trophic cascades and cross-feeding of metabolites can form a network of interactions between the individual populations of any microbial community. In termite guts, the resulting uneven distribution of sources and sinks of microbial metabolites within the system gives rise to metabolic gradients (see the section Physicochemical Gradients in this Chapter), which are indicators of the location of microbial activities in situ. Additionally, the spatial organization of different populations, including intimate associations between prokaryotes and protozoa, underlines the importance of studying the nature of the respective interactions.

Interactions Among Prokaryotes At least three metabolically different groups of microorganisms are involved in the metabolism of hydrogen in the gut of lower termites: the protozoa, which produce H_2 as a product of their fermentative metabolism, and methanogens and homoacetogens, which reduce CO_2 to methane or acetate, respectively (see the section Hydrogen Metabolism in this Chapter). It is generally assumed that hydrogenotrophic methanogens outcompete homoacetogens owing to their higher affinity for the common substrate (Cord-Ruwisch et al., 1988). Nevertheless, both metabolic groups occur simultaneously in the hindgut of termites. In *Reticulitermes flavipes*, where methanogens are mostly restricted to the gut periphery (Leadbetter and Breznak, 1996), in situ rates of reductive acetogenesis surpass those of methanogenesis considerably (Tholen and Brune, 2000), whereas in *Zootermopsis angusticollis*, where methanogens are located mostly inside the gut protozoa (Lee et al., 1987), methanogenesis appears to be the major hydrogenotrophic process (Brauman et al., 1992).

The methanogens in termites seem to be hydrogen-limited in situ, as indicated by the stimulation of methane emission by externally supplied H_2 (Odelson and Breznak, 1983; Messer and Lee, 1989; Ebert and Brune, 1997; Schmitt-Wagner and Brune, 1999). Also, the per cell rates of methanogenesis determined in vitro with *Methanobrevibacter cuticularis*, multiplied by the viable counts of methanogens in *Reticulitermes flavipes*, are much higher than the methane emission rate of living termites (Leadbetter and Breznak, 1996). The relative rates of methane and hydrogen emission by different termites vary considerably (Sugimoto et al., 1998), and evidence is accumulating that the spatial organization of the hydrogen-producing and hydrogen-consuming microorganisms determines the competition and coexistence of different populations (Ebert and Brune, 1997; Tholen and Brune, 2000).

Although reductive acetogenesis is outcompeted as a hydrogen sink by methanogenesis in gut homogenates of soil-feeding termites (Brauman et al., 1992), intact guts of soil-feeding *Cubitermes* spp. show a large potential for reductive acetogenesis (Tholen and Brune, 1999). Taking into account the possibility of an intercompartmental transfer of H_2 (Schmitt-Wagner and Brune, 1999), as has been demonstrated between the midgut and hindgut compartments of cockroaches (Lemke et al., 2001), the contribution of reductive acetogenesis to the overall electron flow in the guts of soil-feeding termites might be larger than expected.

A cross-feeding of hydrogen could also be the basis for the interaction between the prokaryotic epibionts on filamentous bacteria in the gut of *Reticulitermes flavipes* (Breznak and Pankratz, 1977), some of which seem to be methanogenic archaea (Leadbetter and Breznak, 1996). Schultz and Breznak (1979) had demonstrated a cross-feeding of lactate between *Lactococcus lactis* and a propionigenic *Bacteroides* species, to consolidate the predominance of these species among the cultivable bacteria in the hindgut fluid of

Reticulitermes flavipes with the absence of lactate accumulation. Microinjection of ^{14}C-labeled lactate confirmed that lactate is an important intermediate under in vivo conditions but raised several new questions regarding the source of lactate and the role of oxygen in shifting the product spectrum from propionate to acetate (Tholen and Brune, 2000).

An example for cross-feeding of nutrients has been provided by Graber and Breznak (2005), who made a convincing case that *Treponema primitia*, a homoacetogenic spirochete in the gut of *Zootermopsis angusticollis* that requires folate for growth, benefits from the excretion of 5-formyl-tetrahydrofolate by other community members (*Lactococcus lactis* and *Serratia grimesii*).

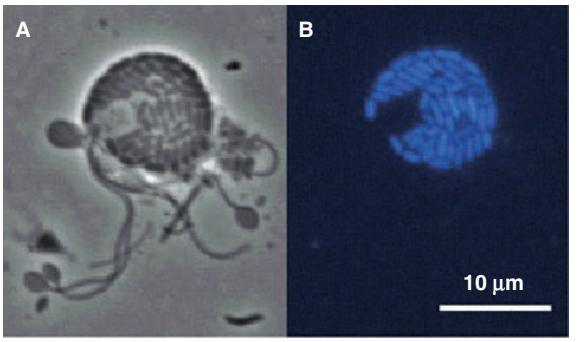

Fig. 10. Phase contrast (A) and epifluorescence (B) photomicrographs of a small trichomonad in *Schedorhinotermes lamanianus* and its attached epibionts, showing the typical F_{420}-autofluorescence of methanogenic archaea. Photomicrographs taken by M. Pester.

Interactions Between Prokaryotes and Protozoa Most of the flagellate protozoa in the guts of lower termites are associated with prokaryotic microorganisms, either as epibionts on their cell surface or as endobionts in the cytoplasm and in the nucleus. The abundance and variety of such associations are documented by numerous electron microscopy studies (for references, see Honigberg [1970], Radek [1999a], Inoue et al. [2000], and Dolan [2001]), but virtually nothing is known about the physiological basis of these associations, especially the metabolic interactions (Brune and Stingl, 2005).

Cook (1932) was the first to observe the emission of a gas other than CO_2 by *Termopsis nevadensis* (syn. *Zootermopsis nevadensis*) and speculated that intestinal protozoa could produce hydrogen, methane, or possibly a mixture of both. Many decades later, termites were shown to emit both hydrogen and methane (Breznak, 1975; Odelson and Breznak, 1983), and axenic cultures of *Trichomitopsis termopsidis* (a gut flagellate from *Zootermopsis*) were shown to produce only hydrogen after methanogenic symbionts were eliminated by treating the cultures with bromoethanesulfonate, an inhibitor of methanogenesis (Odelson and Breznak, 1985a; Odelson and Breznak, 1985b).

Methanogenic symbionts of protozoa can be easily visualized by epifluorescence microscopy (Fig. 10). Lee et al. (1987) reported that methanogenic bacteria in the hindgut of *Zootermopsis angusticollis* are associated only with the small flagellated protozoa *Trichomitopsis termopsidis*, *Tricercomitus termopsidis* and *Hexamastix termopsidis*, whereas they were not observed in the large, hypermastigid protozoa. On the basis of the results of various treatments that selectively eliminated or affected certain protozoa from the gut of this termite, Messer and Lee (1989) concluded that the large protozoa of the genus *Trichonympha* were the most important hydrogen source in the hindgut, whereas the methanogenic symbionts of *Trichomitopsis termopsidis* produced most of the methane.

The large gut flagellates are often colonized by epibiotic bacteria. The presence of special attachment sites on the cell envelope of the flagellates (e.g., Tamm, 1980; Radek et al., 1992; Radek et al., 1996; Dolan and Margulis, 1997; Stingl et al., 2004) indicates a tight association. In some cases, the epibionts seem to be responsible for the locomotion of the host.

The polymastigote flagellate *Mixotricha paradoxa*, which occurs exclusively in the gut of *Mastotermes darwiniensis*, uses its four flagella only for steering. The cell is propelled by the many thousands of spirochetes that cover most of the body surface. The spirochetes are attached to projecting brackets of the cell surface in a manner that allows the helical movement of the individual cells to travel in metachronal waves along the cell surface of the host, resulting in locomotion (Cleveland and Grimstone, 1964). The epibiotic spirochetes were recently identified as members of the *Treponema* cluster by 16S rRNA gene sequence analysis (Wenzel et al., 2003). In addition, a second, rod-shaped epibiont is intimately associated with the cell surface by specific attachment sites (Fig. 11); it is affiliated with other uncultivated Bacteroidales.

The devescovinid flagellate *Caduceia versatilis* (d'Ambrosio et al., 1999) carries two different, rod-shaped and filamentous, epibionts. In this case, the host is propelled by the self-synchronizing movement of the flagella of several thousand rod-shaped bacteria, which are deeply embedded into its the cell surface (Tamm, 1982). The epibionts of *Staurojoenina* flagellates (Fig. 12), recently assigned to the candidate taxon "*Vestibaculum illigatum*" (Stingl et al., 2004), have a similar morphology, although in this flagellate,

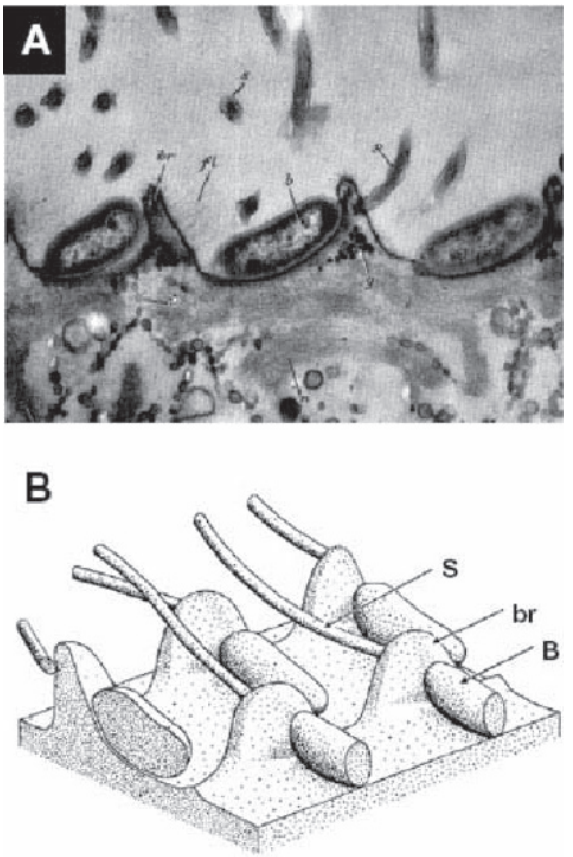

Fig. 11. Transmission electron micrograph (A) of the epibiotic bacteria on the cell surface of *Mixotricha paradoxa*, a large flagellate occurring exclusively in the gut of *Mastotermes darwiniensis* that lives in a motility symbiosis with prokaryotes. The schematic presentation (B) illustrates the regular arrangement of the spirochetal (S) and rod-shaped (B) epibionts and the special attachment brackets (br) at the cell surface. Reproduced from Cleveland and Grimstone (1964).

motility is not due to the bacteria but to their own flagella. Nevertheless, "*Vestibaculum illigatum*" falls into the same cluster of Bacteroidales as the rod-shaped epibiont of *Mixotricha paradoxa* (Stingl et al., 2004), a lineage that also contains numerous clones obtained from other termites (Ohkuma et al., 2002b; Yang et al., 2005).

The larger flagellate species in the gut of *Reticulitermes* species harbor numerous prokaryotic endobionts within their cytoplasm (Bloodgood et al., 1974; Bloodgood and Fitzharris, 1976). The endobionts of *Trichonympha* and *Pyrsonympha* species in *Reticulitermes santonensis* (Fig. 13) belong to the candidate phylum "Endomicrobia", which seem to occur exclusively in termite gut flagellates (Stingl et al., 2005). To date, there is no indication of their possible function. An involvement in hydrogen metabolism is unlikely

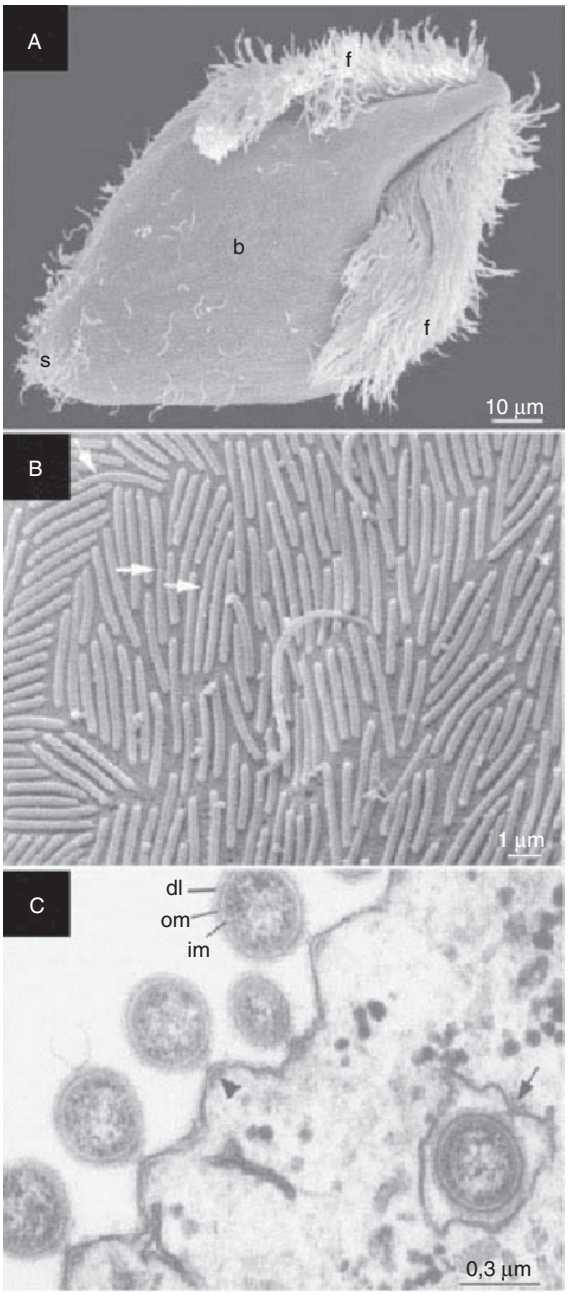

Fig. 12. Scanning (A, B) and transmission (C) electron micrographs of *Staurojoenina* sp. from *Neotermes cubanus*. A) Overview of a cell, showing two of the four flagellar tufts (f), numerous bacterial rods (b), and occasional spirochetes (s) attached to the surface. B) Close-up of the cell surface, showing single spirochetes between the ectobiotic rods. Arrows point to early stages of cell division. C) Cross-section of the epibiotic rods. In addition to an inner membrane (im) and outer membrane (om), the cell is surrounded by a diffuse layer (dl). Electron-dense material supports the plasma membrane of the flagellate below the attachment sites (arrowhead). The arrow points to a phagocytized rod-shaped bacterium with remnants of attachment complex. Reprinted from Stingl et al. (2004).

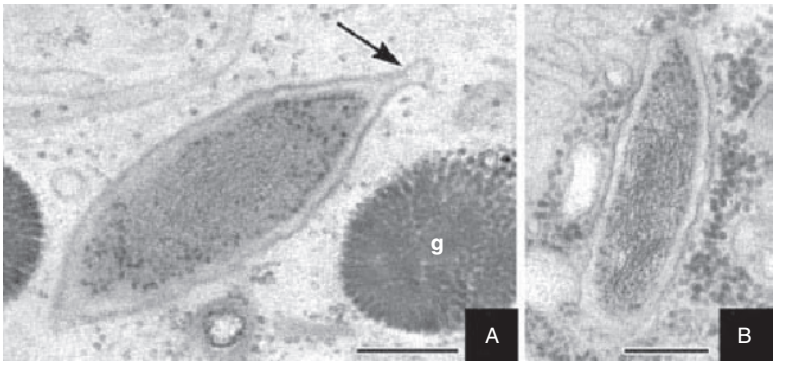

Fig. 13. Transmission-electron micrographs of ultrathin sections of *Trichonympha agilis* (A) and *Pyrsonympha vertens* (B) showing the ultrastructure of the endosymbiotic bacteria in the candidate genus "*Endomicrobium*," which are very abundant in the cytoplasm of these flagellate species. Cells are surrounded by two membranes; the outermost membrane forms tube-like elongations at the tapered cell poles (arrow in A). g, glycogen. Bars = 0.2 µm. Reprinted from Stingl et al. (2005).

since the oxymonadid *Pyrsonympha* species are phylogenetically distant from the hypermastigid flagellates (Moriya et al., 2003; Stingl and Brune, 2003) and do not seem to possess hydrogenosomes (see Brune and Stingl, 2005).

Microbe-Host Interactions

Although the host provides a favorable habitat for the intestinal microbiota and thrives on their metabolic products, there is not always a necessity or clear indication for any specific interactions. However, the intimate associations of bacteria with microvilli of the midgut epithelium or with the epithelial cups in the hindgut cuticle (Breznak and Pankratz, 1977) indicate a closer integration of certain bacteria with the host tissues. Another example suggesting interactions with the gut tissue are the clostridia-related bacteria located in the ectoperitrophic space between the midgut wall and the peritrophic membrane in the mixed segment, closely associated with the mesenteric epithelium (Tokuda et al., 2000; Tokuda et al., 2001).

Pathogens A few reports indicate that the gut microbiota of termites is not always beneficial. *Serratia marcescens*, which has been isolated from the guts of *Reticulitermes hesperus* (Thayer, 1976) and *Mastotermes darwiniensis* (Kuhnigk et al., 1994), causes septicemia in *Coptotermes formosanus* (Osbrink et al., 2001). *Serratia marcescens* seems to form part of the normal microflora of insects since it can be isolated from both healthy and diseased specimens. Usually nonpathogenic when present in the digestive tract in small numbers, it multiplies rapidly once it enters the hemocoel and causes death in 1–3 days (for references, see Lysenko [1985] and Sikorowski and Lawrence [1998]). Also, the fungi associated with *Reticulitermes flavipes* include both cellulolytic species and potential pathogens (Zoberi and Grace, 1990). It is not clear whether entomopathogenic fungi form a part of the natural gut community or are only found among diseased insects (Rath, 2000).

Intracellular Symbionts Termites, like many other insects, are also associated with intracellular prokaryotes that are vertically transmitted via the ovaries (Breznak, 2004). Flavobacteria of the genus *Blattabacterium* reside in specialized cells (bacteriocytes) of cockroaches and the most primitive termite, *Mastotermes darwiniensis* (Bandi et al., 1995). The close phylogenetic relationship between endosymbionts from *Mastotermes darwiniensis* and members of the wood-feeding cockroach genus *Cryptocercus* supports the hypothesis that termites evolved from subsocial, wood-dwelling cockroaches (Lo et al., 2003). All other termites examined carried endocytoplasmic bacteria that are affiliated with the *Wolbachia* group and are located in nonspecialized fat body cells (Bandi et al., 1997). Endonuclear bacteria have been observed in the trophocytes of *Reticulitermes lucifugus* and *Kalotermes flavicollis* (Grandi et al., 1997).

Mutualists or Commensals?

In the hindgut symbiosis, the host creates a rather constant environment for its symbionts, provides a continuous supply of substrates, and by the transfer of the microorganisms to other nestmates, ensures their propagation within the ecosystem. In the symbiosis between lower termites and fiber-digesting flagellates, both partners are indispensable and the mutual advantage is obvious. However, in the case of most prokary-

otic gut symbionts, the symbionts appear to be of little advantage to the host.

If the benefit of the association is unidirectional, a symbiont would be classified as a commensal, and the host might even benefit from its elimination (Brune, 2003). Unfortunately, it is not easy to eliminate specific prokaryotes from the intestinal microbial community of termites selectively, and it is difficult to distinguish between the direct and indirect consequences of their elimination. Removal of the spirochetes from the gut of *Nasutitermes exitiosus* by feeding metronidazole or exposing the termites to pure oxygen kills the termites almost as rapidly as the complete removal of all bacteria by antibiotics (Eutick et al., 1978b).

In view of the constant and massive inoculation with microorganisms in their diet, it is clear that termites cannot keep their gut sterile. The molecular diversity studies have provided ample evidence for a specific microbiota, composed of lineages that occur exclusively in termites (Yang et al., 2005). The absence of a "normal gut microflora" would allow the uncontrolled proliferation of ingested foreign microorganisms and increase the danger of colonization by potential pathogens. *Serratia marcescens* failed to colonize the gut of normal *Coptotermes lacteus*, but transient colonization occurred when the protozoa and spirochetes were killed by exposure to pure oxygen (Veivers et al., 1982).

Under oxygen-limited conditions, the energy loss caused by the activity of the gut microbiota is relatively small (Table 2). The hypothetical, nonsymbiotic oxidation of cellulose to CO_2 would allow the termite to exploit 100% of the free energy of the reaction (Eqn. 1). A homoacetogenic degradation of one glucose equivalent to three acetate molecules by the gut microbiota (Eqn. 2 + Eqn. 3) releases only 10.8% of the free energy contained in the substrate; the rest is still available for the host (Eqn. 4), which also benefits from the nutritionally valuable microbial biomass. However, the situation is quite different if the H_2 formed in the microbial fermentations is converted to methane (Eqn. 2 + Eqn. 5). Although the amount of free energy released in methanogenesis is not much larger than that released in reductive acetogenesis, the host receives only two-thirds of the acetate available under homoacetogenic conditions (Eqn. 6); 28.4% of the free energy in the substrate remains in methane, which cannot be exploited by the host (Eqn. 7) and is lost to the environment.

Such energetic considerations suggest an obvious advantage if a termite uses reductive acetogenesis as the hydrogen-consuming process, and why especially the more highly evolved termites (Termitidae) show a tendency towards increasing methane emission rates is still a mystery (Brauman et al., 1992; Bignell et al., 1997). Nevertheless, considering the added value of metabolic properties such as nitrogen fixation, ammonia assimilation, and provision with vitamins, the advantages for the host may be well worth the investment. The exploitation of the nutritive resources contained in the microbial biomass requires the digestion of the gut symbionts, which is realized by proctodeal trophallaxis (Machida et al., 2001), a behavioral trait that was probably fundamental to both the establishment of the gut microbial community and the evolution of sociality in termites (Nalepa et al., 2001).

Conclusions

Termite guts are minute but efficient bioreactors for the conversion of lignocellulose to short-chain fatty acids and microbial biomass. However, termite guts are not simply anoxic fermentors, but axially and radially structured environments with physicochemically distinct microhabitats, and we are just beginning to understand the complex interactions within the intestinal microbial communities. Microbial diversity in the termite gut is enormous, and the existing isolates represent only a negligible fraction of the untapped diversity of prokaryotes in

Table 2. Free energy of important reactions involved in symbiotic digestion.[a]

Reaction	$\Delta G^{\circ\prime}$ (kJ/mol)[b]	Relative change[c] (%)
(1) Glucose + 6 O_2 → 6 CO_2 + 6 H_2O	–2872	100
(2) Glucose + 2 H_2O → 2 Acetate$^-$ + 2 H^+ + 2 CO_2 + 4 H_2	–216	7.5
(3) 4 H_2 + 2 CO_2 → Acetate$^-$ + H^+ + 2 H_2O	–95	3.3
(4) 3 Acetate$^-$ + 3 H^+ + 6 O_2 → 6 CO_2 + 6 H_2O	–2561	89.2
(5) 4 H_2 + CO_2 → CH_4 + 2 H_2O	–131	4.6
(6) 2 Acetate$^-$ + 2 H^+ + 4 O_2 → 4 CO_2 + 4 H_2O	–1707	59.5
(7) CH_4 + 2 O_2 → CO_2 + 2 H_2O	–818	28.4

[a]For explanations, see text.

[b]Gibbs free energy under standard conditions at pH 7 is calculated after Thauer et al. (1977).

[c]Free energy change relative to the aerobic oxidation of glucose (Reaction 1).

the guts of the more than 2600 described species of termites (Kambhampati and Eggleton, 2000).

Literature Cited

Aanen, D. K., P. Eggleton, C. Rouland-Lefevre, T. Guldberg-Froslev, S. Rosendahl, and J. J. Boomsma. 2002. The evolution of fungus-growing termites and their mutualistic fungal symbionts. Proc. Natl. Acad. Sci. USA 99:14887–14892.

Abo-Khatwa, N. 1978. Cellulase of fungus-growing termites: A new hypothesis on its origin. Experientia 34:559–60.

Amburgey, T. L., G. N. Johnson, and J. L. Etheridge. 1980. A method to mass-produce decayed-wood termite bait blocks. J. Georgia Entomol. Soc. 16:112–115.

Anklin-Mühlemann, R., D. E. Bignell, P. C. Veivers, R. H. Leuthold, and M. Slaytor. 1995. Morphological, microbiological and biochemical studies of the gut flora in the fungus-growing termite Macrotermes subhyalinus. J. Insect Physiol. 41:929–940.

Bakalidou, A., P. Kämpfer, M. Berchtold, T. Kuhnigk, M. Wenzel, and H. König. 2002. Cellulosimicrobium variabile sp. nov., a cellulolytic bacterium from the hindgut of the termite Mastotermes darwiniensis. Int. J. System. Evol. Microbiol. 52:1185–1192.

Bandi, C., M. Sironi, G. Damiani, L. Magrassi, C. A. Nalepa, U. Laudani, and L. Sacchi. 1995. The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. Proc. R. Soc. Lond. Ser. B. Biol. Sci. 259:293–299.

Bandi, C., M. Sironi, C. A. Nalepa, S. Corona, and L. Sacchi. 1997. Phylogenetically distant intracellular symbionts in termites. Parassitologia 39:71–75.

Bauer, S., A. Tholen, J. Overmann, and A. Brune. 2000. Characterization of abundance and diversity of lactic acid bacteria in the hindgut of wood- and soil-feeding termites by molecular and culture-dependent techniques. Arch. Microbiol. 173:126–173.

Béguin, P., and J.-P. Aubert. 1994. The biological degradation of cellulose. FEMS Microbiol. Rev. 13:25–58.

Benemann, J. R. 1973. Nitrogen fixation in termites. Science 181:164–165.

Bentley, B. L. 1984. Nitrogen fixation in termites: Fate of newly fixed nitrogen. J. Insect Physiol. 30:653–655.

Berchtold, M., W. Ludwig, and H. Koenig. 1994. 16S rDNA sequence and phylogenetic position of an uncultivated spirochete from the hindgut of the termite Mastotermes darwiniensis Froggatt. FEMS Microbiol. Lett. 123:269–273.

Berchtold, M., and H. König. 1996. Phylogenetic analysis and in-situ identification of uncultivated spirochetes from the hindgut of the termite Mastotermes darwiniensis. System. Appl. Microbiol. 19:66–73.

Berchtold, M., A. Chatzinotas, W. Schönhuber, A. Brune, R. Amann, D. Hahn, and H. König. 1999. Differential enumeration and in situ localization of micro-organisms in the hindgut of the lower termite Mastotermes darwiniensis by hybridization with rRNA-targeted probes. Arch. Microbiol. 172:407–416.

Bermudes, D., D. Chase, and L. Margulis. 1988. Morphology as a basis for taxonomy of large spirochetes symbiotic in wood-eating cockroaches and termites: Pillotina gen. nov., nom. rev.; Pillotina calotermitidis sp. nov., nom. rev.; Diplocalyx gen. nov., nom. rev.; Diplocalyx calotermitidis sp. nov., nom. rev.; Hollandina gen. nov., nom. rev.; Hollandina pterotermitidis sp. nov., nom. rev.; and Clevelandina reticulitermitidis gen. nov., sp. nov. Int. J. Syst. Bacteriol. 38:291–302.

Bignell, D. E., H. Oskarsson, and J. M. Anderson. 1979. Association of actinomycete-like bacteria with soil-feeding termites (Termitidae, Termitinae). Appl. Environ. Microbiol. 37:339–342.

Bignell, D. E., and J. M. Anderson. 1980a. Determination of pH and oxygen status in the guts of lower and higher termites. J. Insect Physiol. 26:183–188.

Bignell, D. E., H. Oskarsson, and J. M. Anderson. 1980b. Distribution and abundance of bacteria in the gut of a soil-feeding termite Procubitermes aburiensis (Termitidae, Termitinae). J. Gen. Microbiol. 117:393–403.

Bignell, D. E., H. Oskarsson, and J. M. Anderson. 1980c. Specialization of the hindgut wall for the attachment of symbiotic microorganisms in a termite Procubitermes aburiensis. Zoomorphology 96:103–112.

Bignell, D. E., H. Oskarsson, J. M. Anderson, P. Ineson, and T. G. Wood. 1983. Structure, microbial associations and function of the so-called "mixed segment" of the gut in two soil-feeding termites, Procubitermes aburiensis and Cubitermes severus (Termitidae, Termitinae). J. Zool. Lond. 201:445–480.

Bignell, D. E. 1984. The arthropod gut as an environment for microorganisms. *In:* J. M. Anderson, A. D. M. Rayner, and D. W. H. Walton (Eds.) Invertebrate-microbial Interactions. Cambridge University Press. Cambridge, UK. 205–227.

Bignell, D. E., J. M. Anderson, and R. Crosse. 1991. Isolation of facultatively aerobic actinomycetes from the gut, parent soil and mound materials of the termites Procubitermes aburiensis and Cubitermes severus. FEMS Microbiol. Ecol. 85:151–160.

Bignell, D. E. 1994a. Soil-feeding and gut morphology in higher termites. *In:* J. H. Hunt and C. A. Nalepa (Eds.) Nourishment and Evolution in Insect Societies. Westview Press. Boulder, CO. 131–158.

Bignell, D. E., M. Slaytor, P. C. Veivers, R. Mühlemann, and R. H. Leuthold. 1994b. Functions of symbiotic fungus gardens in higher termites of the genus Macrotermes: Evidence against the acquired enzyme hypothesis. Acta Microbiol. Immunol. Hung. 41:391–401.

Bignell, D. E., and P. Eggleton. 1995. On the elevated intestinal pH of higher termites (Isoptera: Termitidae). Insect. Soc. 42:57–69.

Bignell, D. E., P. Eggleton, L. Nunes, and K. L. Thomas. 1997. Termites as mediators of carbon fluxes in tropical forests: Budgets for carbon dioxide and methane emissions. *In:* A. B. Watt, N. E. Stork, and M. D. Hunter (Eds.) Forests and Insects. Chapman and Hall. London, UK. 109–134.

Bignell, D. E. 2000. Introduction to Symbiosis. *In:* T. Abe, D. E. Bignell, and M. Higashi (Eds.) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers. Dordrecht, The Netherlands. 189–208.

Bloodgood, R. A., K. R. Miller, T. P. Fitzharris, and J. R. Mcintosh. 1974. The ultrastructure of Pyrsonympha and its associated microorganisms. J. Morphol. 143:77–106.

Bloodgood, R. A., and T. P. Fitzharris. 1976. Specific association of prokaryotes with symbiotic flagellate protozoa from the hindgut of the termite Reticulitermes and the wood-eating roach, Cryptocercus. Cytobios 17:103–122.

Boga, H. I., and A. Brune. 2003a. Hydrogen-dependent oxygen reduction by homoacetogenic bacteria isolated from termite guts. Appl. Environ. Microbiol. 69:779–786.

Boga, H. I., W. Ludwig, and A. Brune. 2003b. Sporomusa aerivorans sp. nov., an oxygen-reducing homoacetogenic bacterium from the gut of a soil-feeding termite. Int. J. System. Evol. Microbiol. 53:1397–1404.

Brauman, A., J. F. Koenig, J. Dutreix, and J. L. Garcia. 1990a. Characterization of two sulfate-reducing bacteria from the gut of the soil-feeding termite, Cubitermes speciosus. Ant. v. Leeuwenhoek 58:271–275.

Brauman, A., M. Labat, and J. L. Garcia. 1990b. Preliminary studies on the gut microbiota of the soil-feeding termite: Cubitermes speciosus. *In:* R. Lésel (Ed.) Microbiology in Poecilotherms. Elsevier. Amsterdam, The Netherlands. 73–77.

Brauman, A., M. D. Kane, M. Labat, and J. A. Breznak. 1992. Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science 257:1384–1387.

Brauman, A., J. A. Müller, J. L. Garcia, A. Brune, and B. Schink. 1998. Fermentative degradation of 3-hydroxybenzoate in pure culture by a novel strictly anaerobic bacterium, Sporotomaculum hydroxybenzoicum gen. nov., sp. nov. Int. J. System. Bacteriol. 48:215–221.

Brauman, A., D. E. Bignell, and I. Tayasu. 2000. Soil-feeding termites: Biology, microbial associations and digestive mechanisms. *In:* T. Abe, D. E. Bignell, and M. Higashi (Eds.) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers. Dordrecht, The Netherlands. 233–259.

Brauman, A., J. Dore, P. Eggleton, D. Bignell, J. A. Breznak, and M. D. Kane. 2001. Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. FEMS Microbiol. Ecol. 35:27–36.

Brennan, Y., W. N. Callen, L. Christoffersen, P. Dupree, F. Goubet, S. Healey, M. Hernandez, M. Keller, K. Li, N. Palackal, A. Sittenfeld, G. Tamayo, S. Wells, G. P. Hazlewood, E. J. Mathur, J. M. Short, D. E. Robertson, and B. A. Steer. 2004. Unusual microbial xylanases from insect guts. Appl. Environ. Microbiol. 70:3609–3617.

Breznak, J. A., W. J. Brill, J. W. Mertins, and H. C. Coppel. 1973. Nitrogen fixation in termites. Nature 244:577–580.

Breznak, J. A. 1975. Symbiotic relationships between termites and their intestinal microbiota. Symp. Soc. Exp. Biol. 29:559–580.

Breznak, J. A., and H. S. Pankratz. 1977. In situ morphology of the gut microbiota of wood-eating termites [Reticulitermes flavipes (Kollar) and Coptotermes formosanus Shiraki]. Appl. Environ. Microbiol. 33:406–426.

Breznak, J. A. 1984a. Hindgut spirochetes of termites and Cryptocercus punctulatus. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:67–70.

Breznak, J. A., and J. M. Switzer. 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl. Environ. Microbiol. 52:623–630.

Breznak, J. A., J. M. Switzer, and H.-J. Seitz. 1988. Sporomusa termitida sp. nov., an H_2/CO_2-utilizing acetogen isolated from termites. Arch. Microbiol. 150:282–288.

Breznak, J. A., and M. D. Kane. 1990. Microbial H_2/CO_2 acetogenesis in animal guts: Nature and nutritional significance. FEMS Microbiol. Rev. 87:309–314.

Breznak, J. A., and J. Switzer Blum. 1991. Mixotrophy in the termite gut acetogen, Sporomusa termitida. Arch. Microbiol. 156:105–110.

Breznak, J. A. 1994a. Acetogenesis from carbon dioxide in termite guts. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 303–330.

Breznak, J. A., and A. Brune. 1994b. Role of microorganisms in the digestion of lignocellulose by termites. Ann. Rev. Entomol. 39:453–487.

Breznak, J. A. 2000. Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. *In:* T. Abe, D. E. Bignell, and M. Higashi (Eds.) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers. Dordrecht, The Netherlands. 209–231.

Breznak, J. A. 2002. Phylogenetic diversity and physiology of termite gut spirochetes. Integr. Comp. Biol. 42:313–318.

Breznak, J. A. 2004. Invertebrates—Insects. *In:* A. T. Bull (Ed.) Microbial Biodiversity and Bioprospecting. ASM Press. Washington, DC. 191–203.

Brune, A., D. Emerson, and J. A. Breznak. 1995a. The termite gut microflora as an oxygen sink: Microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. 61:2681–2687.

Brune, A., E. Miambi, and J. A. Breznak. 1995b. Roles of oxygen and the intestinal microflora in the metabolism of lignin-derived phenylpropanoids and other monoaromatic compounds by termites. Appl. Environ. Microbiol. 61:2688–2695.

Brune, A., and M. Kühl. 1996. pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. J. Insect Physiol. 42:1121–1127.

Brune, A. 1998. Termite guts: The world's smallest bioreactors. Trends Biotechnol. 16:16–21.

Brune, A., and M. Friedrich. 2000a. Microecology of the termite gut: Structure and function on a microscale. Curr. Opin. Microbiol. 3:263–269.

Brune, A., P. Frenzel, and H. Cypionka. 2000b. Life at the oxic-anoxic interface: Microbial activities and adaptations. FEMS Microbiol. Rev. 24:691–710.

Brune, A., W. Ludwig, and B. Schink. 2002. Propionivibrio limicola sp. nov., a fermentative bacterium specialized in the degradation of hydroaromatic compounds, reclassification of Propionibacter pelophilus as Propionivibrio pelophilus comb. nov. and amended description of the genus Propionivibrio. Int. J. System. Evol. Microbiol. 52:441–444.

Brune, A. 2003. Symbionts aiding digestion. *In:* V. H. Resh and R. T. Cardé (Eds.) Encyclopedia of Insects. Academic Press. New York, NY. 1102–1107.

Brune, A., and U. Stingl. 2005. Prokaryotic symbionts of termite gut flagellates: Phylogenetic and metabolic implications of a tripartite symbiosis. *In:* J. Overmann (Ed.) Molecular Basis of Symbiosis. Springer-Verlag. New York, NY.

Cazemier A. E., J. C. Verdoes, F. A. G. Reubsaet, J. H. P. Hackstein, C. van der Drift, and H. J. M. Op den Camp. 2003. Promicromonospora pachnodae sp. nov., a member of the (hemi)cellulolytic hindgut flora of larvae of the scarab beetle Pachnoda marginata. Ant. v. Leeuwenhoek 83:135–148.

Cleveland, L. R. 1925a. The effects of oxygenation and starvation on the symbiosis between the termite, Termopsis, and its intestinal flagellates. Biol. Bull. 48:309–327.

Cleveland, L. R. 1925b. Toxicity of oxygen for protozoa in vivo and in vitro: Animals defaunated without injury. Biol. Bull. 48:455–468.

Cleveland, L. R. 1926. Symbiosis among animals with special reference to termites and their intestinal flagellates. Quart. Rev. Biol. 1:51–64.

Cleveland, L. R., and A. V. Grimstone. 1964. The fine structure of the flagellate Mixotricha paradoxa and its associated micro-organisms. Proc. R. Soc. Lond. Ser. B. Biol. Sci. 159:668–686.

Collins, N. M. 1983. The utilization of nitrogen resources by termites (Isoptera). *In:* J. A. Lee, S. McNeill, and I. C. H. Rorison (Eds.) Nitrogen as an Ecological Factor. Blackwell Scientific Publications. Oxford, UK. 381–412.

Collins, M. D., and H. N. Shah. 1986. Reclassification of Bacteroides termitidis Sebald (Holdeman and Moore) in a new genus Sebaldella, as Sebaldella termitidis comb. nov. Int. J. System. Bacteriol. 36:349–350.

Cook, S. F. 1932. The respiratory gas exchange in Termopsis nevadensis. Biol. Bull. 63:246–257.

Cook, S. F. 1943. Nonsymbiotic utilization of carbohydrates by the termite Zootermopsis angusticollis. Physiol. Zool. 16:123–128.

Cook, T. J., and R. E. Gold. 2000. Effects of different cellulose sources on the structure of the hindgut flagellate community in Reticulitermes virginicus (Isoptera: Rhinotermitidae). Sociobiology 35:119–130.

Cord-Ruwisch, R., H. J. Seitz, and R. Conrad. 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Arch. Microbiol. 149:350–357.

Cornelius, M. L., D. J. Daigle, W. J. Connick Jr., A. Parker, and K. Wunch. 2002. Responses of Coptotermes formosanus and Reticulitermes flavipes (Isoptera: Rhinotermitidae) to three types of wood rot fungi cultured on different substrates. J. Econ. Entomol. 95:121–128.

Crosland, M. W. J., L. K. Chan, and J. A. Buswell. 1996. Symbiotic fungus and enzymatic digestion in the gut of the termite, Macrotermes barneyi (Light) (Isoptera: Termitidae). J. Entomol. Sci. 31:132–137.

Curtis, A. D., and D. A. Waller. 1995. Changes in nitrogen fixation rates in termites (Isoptera: Rhinotermitidae) maintained in the laboratory. Ann. Entomol. Soc. Am. USA 88:764–767.

Curtis, A. D., and D. A. Waller. 1996. The effects of decreased pO_2 and increased pCO_2 on nitrogen fixation rates in termites (Isoptera: Rhinotermitidae). J. Insect Physiol. 42:867–872.

Curtis, A. D., and D. A. Waller. 1998. Seasonal patterns of nitrogen fixation in termites. Funct. Ecol. 12:803–807.

Cypionka, H. 2000. Oxygen respiration by Desulfovibrio species. Ann. Rev. Microbiol. 54:827–848.

Czolij, R., M. Slaytor, P. C. Veivers, and R. W. O'Brien. 1984. Gut morphology of Mastotermes darwiniensis Froggatt (Isoptera: Mastotermitidae). Int. J. Insect Morphol. Embryol. 13:337–355.

Czolij, R., M. Slaytor, and R. W. O'Brien. 1985. Bacterial flora of the mixed segment and the hindgut of the higher termite Nasutitermes exitiosus Hill (Termitidae, Nasutitermitinae). Appl. Environ. Microbiol. 49:1226–1236.

d'Ambrosio, U., M. Dolan, A. M. Wier, and L. Margulis. 1999. Devescovinid trichomonad with axostyle-based rotary motor ("Rubberneckia"): Taxonomic assignment as Caduceia versatilis sp. nov. Eur. J. Protistol. 35:327–337.

Darlington, J. P. E. C. 1994. Nutrition and evolution in fungus-growing termites. *In:* J. H. Hunt and C. A. Nalepa (Eds.) Nourishment and Evolution in Insect Societies. Westview Press. Boulder, CO. 105–130.

Davison, A., and M. Blaxter. 2005. Ancient Origin of Glycosyl Hydrolase Family 9 Cellulase Genes. Mol. Biol. Evol. 22:1273–1284.

de Bary, A. 1878. Ueber Symbiose. Bericht der Versammlung Deutscher Naturforscher und Aerzte 121–126.

Derakshani, M., L. Lukow, and W. Liesack. 2001. Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. Appl. Environ. Microbiol. 67:623–631.

Dolan, M., and L. Margulis. 1997. Staurojoenia and other symbionts in Neotermes from San Salvador Island, Bahamas. Symbiosis 22:229–239.

Dolan, M. F. 2001. Speciation of termite gut protists: The role of bacterial symbionts. Int. Microbiol. 4:203–208.

Donovan, S. E., P. Eggleton, and D. E. Bignell. 2001. Gut content analysis and a new feeding group classification of termites. Ecol. Entomol. 26:356–366.

Donovan, S. E., K. J. Purdy, M. D. Kane, and P. Eggleton. 2004. Comparison of Euryarchaea strains in the guts and food-soil of the soil-feeding termite Cubitermes fungifaber across different soil types. Appl. Environ. Microbiol. 70:3884–3892.

Ebert, A., and A. Brune. 1997. Hydrogen concentration profiles at the oxic-anoxic interface: A microsensor study of the hindgut of the wood-feeding lower termite Reticulitermes flavipes (Kollar). Appl. Environ. Microbiol. 63:4039–4046.

Eutick, M. L., R. W. O'Brien, and M. Slaytor. 1976. Aerobic state of gut of Nasutitermes exitiosus and Coptotermes lacteus, high and low caste termites. J. Insect Physiol. 22:1377–1380.

Eutick, M. L., R. W. O'Brien, and M. Slaytor. 1978a. Bacteria from the gut of Australian termites. Appl. Environ. Microbiol. 35:823–828.

Eutick, M. L., P. C. Veivers, R. W. O'Brien, and M. Slaytor. 1978b. Dependence of the higher termite Nasutitermes exitiosus and the lower termite Coptotermes lacteus on their gut flora. J. Insect Physiol. 24:363–368.

French, J. R. J., G. L. Turner, and J. F. Bradbury. 1976. Nitrogen fixation by bacteria from the hindgut of termites. J. Gen. Microbiol. 95:202–206.

Friedrich, M. W., D. Schmitt-Wagner, T. Lueders, and A. Brune. 2001. Axial differences in community structure of Crenarchaeota and Euryarchaeota in the highly compartmentalized gut of the soil-feeding termite Cubitermes orthognathus. Appl. Environ. Microbiol. 67:4880–4890.

Fröhlich, J., and H. König. 1999a. Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. System. Appl. Microbiol. 22:249–257.

Fröhlich, J., H. Sass, H.-D. Babenzien, T. Kuhnigk, A. Varma, S. Saxena, C. Nalepa, P. Pfeiffer, and H. König. 1999b. Isolation of Desulfovibrio intestinalis sp. nov. from the hindgut of the lower termite Mastotermes darwiniensis. Can. J. Microbiol. 45:145–152.

Fujita, A., I. Shimizu, and T. Abe. 2001. Distribution of lysozyme and protease, and amino acid concentration in the guts of a wood-feeding termite, Reticulitermes speratus (Kolbe): Possible digestion of symbiont bacteria transferred by trophallaxis. Physiol. Entomol. 26:116–123.

Fujita, A., and T. Abe. 2002a. Amino acid concentration and distribution of lysozyme and protease activities in the guts of higher termites. Physiol. Entomol. 27:76–78.

Fujita, A., T. Minamoto, I. Shimizu, and T. Abe. 2002b. Molecular cloning of lysozyme-encoding cDNAs expressed in the salivary gland of a wood-feeding termite, Reticulitermes speratus. Insect Biochem. Molec. Biol. 32:1615–1624.

Fujita, A. 2004. Lysozymes in insects: what role do they play in nitrogen metabolism? Physiol. Entomol. 299:305–310.

Graber, J. R., and J. A. Breznak. 2004a. Physiology and nutrition of Treponema primitia, an H_2/CO_2-acetogenic spirochete from termite hindguts. Appl. Environ. Microbiol. 70:1307–1314.

Graber, J. R., J. R. Leadbetter, and J. A. Breznak. 2004b. Description of Treponema azotonutricium sp. nov. and Treponema primitia sp. nov., the first spirochetes isolated from termite guts. Appl. Environ. Microbiol. 70:1307–1314.

Graber, J. R., and J. A. Breznak. 2005. Folate cross-feeding supports symbiotic homoacetogenic spirochetes. Appl. Environ. Microbiol. 71:1883–1889.

Grandi, G., L. Guidi, and M. Chicca. 1997. Endonuclear bacterial symbionts in two termite species: An ultrastructural study. J. Submicrosc. Cytol. Pathol. 29:281–292.

Grassé, P.-P., and C. Noirot. 1958. Le meule des termites champignonnistes et sa signification symbiotique. Ann. Sci. Nat. Ser. 11, Zool. Biol. Animale 11:113–128.

Grech-Mora, I., M.-L. Fardeau, B. K. C. Patel, B. Ollivier, A. Rimbault, G. Prensier, G., J.-L. Garcia, and E. Garnier-Sillam. 1996. Isolation and characterization of Sporobacter termitidis gen. nov., sp. nov., from the digestive tract of the wood-feeding termite Nasutitermes lujae. Int. J. Syst. Bacteriol. 46:512–518.

Guo, L., D. R. Quicili, J. Chase, and G. J. Blomquist. 1991. Gut tract microorganisms supply the precursors for methyl-branched hydrocarbon biosynthesis in the termite, Zootermopsis nevadensis. Insect Biochem. 21:327–333.

Harazono, K., N. Yamashita, N. Shinzato, Y. Watanabe, T. Fukatsu, and R. Kurane. 2003. Isolation and characterization of aromatics-degrading microorganisms from the gut of the lower termite Coptotermes formosanus. Biosci. Biotechnol. Biochem. 67:889–892.

Hethener, P., A. Brauman, and J. L. Garcia. 1992. Clostridium termitidis sp. nov., a cellulolytic bacterium from the gut of the wood-feeding termite, Nasutitermes lujae. System. Appl. Microbiol. 5:52–58.

Higashi, M., T. Abe, and T. P. Burns. 1992. Carbon-nitrogen balance and termite ecology. Proc. R. Soc. Lond. Ser. B. Biol. Sci. 249:303–308.

Hirai, H., N. Shinzato, A. Nakagawa, Y. Watanabe, and R. Kurane. 2000. Degradation of lignin model compounds by various termites [in Japanese]. Mokuzai Gakkaishi 46:63–67.

Hollande A., and J. Valentin. 1969. Appareil de Golgi, pinocytose, lysosomes, mitochondries, bactéries symbiontiques, atractophores et pleuromitose chez les Hypermastigines du genre Joenia: Affinités entre Joenidae et Trichomonadines. Protistologica 5:39–86.

Hongoh, Y., M. Ohkuma, and T. Kudo. 2003a. Molecular analysis of bacterial microbiota in the gut of the termite Reticulitermes speratus (Isoptera: Rhinotermitidae). FEMS Microbiol. Ecol. 44:231–242.

Hongoh Y., H. Yuzawa, M. Ohkuma, and T. Kudo. 2003b. Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. FEMS Microbiol. Lett. 221:299–304.

Honigberg, B. M. 1970. Protozoa associated with termites and their role in digestion. *In:* K. Krishna and F. M. Weesner (Eds.) Biology of Termites. Academic Press. New York, NY. 2:1–36.

Hopkins, D. W., J. A. Chudek, D. E. Bignell, J. Frouz, E. A. Webster, and T. Lawson. 1998. Application of ^{13}C NMR to investigate the transformations and biodegradation of organic materials by wood- and soil-feeding termites, and a coprophagous litter-dwelling dipteran larva. Biodegradation 9:423–431.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180:4765–4774.

Hungate, R. E. 1939. Experiments on the nutrition of Zootermopsis. III: The anaerobic carbohydrate dissimilation by the intestinal protozoa. Ecology 20:230–245.

Hungate, R. E. 1943. Quantitative analyses of the cellulose fermentation by termite protozoa. Ann. Entomol. Soc. Am. 36:730–739.

Hungate, R. E. 1946. Studies on cellulose fermentation. II: An anaerobic cellulose-decomposing actinomycete, Micromonospora propionici, n. sp. J. Bacteriol. 51:51–56.

Hungate, R. E. 1955. Mutualistic intestinal protozoa. *In:* S. H. Hutner and A. Lwoff (Eds.) Biochemistry and Physiology of Protozoa. Academic Press. New York, NY. 2:159–199.

Hyodo F., J. Azuma, and T. Abe. 1999. Estimation of effect of passage through the gut of a lower termite, Coptotermes formosanus Shiraki, on lignin by solid-state CP MAS ^{13}C- NMR. Holzforschung 53:244–246.

Hyodo F., T. Inoue, J. I. Azuma, I. Tayasu, and T. Abe. 2000. Role of the mutualistic fungus in lignin degradation in the fungus-growing termite Macrotermes gilvus (Isoptera: Macrotermitinae). Soil Biol. Biochem. 32:653–658.

Iida, T., M. Ohkuma, K. Ohtoko, and T. Kudo. 2000. Symbiotic spirochetes in the termite hindgut: Phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol. Ecol. 34:17–26.

Inoue, T., K. Murashima, J.-I. Azuma, A. Sugimoto, and M. Slaytor. 1997. Cellulose and xylan utilization in the lower termite Reticulitermes speratus. J. Insect Physiol. 43:235–242.

Inoue, T., O. Kitade, T. Yoshimura, and I. Yamaoka. 2000. Symbiotic associations with protists. *In:* T. Abe, D. E. Bignell, and M. Higashi (Eds.) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers. Dordrecht, The Netherlands. 275–288.

Inoue, T., S. Moriya, M. Ohkuma, and T. Kudo. 2005. Molecular cloning and charactrization of a cellulase gene from a symbiotic protist of the lower termite, Coptotermes formosanus. Gene. 349:67–75.

Itakura, S., H. Tanaka, and A. Enoki. 1999. Occurrence and metabolic role of the pyruvate dehydrogenase complex in the lower termite Coptotermes formosanus (Shiraki). Insect Biochem. Molec. Biol. 29:625–633.

Itakura, S., H. Tanaka, A. Enoki, D. J. Chappell, and M. Slaytor. 2003. Pyruvate and acetate metabolism in termite mitochondria. J. Insect Physiol. 49:917–926.

Jeffries, T. W. 1994. Biodegradation of lignin and hemicelluloses. *In:* C. Ratledge (Ed.) Biochemistry of Microbial Degradation. Kluwer Academic Publishers. Dordrecht, The Netherlands. 233–277.

Ji, R., A. Kappler, and A. Brune. 2000. Transformation and mineralization of synthetic ^{14}C-labeled humic model compounds by soil-feeding termites. Soil Biol. Biochem. 32:1281–1291.

Ji, R., and A. Brune. 2001. Transformation and mineralization of ^{14}C-labeled cellulose, peptidoglycan, and protein by the soil-feeding termite Cubitermes orthognatus. Biol. Fertil. Soils 33:166–174.

Ji, R., and A. Brune. 2005. Digestion of peptidic residues in humic substances by an alkali-stable and humic-acid-tolerant proteolytic activity in the gut of soil-feeding termites. Soil. Biol. Biochem. 37:1648–1655.

Johjima, T., M. Ohkuma, and T. Kudo. 2003. Isolation and cDNA cloning of novel hydrogen peroxide-dependent phenol oxidase from the basidiomycete Termitomyces albuminosus. Appl. Microbiol. Biotechnol. 61:220–225.

Kambhampati, S., and P. Eggleton. 2000. Taxonomy and phylogenetics of Isoptera. *In:* T. Abe, D. E. Bignell, and M. Higashi (Eds.) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers. Dordrecht, The Netherlands. 1–23.

Kane, M. D., and J. A. Breznak. 1991a. Acetonema longum gen. nov. sp.nov., an H_2/CO_2 acetogenic bacterium from the termite, Pterotermes occidentis. Arch. Microbiol. 156:91–98.

Kane, M. D., A. Brauman, and J. A. Breznak. 1991b. Clostridium mayombei sp. nov., an H_2/CO_2 acetogenic bacterium from the gut of the African soil-feeding termite, Cubitermes speciosus. Arch. Microbiol. 156:99–104.

Kane, M. D. 1997. Microbial fermentation in insect guts. *In:* R. I. Mackie and B. A. White (Eds.) Gastrointestinal Microbiology. Chapman and Hall. New York, NY. 1:231–265.

Kappler, A., and A. Brune. 1999. Influence of gut alkalinity and oxygen status on mobilization and size-class distribution of humic acids in the hindgut of soil-feeding termites. Appl. Soil Ecol. 13:219–229.

Kappler A., and A. Brune. 2002. Dynamics of redox potential and changes in redox state of iron and humic acids during gut passage in soil-feeding termites (Cubitermes spp.). Soil Biol. Biochem. 34:221–227.

Katoh, H., T. Miura, K. Maekawa, N. Shinzato, and T. Matsumoto. 2002. Genetic variation of symbiotic fungi cultivated by the macrotermitine termite Odontotermes formosanus (Isoptera: Termitidae) in the Ryukyu Archipelago. Molec. Ecol. 11:1565–1572.

Katzin, L. I., and H. Kirby. 1939. The relative weight of termites and their protozoa. J. Parasitol. 25:444–445.

Koidzumi, M. 1921. Studies on the intestinal protozoa found in the termites of Japan. Parasitology 13:235–305.

Kovoor, J. 1967. Le pH intestinal d'un termite supérieur, Microcerotermes edentatus (Was., Amitermitinae). Insect. Soc. 14:157–160.

Kovoor, J. 1968. L'intestin d'un termite supérieur (Microcerotermes edentatus, Wasman, Amitermitinae). Histophysiologie et flore bacterienne symbiotique. Bull. Biol. Fr. Belg. 102:45–84.

Kudo, T., M. Ohkuma, S. Moriya, S. Noda, and K. Ohtoko. 1998. Molecular phylogenetic identification of the intestinal anaerobic microbial community in the hindgut of the termite, Reticulitermes speratus, without cultivation. Extremophiles 2:155–161.

Kuhnigk, T., E.-M. Borst, A. Ritter, P. Kämpfer, A. Graf, H. Hertel, and H. König. 1994. Degradation of lignin monomers by the hindgut flora of xylophagous termites. System. Appl. Microbiol. 17:76–85.

Kuhnigk, T., E.-M. Borst, and A. Breunig. 1995. Bacillus oleronius sp. nov., a member of the hindgut flora of the termite Reticulitermes santonensis (Feytaud). Can. J. Microbiol. 41:699–706.

Kuhnigk, T., J. Branke, D. Krekeler, H. Cypionka, and H. König. 1996. A feasible role of sulfate-reducing bacteria in the termite gut. System. Appl. Microbiol. 19:139–149.

Kuhnigk, T., and H. König. 1997. Degradation of dimeric lignin model compounds by aerobic bacteria isolated from the hindgut of xylophagous termites. J. Basic Microbiol. 37:205–211.

La Fage, J. P., and W. L. Nutting. 1978. Nutrient dynamics of termites. *In:* M. V. Brian (Ed.) Production Ecology of Ants and Termites. Cambridge University Press. Cambridge, UK. 165–232.

Leadbetter, J. R., and J. A. Breznak. 1996. Physiological ecology of Methanobrevibacter cuticularis sp. nov. and Methanobrevibacter curvatus sp. nov., isolated from the hindgut of the termite Reticulitermes flavipes. Appl. Environ. Microbiol. 62:3620–3631.

Leadbetter, J. R., L. D. Crosby, and J. A. Breznak. 1998. Methanobrevibacter filiformis sp. nov., a filamentous methanogen from termite hindguts. Arch. Microbiol. 169:287–292.

Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283:686–689.

Leander, B. S., and P. J. Keeling. 2004. Symbiotic innovation in the oxymonad Streblomastix strix. J. Euk. Microbiol. 51:291–300.

Leaphart, A. B., and C. R. Lovell. 2001. Recovery and analysis of formyltetrahydrofolate synthetase gene sequences from natural populations of acetogenic bacteria. Appl. Environ. Microbiol. 67:1392–1395.

Leaphart, A. B., M. J. Friez, and C. R. Lovell. 2003. Formyltetrahydrofolate synthetase sequences from salt marsh plant roots reveal a diversity of acetogenic bacteria and other bacterial functional groups. Appl. Environ. Microbiol. 69:693–696.

Lee, M. J., P. J. Schreurs, A. C. Messer, and S. H. Zinder. 1987. Association of methanogenic bacteria with flagellated protozoa from a termite hindgut. Curr. Microbiol. 15:337–341.

Leidy, J. 1849. [no title]. Proc. Acad. Nat. Sci. (Phila.) 4:225–233.

Leidy, J. 1881. The parasites of the termites. J. Acad. Nat. Sci. (Phila.), 2nd Ser. 8:425–447.

Lemke T., T. van Alen, J. H. P. Hackstein, and A. Brune. 2001. Cross-epithelial hydrogen transfer from the midgut compartment drives methanogenesis in the hindgut of cockroaches. Appl. Environ. Microbiol. 67:4657–4661.

Li, L., J. Fröhlich, P. Pfeiffer, and H. König. 2003. Termite gut symbiotic archaezoa are becoming living metabolic fossils. Eukar. Cell 2:1091–1098.

Lilburn, T. G., T. M. Schmidt, and J. A. Breznak. 1999. Phylogenetic diversity of termite gut spirochaetes. Environ. Microbiol. 1:331–345.

Lilburn, T. G., K. S. Kim, N. E. Ostrom, K. R. Byzek, J. R. Leadbetter, and J. A. Breznak. 2001. Nitrogen fixation by symbiotic and free-living spirochetes. Science 292:2495–2498.

Lo, N., C. Bandi, H. Watanabe, C. Nalepa, and T. Beninati. 2003a. Evidence for co-cladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. Molec. Biol. Evol. 20:907–913.

Lo, N., H. Watanabe, and M. Sugimura. 2003b. Evidence for the presence of a cellulase gene in the last common ancestor of bilaterian animals. Proc. R. Soc. Lond. Ser. B. Biol. Sci. 270, Suppl. 1:S69–S72.

Lysenko, O. 1985. Non-sporeforming bacteria pathogenic to insects: Incidence and mechanisms. Ann. Rev. Microbiol. 39:673–695.

Machida, M., O. Kitade, T. Miura, and T. Matsumoto. 2001. Nitrogen recycling through proctodeal trophallaxis in the Japanese damp-wood termite Hodotermopsis japonica (Isoptera, Termopsidae). Insect. Soc. 48:52–56.

Mannesmann, R., and B. Piechowski. 1989. Verteilungsmuster von Gärkammerbakterien einiger Termitenarten. Mater. Org. 24:161–178.

Margulis, L., A. Olendzenski, and B. A. Afzelius. 1990. Endospore-forming filamentous bacteria symbiotic in termites: Ultrastructure and growth in culture of Arthromitus. Symbiosis 8:95–116.

Margulis, L., J. Z. Jorgensen, S. Dolan, R. Kolchinsky, F. A. Rainey, and S. C. Lo. 1998. The Arthromitus stage of Bacillus cereus: Intestinal symbionts of animals. Proc. Natl. Acad. Sci. USA 95:1236–1241.

Martin, M. M., and J. S. Martin. 1978. Cellulose digestion in the midgut of the fungus-growing termite Macrotermes natalensis: The role of acquired digestive enzymes. Science 199:1453–1455.

Martin, M. M., and J. S. Martin. 1979. The distribution and origins of the cellulolytic enzymes of the higher termite, Macrotermes natalensis. Physiol. Zool. 52:11–21.

Martin, M. M. 1983. Cellulose digestion in insects. Comp. Biochem. Physiol. 75A:313–324.

Matoub, M., and C. Rouland. 1995. Purification and properties of the xylanases from the termite Macrotermes bellicosus and its symbiotic fungus Termitomyces sp. Comp. Biochem. Physiol. 112B:629–635.

Matsuura, K. 2001. Nestmate recognition mediated by intestinal bacteria in a termite, Reticulitermes speratus. Oikos 92:20–26.

McSweeney C. S., A. Dulieu, R. I. Webb, T. Del Dot, and L. L. Blackall. 1999. Isolation and characterization of a Clostridium sp. with cinnamoyl esterase activity and unusual cell envelope ultrastructure. Arch. Microbiol. 172:139–149.

Messer, A. C., and M. J. Lee. 1989. Effect of chemical treatments on methane emission by the hindgut microbiota in the termite Zootermopsis angusticollis. Microb. Ecol. 18:275–284.

Moriya, S., J. B. Dacks, A. Takagi, S. Noda, M. Ohkuma, W. F. Doolittle, and T. Kudo. 2003. Molecular phylogeny of three oxymonad genera: Pyrsonympha, Dinenympha and Oxymonas. J. Eukar. Microbiol. 50:190–197.

Nakashima, K., H. Watanabe, and J.-I. Azuma. 2002a. Cellulase genes from the parabasalian symbiont Pseudotrichonympha grassii in the hindgut of the wood-feeding termite Coptotermes formosanus. Cell. Mol. Life. Sci. 59:1554–1560.

Nakashima, K., H. Watanabe, H. Saitoh, G. Tokuda, and J.-I. Azuma. 2002. Dual cellulose-digesting system of the wood-feeding termite, Coptotermes formosanus Shiraki. Insect Biochem. Molec. Biol. 32:777–784.

Nalepa, C. A., D. E. Bignell, and C. Bandi. 2001. Detritivory, coprophagy, and the evolution of digestive mutualisms in Dictyoptera. Insect. Soc. 48:194–201.

Ndiaye, D., R. Lensi, M. Lepage, and A. Brauman. 2004. The effect of the soil-feeding termite Cubitermes niokoloensis on soil microbial activity in a semi-arid savanna in West Africa. Plant Soil 259:277–286.

Noda, S., M. Ohkuma, R. Usami, K. Horikoshi K., and T. Kudo. 1999. Culture-independent characterization of a gene responsible for nitrogen fixation in the symbiotic microbial community in the gut of the termite Neotermes koshunensis. Appl. Environ. Microbiol. 65:4935–4942.

Noda, S., M. Ohkuma, A. Yamada, Y. Hongoh, and T. Kudo. 2003. Phylogenetic position and in situ identification of ectosymbiotic spirochetes on protists in the termite gut. Appl. Environ. Microbiol. 69:625–633.

Noirot, C. 1992. From wood- to humus-feeding: An important trend in termite evolution. *In:* J. Billen (Ed.) Biology and Evolution of Social Insects. Leuven University Press. Leuven, Belgium. 107–119.

Noirot, C. 1995. The gut of termites (isoptera): Comparative anatomy, systematics, phylogeny. I: Lower termites. Ann. Soc. Entomol. Fr. (N.S.) 31:197–226.

Noirot, C. 2001. The gut of termites (isoptera). Comparative anatomy, systematics, phylogeny. II: Higher termites (termitidae). Ann. Soc. Entomol. Fr. (N.S.) 37:431–471.

O'Brien, R. W., and M. Slaytor. 1982. Role of microorganisms in the metabolism of termites. Australian J. Biol. Sci. 35:239–262.

O'Brien, R. W., and J. A. Breznak. 1984. Enzymes of acetate and glucose metabolism in termites. Insect Biochem. 14:639–643.

Odelson, D. A., and J. A. Breznak. 1983. Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl. Environ. Microbiol. 45:1602–1613.

Odelson, D. A., and J. A. Breznak. 1985a. Cellulase and other polymer-hydrolyzing activities of Trichomitopsis termopsidis, a symbiotic protozoan from termites. Appl. Environ. Microbiol. 49:622–626.

Odelson, D. A., and J. A. Breznak. 1985b. Nutrition and growth characteristics of Trichomitopsis termopsidis, a cellulolytic protozoan from termites. Appl. Environ. Microbiol. 49:614–621.

Ohtoko K., M. Ohkuma, S. Moriya, T. Inoue, R. Usami, and T. Kudo. 2000. Diverse genes of cellulase homologues of glycosyl hydrolase family 45 from the symbiotic protists in the hindgut of the termite Reticulitermes speratus. Extremophiles 4:343–349.

Ohkuma, M., S. Noda, K. Horikoshi, and T. Kudo. 1995. Phylogeny of symbiotic methanogens in the gut of the termite Reticulitermes speratus. FEMS Microbiol. Lett. 134:45–50.

Ohkuma, M., and T. Kudo. 1996a. Phylogenetic diversity of the intestinal bacterial community in the termite Reticulitermes speratus. Appl. Environ. Microbiol. 62:461–468.

Ohkuma, M., S. Noda, R., Usami, K. Horikoshi, and T. Kudo. 1996b. Diversity of nitrogen-fixation genes in the symbiotic intestinal microflora of the termite Reticulitermes speratus. Appl. Environ. Microbiol. 62:2747–2752.

Ohkuma, M., and T. Kudo. 1998. Phylogenetic analysis of the symbiotic intestinal microflora of the termite Cryptotermes domesticus. FEMS Microbiol. Lett. 164:389–395.

Ohkuma, M., T. Iida, and T. Kudo. 1999a. Phylogenetic relationships of symbiotic spirochetes in the gut of diverse termites. FEMS Microbiol. Lett. 181:123–129.

Ohkuma M., S. Noda, and T. Kudo. 1999b. Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. Appl. Environ. Microbiol. 65:4926–4934.

Ohkuma, M., S. Noda, and T. Kudo. 1999c. Phylogenetic relationships of symbiotic methanogens in diverse termites. FEMS Microbiol. Lett. 171:147–153.

Ohkuma, M. 2002a. Symbiosis in the termite gut: Culture-independent molecular approaches. *In:* J. Seckbach (Ed.) Symbiosis: Mechanisms and Model Systems. Kluwer Academic Publishers. Dordrecht, The Netherlands. 717–730.

Ohkuma, M., S. Noda, Y. Hongoh, and T. Kudo. 2002b. Diverse bacteria related to the bacteroides subgroup of the CFB phylum within the gut symbiotic communities of various termites. Biosci. Biotechnol. Biochem. 66:78–84.

Ohkuma, M. 2003. Termite symbiotic systems: Efficient bio-recycling of lignocellulose. Appl. Microbiol. Biotechnol. 61:1–9.

Ohkuma, M., H. Shimizu, T. Thongaram, S. Kosono, K. Moriya, S. Trakulnaleamsai, N. Noparatnaraporn, and T. Kudo. 2003. An alkaliphilic and xylanolytic Paenibacillus species isolated from the gut of a soil-feeding termite. Microb. Environ. 18:145–151.

Osbrink, W. L. A., K. S. Williams, W. J. Connick, Jr., M. S. Wright, and A. R. Lax. 2001. Virulence of bacteria associated with the formosan subterranean termite (Isoptera: Rhinotermitidae) in New Orleans, La. Environ. Entomol. 30:443–448.

Paster, B. J., W. Ludwig, W. G. Weisburg, E. Stackebrandt, R. B. Hespell, C. M. Hahn, H. Reichenbach, K. O. Stetter, and C. R. Woese. 1985. A phylogenetic grouping of the bacteroides, cytophagas, and certain flavobacteria. System. Appl. Microbiol. 6:34–42.

Paster, B. J., F. E. Dewhirst, S. M. Cooke, V. Fussing, L. K. Poulsen, and J. A. Breznak. 1996. Phylogeny of not-yet-cultured spirochetes from termite guts. Appl. Environ. Microbiol. 62:347–352.

Pasti, M. B., and M. L. Belli. 1985. Cellulolytic activity of actinomycetes isolated from termite (Termitidae) gut. FEMS Microbiol. Lett. 26:107–112.

Pasti, M. B., A. L. Pometto III, M. P. Nuti, and D. L. Crawford. 1990. Lignin-solubilizing ability of actinomycetes isolated from termite (Termitidae) gut. Appl. Environ. Microbiol. 56:2213–2218.

Patricolo, E., L. Villa, and M. Arizzi. 2001. TEM observations on symbionts of Joenia annectens (Flagellata Hypermastigida). J. Nat. Hist. 35:471–480.

Paul, J., A. Sarkar, and A. K. Varma. 1986. In vitro studies of cellulose digesting properties of Staphylococcus saprophyticus isolated from termite gut. Curr. Sci. 55:710–714.

Paul, J., S. Saxena, and A. Varma. 1993. Ultrastructural studies of the termite (Odontotermes obesus) gut microflora and its cellulolytic properties. World J. Microbiol. Biotechnol. 9:108–112.

Pierantoni, U. 1936. La simbiosi fisiologica nei termitidi xilophagi e nei loro flagellati intestinali. Arch. Zool. Ital. 22:135–173.

Potrikus, C. J., and J. A. Breznak. 1977. Nitrogen-fixing Enterobacter agglomerans isolated from guts of wood-eating termites. Appl. Environ. Microbiol. 33:392–399.

Potrikus, C. J., and J. A. Breznak. 1980. Anaerobic degradation of uric acid by gut bacteria of termites. Appl. Environ. Microbiol. 40:125–132.

Potrikus, C. J., and J. A. Breznak. 1981. Gut bacteria recycle uric acid nitrogen in termites: A strategy for nutrient conservation. Proc. Natl. Acad. Sci. USA 78:4601–4605.

Potts, R. C., and P. H. Hewitt. 1973. The distribution of intestinal bacteria and cellulase activity in the harvester termite Trinervitermes trinervoides (Nasutitermitidae). Insect. Soc. 20:215–220.

Radek, R., K. Hausmann, and A. Breunig. 1992. Ectobiotic and endocytobiotic bacteria associated with the termite flagellate Joenia annectens. Acta Protozool. 31:93–107.

Radek, R., J. Rösel, and K. Hausmann. 1996. Light and electron microscopic study of the bacterial adhesion to termite flagellates applying lectin cytochemistry. Protoplasma 193:105–122.

Radek, R. 1999a. Flagellates, bacteria, and fungi associated with termites: Diversity and function in nutrition—a review. Ecotropica 5:183–196.

Radek, R., and G. Tischendorf. 1999b. Bacterial adhesion to different termite flagellates: Ultrastructural and functional evidence for distinct molecular attachment modes. Protoplasma 207:43–53.

Rasmussen, R. A., and M. A. K. Khalil. 1983. Global production of methane by termites. Nature 301:704–705.

Rath, A. C. 2000. The use of entomopathogenic fungi for control of termites. Biocontrol Sci. Technol. 10:563–581.

Rohrmann, G. F., and A. Y. Rossman. 1980. Nutrient strategies of Macrotermes ukuzii (Isoptera: Termitidae). Pedobiologia 20:61–73.

Rother, A., R. Radek, and K. Hausmann. 1999. Characterization of surface structures covering termite flagellates of the family Oxymonadidae and ultrastructure of two oxymonad species, Microrhopalodina multinucleata and Oxymonas sp. Eur. J. Protistol. 35:1–16.

Rouland, C., C. Chararas, and J. Renoux. 1989. Les osidases digestives présentes dans l'intestin moyen, l'intestin postérieur et les glandes salivaires du termite humivore Crenetermes albotarsalis. C. R. Acad. Sci. Paris Série III 308:281–285.

Rouland, C. 2000. Symbiosis with fungi. *In:* T. Abe, D. E. Bignell, and M. Higashi (Eds.) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers. Dordrecht, The Netherlands. 289–306.

Rouland-Lefevre C., M. N. Diouf, A. Brauman, and M. Neyra. 2002. Phylogenetic relationships in Termitomyces (family Agaricaceae) based on the nucleotide sequence of ITS: A first approach to elucidate the evolutionary history of the symbiosis between fungus-growing termites and their fungi. Molec. Phylogenet. Evol. 22:423–429.

Sacchi, L., C. A. Nalepa, M. Lenz, C. Bandi, S. Corona, A. Grigolo, and E. Bigliardi. 2000. Transovarial transmission of symbiotic bacteria in Mastotermes darwiniensis (Isoptera: Mastotermitidae): Ultrastructural aspects and phylogenetic implications. Ann. Entomol. Soc. Am. 93:1308–1313.

Salmassi, T. M., and J. R. Leadbetter. 2003. Molecular aspects of CO_2-reductive acetogenesis in cultivated spirochetes and the gut community of the termite Zootermopsis angusticollis. Microbiology 149:2529–2537.

Sanderson, M. G. 1996. Biomass of termites and their emissions of methane and carbon dioxide: A global database. Global Biogeochem. Cycles 10:543–557.

Sands, W. A. 1969. The association of termites and fungi. *In:* K. Krishna and F. M. Weesner (Eds.) Biology of Termites. Academic Press. New York, NY. 1:495–524.

Schäfer, A., R. Konrad, T. Kuhnigk, P. Kämpfer, H. Hertel, and H. König. 1996. Hemicellulose-degrading bacteria

and yeasts from the termite gut. J. Appl. Bacteriol. 80:471–478.

Schmitt-Wagner, D., and A. Brune. 1999. Hydrogen profiles and localization of methanogenic activities in the highly compartmentalized hindgut of soil-feeding higher termites (Cubitermes spp.). Appl. Environ. Microbiol. 65:4490–4496.

Schmitt-Wagner, D., M. Friedrich, B. Wagner, and A. Brune. 2003a. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (Cubitermes spp.). Appl. Environ. Microbiol. 69:6018–6024.

Schmitt-Wagner, D., M. Friedrich, B. Wagner, and A. Brune. 2003b. Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (Cubitermes spp.). Appl. Environ. Microbiol. 69:6007–6017.

Schultz, J. E., and J. A. Breznak. 1978. Heterotrophic bacteria present in hindguts of wood-eating termites [Reticulitermes flavipes (Kollar)]. Appl. Environ. Microbiol. 35:930–936.

Schultz, J. E., and J. A. Breznak. 1979. Cross-feeding of lactate between Streptococcus lactis and Bacteroides sp. isolated from termite hindguts. Appl. Environ. Microbiol. 37:1206–1210.

Seedorf, H., A. Dreisbach, R. Hedderich, S. Shima, and R. K. Thauer. 2004. $F_{420}H_2$ oxidase (FprA) from Methanobrevibacter arboriphilus, a coenzyme F_{420}-dependent enzyme involved in O_2 detoxification. Arch. Microbiol. 182:126–137.

Shima, S., M. Sordel-Klippert, A. Brioukhanov, A. Netrusov, D. Linder, and R. K. Thauer. 2001. Characterization of a heme-dependent catalase from Methanobrevibacter arboriphilus. Appl. Environ. Microbiol. 67:3041–3045.

Shinzato, N., T. Matsumoto, I. Yamaoka, T. Oshima, and A. Yamagishi. 1999. Phylogenetic diversity of symbiotic methanogens living in the hindgut of the lower termite Reticulitermes speratus analyzed by PCR and in situ hybridization. Appl. Environ. Microbiol. 65: 837–840.

Shinzato, N., T. Matsumoto, I. Yamaoka, T. Oshima, and A. Yamagishi. 2001. Methanogenic symbionts and the locality of their host lower termites. Microb. Environ. 16:43–47.

Sikorowski, P. P., and A. M. Lawrence. 1998. Transmission of Serratia marcescens (Enterobacteriaceae) in adult Heliothis virescens (Lepidoptera: Noctuidae) laboratory colonies. Biol. Control 12:50–55.

Slaytor, M. 1992. Cellulose digestion in termites and cockroaches: What role do symbionts play? Comp. Biochem. Physiol. 103B:775–784.

Slaytor, M., and D. J. Chappell. 1994. Nitrogen metabolism in termites. Comp. Biochem. Physiol. 107:1–10.

Slaytor, M., P. C. Veivers, and N. Lo. 1997. Aerobic and anaerobic metabolism in the higher termite Nasutitermes walkeri (Hill). Insect Biochem. Molec. Biol. 27:291–303.

Slaytor, M. 2000. Energy metabolism in the termite gut and its gut microbiota. *In:* T. Abe, D. E. Bignell, and M. Higashi, M. (Eds.) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers. Dordrecht, The Netherlands. 307–332.

Snel, J., H. J. Blok, H. M. P. Kengen, W. Ludwig, F. G. J. Poelma, J. P. Koopman, and A. D. L. Akkermans. 1994. Phylogenetic characterization of Clostridium related segmented filamentous bacteria in mice based on 16S ribosomal RNA analysis. System. Appl. Microbiol. 17:172–179.

Snel, J., P. P. Heinen, H. J. Blok, R. J. Carman, A. J. Duncan, P. C. Allen, and M. D. Collins. 1995. Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "Candidatus Arthromitus". Int. J. Syst. Bacteriol. 45:780–782.

Stackebrandt, E., P. Schumann, and X. L. Cui. 2004. Reclassification of Cellulosimicrobium variabile Bakalidou et al. 2002 as Isoptericola variabilis gen. nov., comb. nov. Int. J. System. Evol. Microbiol. 54:685–688.

Stingl, U., and A. Brune. 2003. Phylogenetic diversity and whole-cell hybridization of oxymonad flagellates from the hindgut of the wood-feeding lower termite Reticulitermes flavipes. Protist 154:147–155.

Stingl, U., A. Maass, R. Radek, and A. Brune. 2004. Symbionts of the gut flagellate Staurojoenina sp. from Neotermes cubanus represent a novel, termite-associated lineage of Bacteroidales: Description of "Candidatus Vestibaculum illigatum." Microbiology 150:2229–2235.

Stingl, U., R. Radek, and A. Brune. 2005. "Endomicrobia": Cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. Appl. Environ. Microbiol. 71:1473–1479.

Sugimoto, A., T. Inoue, I. Tayasu, L. Miller, S. Takeichi, and T. Abe. 1998. Methane and hydrogen production in a termite-symbiont system. Ecol. Res. 13:241–257.

Tamm, S. L. 1980. The ultrastructure of prokaryotic-eukaryotic cell junctions. J. Cell. Sci. 44:335–352.

Tamm, S. L. 1982. Flagellated epibiotic bacteria propel a eucaryotic cell. J. Cell. Biol. 94:697–709.

Taprab, Y., M. Ohkuma, T. Johjima, Y. Maeda, S. Moriya, T. Inoue, P. Suwanarit, N. Noparatnaraporn, and T. Kudo. 2002. Molecular phylogeny of symbiotic basidiomycetes of fungus-growing termites in Thailand and their relationship with the host. Biosci. Biotechnol. Biochem. 66:1159–1163.

Tayasu, I., A. Sugimoto, E. Wada, and T. Abe. 1994. Xylophagous termites depending on atmospheric nitrogen. Naturwissenschaften 81:229–231.

Tayasu, I., T. Abe, P. Eggleton, and D. E. Bignell. 1997. Nitrogen and carbon isotope ratios in termites: An indicator of trophic habit along the gradient from wood-feeding to soil-feeding. Ecol. Entomol. 22:343–351.

Tayasu, I. 1998. The use of carbon and nitrogen isotope ratios in termite research. Ecol. Res. 13:377–387.

Terra, W. R. 1990. Evolution of digestive systems of insects. Ann. Rev. Entomol. 35:181–200.

Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100–180.

Thayer, D. W. 1976. Facultative wood-digesting bacteria from the hind-gut of the termite Reticulitermes hesperus. J. Gen. Microbiol. 95:287–296.

Thayer, D. W. 1978. Carboxymethylcellulase produced by facultative bacteria from the hind-gut of the termite Reticulitermes hesperus. J. Gen. Microbiol. 106:13–18.

Tholen, A., B. Schink, and A. Brune. 1997. The gut microflora of Reticulitermes flavipes, its relation to oxygen, and evidence for oxygen-dependent acetogenesis by the most abundant Enterococcus sp. FEMS Microbiol. Ecol. 24:137–149.

Tholen, A., and A. Brune. 1999. Localization and in situ activities of homoacetogenic bacteria in the highly com-

partmentalized hindgut of soil-feeding higher termites (Cubitermes spp.). Appl. Environ. Microbiol. 65:4497–4505.

Tholen, A., and A. Brune. 2000. Impact of oxygen on metabolic fluxes and in situ rates of reductive acetogenesis in the hindgut of the wood-feeding termite Reticulitermes flavipes. Environ. Microbiol. 2:436–449.

Thongaram, T., S. Kosono, M. Ohkuma, Y. Hongoh, M. Kitada, T. Yoshinaka, S. Trakulnaleamsai, N. Noparatnaraporn, and T. Kudo. 2003. Gut of higher termites as a niche for alkaliphiles as shown by culture-based and culture-independent studies. Microb. Environ. 18:152–159.

Thongaram, T., Y. Hongoh, S. Kosono, M. Ohkuma, S. Trakulnaleamsai, N. Noparatnaraporn, and T. Kudo. 2005. Comparison of bacterial communities in the alkaline gut segment among various species of higher termites. Extremophiles. 9:229–238.

To, L., L. Margulis, and A. T. W. Cheung. 1978. Pillotinas and hollandinas: Distribution and behaviour of large spirochaetes symbiotic in termites. Microbios 22:103–133.

To, L. P., L. Margulis, D. Chase, and W. L. Nutting. 1980. The symbiotic microbial community of the Sonoran desert termite: Pterotermes occidentis. Biosystems 13:109–137.

Tokuda, G., I. Yamaoka, and H. Noda. 2000. Localization of symbiotic clostridia in the mixed segment of the termite Nasutitermes takasagoensis (Shiraki). Appl. Environ. Microbiol. 66:2199–2207.

Tokuda, G., T. Nakamura, R. Murakami, and I. Yamaoka. 2001. Morphology of the digestive system in the wood-feeding termite Nasutitermes takasagoensis (Shiraki) [Isoptera: Termitidae]. Zool. Sci. 18:869–877.

Tokuda, G., N. Lo, H. Watanabe, G. Arakawa, T. Matsumoto, and H. Noda. 2004. Major alteration of the expression site of endogenous cellulases in members of an apical termite lineage. Mol. Ecol. 13:3219–3228.

Tokura, M., M. Ohkuma, and T. Kudo. 2000. Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol. Ecol. 33:233–240.

Trinkerl, M., A. Breunig, R. Schauder, and H. König. 1990. Desulfovibrio termitidis sp. nov., a carbohydrate-degrading sulfate-reducing bacterium from the hindgut of a termite. System. Appl. Microbiol. 13:372–377.

Veivers, P. C., R. W. O'Brien, and M. Slaytor. 1980. The redox state of the gut of termites. J. Insect Physiol. 26:75–77.

Veivers, P. C., R. W. O'Brien, and M. Slaytor. 1982. Role of bacteria in maintaining the redox potential in the hindgut of termites and preventing entry of foreign bacteria. J. Insect Physiol. 28:947–951.

Vu, A. T., N. C. Ngyen, and J. R. Leadbetter. 2004. Iron reduction in the metal-rich guts of wood-feeding termites. Geobiology. 2:239–247.

Watanabe, H., and G. Tokuda. 2001. Animal cellulases. Cell. Molec. Life Sci. 58:1167–1178.

Watanabe, H., K. Nakashima, H. Saito, and M. Slaytor. 2002. New endo-beta-1,4-glucanases from the parabasalian symbionts, Pseudotrichonympha grassii and Holomastigotoides mirabile of Coptotermes termites. Cell. Mol. Life Sci. 59:1983–1992.

Watanabe, Y., N. Shinzato, and T. Fukatsu. 2003. Isolation of actinomycetes from termites' guts. Biosci. Biotechnol. Biochem. 67:1797–1801.

Wenzel, M., I. Schönig, M. Berchtold, P. Kämpfer, and H. König. 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite Zootermopsis angusticollis. J. Appl. Microbiol. 92:32–40.

Wenzel, M., R. Radek, G. Brugerolle, and H. König. 2003. Identification of the ectosymbiotic bacteria of Mixotricha paradoxa involved in movement symbiosis. Eur. J. Protistol. 39:11–24.

Wier, A., J. Ashen, and L. Margulis. 2000. Canaleparolina darwiniensis, gen. nov., sp. nov., and other pillotinaceous spirochetes from insects. Int. Microbiol. 3:213–223.

Williams, C. M., P. C. Veivers, M. Slaytor, and S. V. Cleland. 1994. Atmospheric carbon dioxide and acetogenesis in the termite Nasutitermes walkeri (Hill). Comp. Biochem. Physiol. 107A:113–118.

Yamin, M. A. 1979. Termite flagellates. Sociobiology 4:1–119.

Yang, H., D. Schmitt-Wagner, U. Stingl, and A. Brune. 2005. Niche heterogeneity determines bacterial community structure in the in the termite gut (Reticulitermes santonensis). Environ. Microbiol. 7(7):916–932.

Yara, K., K. Jahana, and H. Hayashi. 1989. In situ morphology of the gut microbiota of the fungus-growing termite Odontotermes formosanus (Termitidae: Macrotermitinae). Sociobiology 15:247–260.

Yoshimura, T., T. Fujino, T. Ito, K. Tsunoda, and M. Takahashi. 1996. Ingestion and decomposition of wood and cellulose by the protozoa in the hindgut of Coptotermes formosanus Shiraki (Isoptera: Rhinotermitidae) as evidenced by polarizing and transmission electron microscopy. Holzforschung 50:99–104.

Zoberi, M. H., and J. K. Grace. 1990. Fungi associated with the subterranean termite Reticulitermes flavipes in Ontario. Mycologia 82:289–294.

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CHAPTER 2.5

Marine Chemosynthetic Symbioses

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Introduction

Bacteria and marine eukaryotes often coexist in symbioses that significantly influence the ecology, physiology and evolution of both partners. De Bary (1879) defined symbiosis as "the living together of differently named organisms," implying that the term encompasses both positive (e.g., mutualism) and negative (e.g., parasitism) associations. Many researchers now view symbiotic interactions as those that persist over the majority of the lifespan of the organisms involved and that provide benefits to each partner beyond those obtained in the absence of association. This chapter describes such symbioses, specifically those between marine invertebrate and protist hosts and chemosynthetic bacterial symbionts.

These bacteria, which cluster primarily within the Gammaproteobacteria (Fig. 1), are chemoautotrophs or methanotrophs. In both chemoautotrophic and methanotrophic symbioses, the hosts, through an astonishing array of physiological and behavioral adaptations, provide the symbiont access to the substrates (i.e., electron donors and acceptors) necessary for the generation of energy and bacterial biomass. In exchange, a portion of the carbon fixed by the symbiont is used, either directly or indirectly, for host energy and biosynthesis. These symbioses thereby increase the metabolic capabilities, and therefore the number of ecological niches, of both the host and the bacterial symbiont.

In those symbioses for which the electron donor has been explicitly identified, sulfide and other inorganic reduced sulfur compounds (e.g., thiosulfate) fuel energy generation by the chemoautotrophic symbionts, serving as electron sources for oxidative phosphorylation. In these symbioses, the ATP produced in electron transport fuels autotrophic CO_2 fixation via the Calvin cycle. In contrast, bacteria in marine invertebrate-methanotroph symbioses use methane (CH_4) as an energy, electron, and carbon source. Unlike their protist or metazoan hosts, chemoautotrophs and methanotrophs share the ability to use reduced inorganic compounds or methane for energy generation and carbon dioxide or methane for carbon fixation and utilization. On the basis of these unique biosynthetic capacities, notably the ability to synthesize C_3 compounds from C_1 compounds, we refer collectively to these bacterial symbionts as "chemosynthetic."

Given the sulfide-rich habitats in which chemoautotrophic symbioses occur, researchers infer that the bacterial symbionts oxidize reduced inorganic sulfur compounds to obtain energy and reducing power for autotrophic carbon fixation. While some endosymbionts (such as those in the protobranchs *Solemya velum* and *S. reidi*; Cavanaugh, 1983; Anderson et al., 1987) utilize thiosulfate ($S_2O_3^{\mu}$), an intermediate in sulfide oxidation, hydrogen sulfide is inferred to be the preferred energy source in a variety of symbioses (see review in Van Dover, 2000). But for many symbioses the actual energy source has not been identified definitively; rather, only an autotrophic metabolism has been confirmed. Indeed, chemosynthetic bacteria utilizing other energy sources (e.g., hydrogen or ammonia) could also serve similar nutritional roles in symbiotic associations. In this review, bacterial symbionts that have been shown to use reduced sulfur compounds (H_2S, HS^{μ}, $S^{\mu 2}$, $S_2O_3^{\mu}$, S^o) for energy metabolism are referred to as thioautotrophs, while the more general term "chemoautotroph" is used to describe symbionts for which data supporting autotrophic CO_2 fixation exist but for which the lithotrophic energy source is unknown.

History

The discovery of deep-sea hydrothermal vents and the flourishing ecosystems associated with them significantly advanced scientific understanding of chemosynthetic symbioses. Oceanographers in the research submersible *Alvin* discovered hydrothermal vents along the Galapagos Rift in 1977. In stark contrast to perceptions of the deep benthos as a cold, food-limited habitat incapable of supporting substantial biomass, hydrothermal vents are oases, characterized by high concentrations of free-living

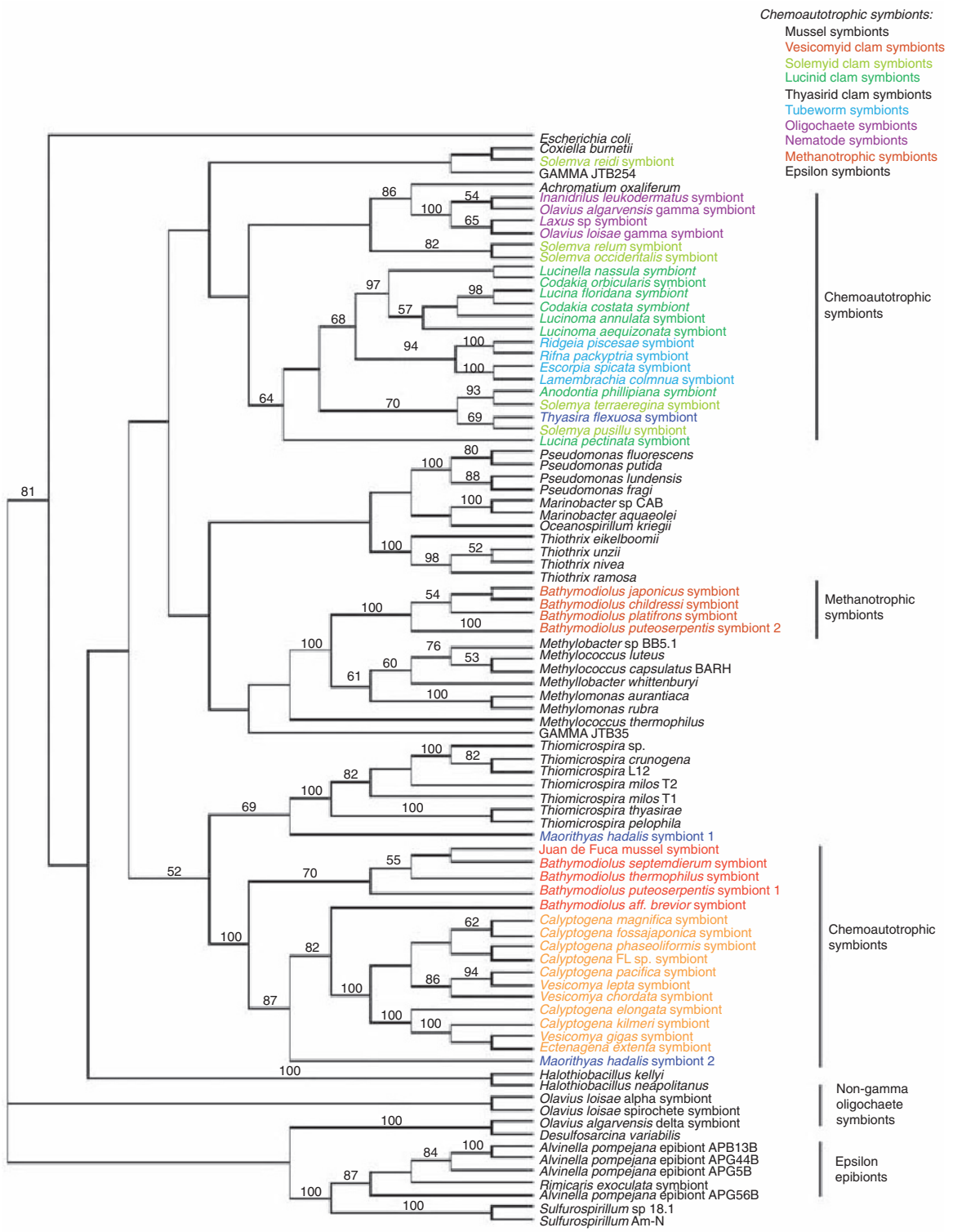

Fig. 1. Phylogeny showing the strict consensus of 46 trees obtained via parsimony analyses of 16S rRNA gene sequences (1456 bp) from symbiotic and free-living bacteria. Results greater than 50% from a 500 replicate bootstrap analysis are reported above respective branches. Chemosynthetic symbiont taxa are color-coded (see key on Figure).

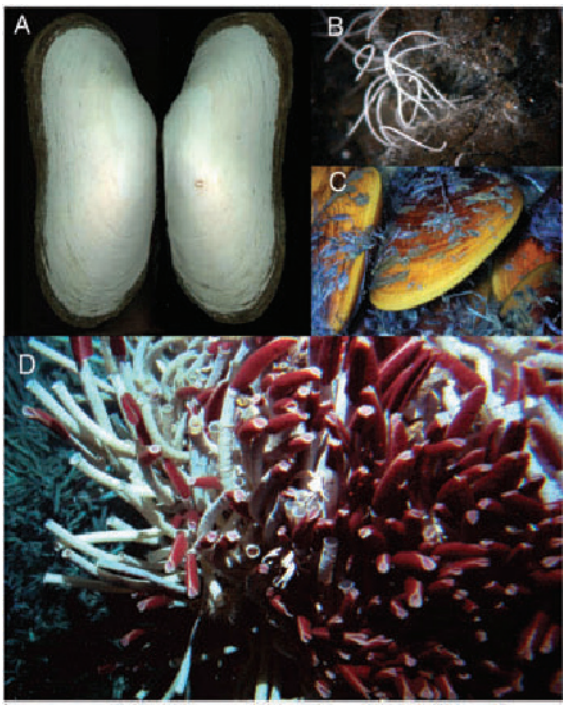

Fig. 2. Symbiont-containing host organisms from hydrothermal vents and cold seeps. (A) *Calyptogena magnifica* shell; courtesy of Emilio Jorge Power. (B) Filamentous bacteria on sulfide deposits from the East Pacific Rise. (C) *Bathymodiolus childressi* from the Gulf of Mexico; courtesy of the National Oceanic and Atmospheric Administration. (D) Riftia pachyptila at the East Pacific Rise.

microorganisms and dense aggregations of invertebrates (Lonsdale, 1977; Grassle, 1985; Van Dover, 2000). Researchers first argued that vent invertebrates achieved high densities by filtering organic matter, which was presumably transported to vent sites in hydrothermally-driven convection cells (Lonsdale, 1977). A second hypothesis suggested that the invertebrate community fed directly on locally dense populations of free-living chemoautotrophic bacteria (Lonsdale, 1977; Corliss et al., 1979).

However, studies of the giant tubeworm, *Riftia pachyptila* (Fig. 2), whose lack of mouth and gut precludes suspension feeding, suggested that sulfide-oxidizing endosymbiotic bacteria might contribute substantially to the vent food web. Cavanaugh et al. (1981) proposed that symbiotic chemosynthetic bacteria occurred in *R. pachyptila*. Microscopic and biochemical evidence indicated Gram-negative bacteria were present, packed within the tubeworm trophosome (Cavanaugh, 1981; Fig. 3), a highly vascularized organ in the tubeworm trunk in which activities of enzymes involved in sulfide oxidation and carbon fixation were also detected (Felbeck, 1981a). In addition, Rau (1981) used stable isotope signatures to show a nonphotosynthetic carbon source for *R. pachyptila*, implying a role for chemoautotrophy in tubeworm metabolism. Following confirmation of a chemosynthetic endosymbiosis within the giant tubeworm, researchers questioned the putative reliance on filter feeding by other vent invertebrates. Ultimately, anatomical, enzymological, and isotopic analyses revealed the presence of sulfur-oxidizing bacterial symbionts either within the tissues (endosymbiotic) or attached to the surfaces (episymbiotic) of most vent taxa, including vesicomyid clams, mytilid mussels, shrimp, and polychaete worms (Cavanaugh, 1994; Nelson and Fisher, 1995a; Van Dover, 2000).

Recognizing the ubiquity of chemoautotrophic symbioses at hydrothermal vents, researchers searched for and discovered similar symbiotic associations in other marine habitats, including coastal and subtidal reducing sediments (e.g., Felbeck et al., 1981b; Southward et al., 1981; Southward, 1982; Cavanaugh, 1983; Giere, 1985; Bauer-Nebelsick et al., 1996), brine and hydrocarbon seeps (Sibuet and Olu, 1998), and whale skeletons (Bennet et al., 1994; Smith and Baco, 2003), thereby extending the host taxa to include solemyid and lucinid bivalves, pogonophoran tubeworms, echinoids and ciliates. In addition, methanotrophic bacteria were detected in a marine sponge (Vacelet et al., 1995), a pogonophoran tubeworm (Schmaljohann and Flügel, 1987), and in vent and seep mussels, sometimes co-occurring with sulfur-oxidizing chemoautotrophs in a "dual symbiosis" (Childress et al., 1986; Cavanaugh et al., 1987; Cavanaugh, 1993).

Habitat Chemistry

The seemingly disparate ecological niches where these symbioses are found all possess a chemical gradient, or chemocline, which chemosynthetic bacteria exploit for energy production. Chemoclines form where reduced, high-energy compounds such as sulfide or methane (typically produced in anoxic habitats, including vent fluids and sediments) underlie an oxic water column. Chemosynthetic microorganisms must bridge the oxic-anoxic interface to access both the reduced compounds (e.g., H_2S) used as an energy source and the oxygen to which electrons are shuttled in aerobic energy metabolism.

The source of reduced compounds used in chemoautotrophic energy metabolism differs among habitats. In marine sediments microbial sulfate reduction (in which $SO_4^{\mu 2}$ is used as an electron acceptor during the oxidation of organic matter) dominates, and the sulfide utilized by thioautotrophic symbioses (e.g., involving lucinid clams or solemyid protobranchs) in these habitats is of biogenic origin. In contrast, sulfide at

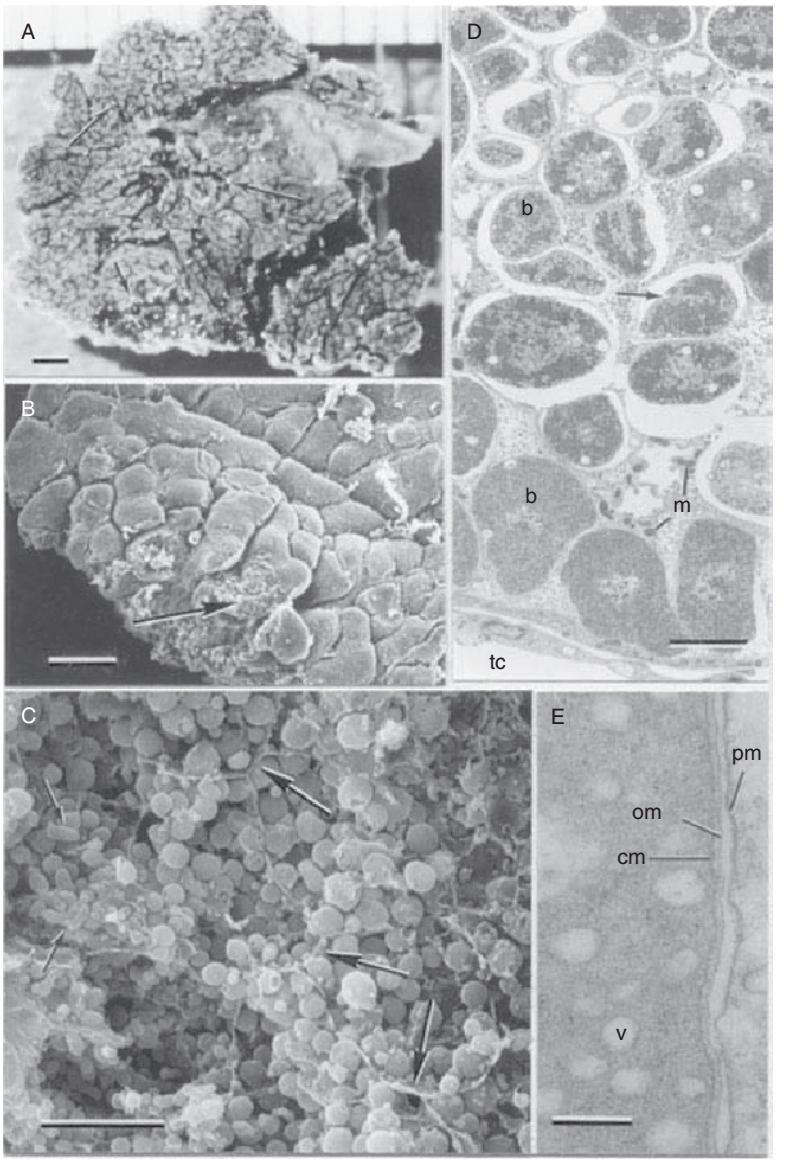

Fig. 3. *Riftia pachyptila* Jones (A–C: Galapagos Rift; D, E: 21°N, East Pacific Rise). (A) Photograph showing elemental sulfur crystals (arrows) scattered throughout trophosome; courtesy of M. L. Jones. (B) Scanning electron micrograph showing lobules of trophosome; arrow indicates area of C (below) where surface epithelium was removed to reveal symbionts within trophosome. (C) Same, higher magnification, showing symbionts within trophosome; note spherical cells as well as rod-shaped cells (small arrows); large arrows indicate likely host cell membranes. (D) Cross-section of portion of trophosome lobule showing variable fine structure of symbionts, including membrane-bound vesicles in many cells; all symbionts contained within membrane-bound vacuoles, either singly or in groups of two or more; arrow: dividing bacterium; b: bacteria; m: mitochondria; tc: trunk coelomic cavity. (E) Same, higher magnification, showing cell envelope of symbiont (resembling that of Gram-negative bacteria), intracytoplasmic vesicles, and peribacterial membrane; v: vesicle; cm: symbiont cytoplasmic membrane; om: symbiont outer membrane; pm: peribacterial membrane. Scale bars: A, 1 μm; B, 250 μm; C, 10 μm; D, 3 μm; and E, 0.2 μm. From Cavanaugh (1985), with permission.

hydrothermal vents is produced by the geothermal reduction of seawater sulfate and by the interaction between geothermally heated water and sulfur-containing rocks (e.g., basalt; Alt, 1995; Elderfield and Schultz, 1996; Rouxel et al., 2004). Seawater that percolates into the developing crust becomes heated and reacts with oceanic basalt, becoming enriched with metals and sulfide and charged with volcanic gases such as methane and carbon dioxide. Heated vent water then exits with concentrations of reduced compounds orders of magnitude higher than in ambient seawater. Hydrothermal effluent is hot (temperatures up to 350–400°C), acidic (pH ~ 3), anoxic, and can contain 3–12 mmol/kg of H_2S, 25–100 μmol/kg of CH_4, and 0.05–1 mmol/kg of H_2, as well as 360–1140 μmol/kg of Mn and 750–6500 μmol/kg of Fe (Elderfield and Schultz, 1996). As it exits the seafloor and mixes with the ambient bottom oxygenated seawater (pH, ca. 8; temperature = 1.8°C; [O_2], ca. 110 μM), metallic sulfides precipitate out resulting in "black smokers" (reviewed in Elderfield and Schultz, 1996). Vent organisms are typically found clustered around more diffuse or low flow vents, which are caused by ambient seawater mixing in the shallow subsurface with vent fluid. These vents are characterized by a higher pH (ca. 6), lower temperatures (1.8 to ca. 40°C), and, consequently, lower concentrations of reduced chemicals (Van Dover, 2000).

The relative acidity of vent fluid (black smokers: pH, ca. 3; diffuse flow vents: pH, ca. 6) significantly impacts the concentration of inorganic

chemicals available to chemosynthetic symbioses. For example, carbon dioxide (CO_2), bicarbonate (HCO_3^{μ}), and carbonate ($CO_3^{\mu 2}$), the three distinct chemical species of the dissolved inorganic carbon (DIC) pool, vary in relative abundance depending on pH; pK_a values for these compounds are 6.4 for $CO_2 : HCO_3^{\mu}$ and 10.3 for $HCO_3^{\mu} : CO_3^{\mu 2}$ at 25°C (Stumm and Morgan, 1996). Thus CO_2, which diffuses freely across biological membranes and is the DIC species fixed by chemoautotrophic symbionts utilizing the Calvin cycle, is readily available at vents (Cavanaugh and Robinson, 1996; Goffredi et al., 1997b). In addition, sulfide exists at three levels of dissociation (H_2S, HS^{μ}, and $S^{\mu 2}$) depending on pH, with pK_a values of 7.0 for $H_2S : HS^{\mu}$ and 12.9 for $HS^{\mu} : S^{\mu 2}$ at 25°C (Stumm and Morgan, 1996). Therefore, in the relatively acidic vent fluids sulfide occurs predominantly as H_2S. In such effluent, total sulfide (H_2S, HS^{μ}, and $S^{\mu 2}$) concentration correlates positively with temperature (Johnson et al., 1988); conversely, higher temperatures (>30°C) may facilitate reactions between sulfide and other dissolved elements (such as iron) that reduce free sulfide availability (Luther et al., 2001). The chemical environment (i.e., concentrations of CO_2, O_2, H_2S, CH_4, H^+, and dissolved metals) is therefore expected to significantly influence the ecology and evolution of chemosynthetic symbioses.

Methods for Studying Chemosynthetic Symbioses

To date, the bacteria involved in these symbiotic associations have not yet been isolated and grown in pure culture—perhaps because the unique environment encountered in situ by chemosynthetic symbionts has not been recreated or because a reduced genome, characteristic of many endosymbionts, has precluded growth outside of the host. Symbiotic bacteria are therefore studied indirectly, using methods to assess their physiology, ecology and phylogeny within the context of the intact symbiosis. Traditionally, researchers identify chemosynthetic symbioses using a combination of microscopy (light, confocal, scanning and transmission electron), which provides visual information on the location, morphology and ultrastructure of symbionts, and enzyme assays, which detect and quantify the activity of key proteins involved in chemoautotrophic (e.g., ribulose 1,5-bisphosphate carboxylase-oxygenase) or methanotrophic (e.g., methanol dehydrogenase) metabolism. In addition, tracing the incorporation of radiolabeled substrates (e.g., carbon dioxide, methane and nitrogen species) within the host helps define the physiology of the host-bacteria partnership. Such physiological assays, in conjunction with analyses of stable isotope signatures of symbiont-containing and symbiont-free host tissue, provide valuable insight into the trophic dynamics of symbiont-based communities. Molecular approaches, such as polymerase chain reaction (PCR)-based gene probing, 16S rRNA gene analysis, and fluorescent in situ hybridization (FISH), are increasingly used to characterize the systematic relationships of symbiont and host species (e.g., Distel et al., 1995; Peek et al., 1998; Dubilier et al., 1999) and the metabolism and gene flow of the bacterial symbionts (Robinson et al., 1998; Lee et al., 1999; Millikan et al., 1999; Podar et al., 2002). Molecular techniques have also been used to detect symbiont transmission modes (Cary and Giovannoni, 1993a; Krueger, 1996a) as well as symbiont abundance (Polz and Cavanaugh, 1995).

Summary

This chapter reviews symbiotic associations between chemosynthetic bacteria and marine invertebrate and protist hosts. A bias toward symbioses between chemoautotrophic bacteria and hydrothermal vent invertebrates is evident, primarily because our knowledge of marine bacterial symbioses stems largely from studies of vent fauna done in the 27 years following the discovery of these unique organisms. But despite this impressive body of research, much about these marine symbioses remains to be revealed. In conjunction with several earlier reviews that provide a thorough and thoughtful treatment of symbioses occurring at hydrothermal vents and cold seeps (Fisher, 1990; Felbeck and Distel, 1991; Childress and Fisher, 1992; Cavanaugh, 1994; Nelson and Fisher, 1995a), the following chapter presents an overview of the ecology, physiology and evolution of chemosynthetic symbioses.

Host Diversity

Chemosynthetic bacteria are known to associate with a diversity of invertebrate hosts (six phyla), as well as with ciliate protists (Table 1 and references therein). To date, the majority of the symbionts characterized via 16S rRNA phylogenetic analyses fall within the Gammaproteobacteria division (Fig. 1; discussed further below). The intimacy of these associations varies among taxa. The bacterial partners may be episymbionts living on the surface of the host (e.g., on shrimp, nematodes, sponges, limpets and ciliates; Figs. 4–6), or endosymbionts living either extracellularly within host tissue (e.g., in oligochaetes; Fig. 7) or in specialized host cells and organs (e.g., in bivalves and

Table 1. List of invertebrate taxa hosting chemoautotrophic or methanotrophic bacterial symbionts.[a]

Group	Common name	Symbiont-containing tissue	Location	Habitat	Symbiont type	References
Protozoa						
Class Ciliata	Ciliate	NA	Epibiotic	Cold seeps, mangrove swamp	Chemoautotroph	Bauer-Nebelsick et al., 1996 Ott et al., 1998 Buck et al., 2000 Fenchel and Finlay, 1989
Porifera						
Class Demospongiae Family Cladorhizidae	Sponge	NA	Extracellular	Cold seeps	Methanotroph	Vacelet et al., 1995, 1996
Nemata						
Subfamily Stilbonematinae	Nematode	Cuticle	Epibiotic	Reducing sediments	Chemoautotroph	Schiemer et al., 1990 Polz et al., 1992
Mollusca						
Class Bivalvia						
Subclass Protobranchia						
Family Solemyidae	Clam	Gills	Intracellular	Reducing sediments, hydrothermal vents,[b] cold seeps[c]	Chemoautotroph	Cavanaugh, 1983 Fisher and Childress, 1986 Conway et al., 1989
Subclass Heterodonta						
Family Lucinidae	Clam	Gills	Intracellular	Reducing sediments, cold seeps[c]	Chemoautotroph	Giere, 1985 Schweimanns and Felbeck, 1985
Family Thyasiridae	Clam	Gills	Intracellular	Reducing sediments, cold seeps[c]	Chemoautotroph	Dando and Southward, 1986 Herry and Le Pennec, 1987
Family Vesicomyidae	Clam	Gills	Intracellular	Hydrothermal vents, cold seeps	Chemoautotroph	Cavanaugh, 1983 Rau, 1981
Subclass Pteriomorphia						
Family Mytilidae	Mussel	Gills	Extracellular Intracellular	Hydrothermal vents, cold seeps	Chemoautotroph and/or methanotroph	Childress et al., 1986 Cavanaugh et al., 1987 Fisher et al., 1988 Cavanaugh et al., 1992
Class Gastropoda						
Family Provannidae	Snail	Gills	Intracellular	Hydrothermal vents	Chemoautotroph	Stein et al., 1988 Endow and Ohta, 1989

Family Lepetodrilidae	Limpet	Gills	Epibiotic	Hydrothermal vents	Chemoautotroph	de Burgh and Singla, 1984 Fox et al., 2002 Bates et al., 2004
Annelida[d]						
Class Polychaeta	Worm	Dorsal surface	Epibiotic	Hydrothermal vents	Chemoautotroph	Desbruyeres et al., 1983, 1985 Cary et al., 2003
Family Alvinellidae						
Family Siboglinidae (Vestimentifera and Pogonophora)	Tubeworm	Trophosome	Intracellular	Deep-sea hydrothermal vents, cold seeps, reducing sediments, fjords	Chemoautotroph	Cavanaugh et al., 1981 Felbeck, 1981 Southward et al., 1981 Brooks et al., 1987 Schmaljohann and Flügel, 1987 Southward and Southward, 1988 de Burgh et al., 1989
Class Clitellata						
Subfamily Phallodrilinae	Oligochaete	Subcuticular	Extracellular	Coralline sands	Chemoautotroph[e]	Felbeck et al., 1983 Giere, 1981, 1985 Giere and Langheld, 1987
Arthropoda						
Class Crustacea						
Family Alvinocarididae	Shrimp	Carapace	Epibiotic	Hydrothermal vents	Chemoautotroph	Van Dover et al., 1988 Polz and Cavanaugh, 1995 Polz et al., 1999
Echinodermata						
Class Echinoidea	Sea urchin	Gut	Extracellular	Reducing sediments	Chemoautotroph[f]	Temara et al., 1993 Brigmon and de Ridder, 1998

Abbreviation: NA, not applicable.

[a]Chemosynthetic status of symbionts inferred from ultrastructural, physiological, enzymatic, and molecular data.

[b]A solemyid protobranch, *Acharax alinae*, has been described from the Lau Basin hydrothermal vents, but the symbiosis has not been characterized (Metivier and Voncosel, 1993).

[c]Solemyid clams have been collected from cold seeps in the eastern Pacific, and lucinid and thyasirid clams have been collected from cold seeps in the Gulf of Mexico, Sagami Bay, and Barbados Prism, though the presence of symbionts has not been formally described (Sibuet and Olu, 1998).

[d]Though it is now accepted that the pogonophoran and vestimentiferan worms are not separate phyla but members of phylum Annelida, they are listed as separate groups for identification purposes.

[e]The oligochaete *Olavius algarvensis* has been shown to have an additional symbiont which is a sulfur reducing bacterium and member of the delta Proteobacteria (Dubilier et al., 2001). *Olavius loisae* has been shown to host an alpha proteobacterium and a spirochete as well as a chemoautotroph (Dubilier et al., 1999).

[f]This symbiont has been described as *Thiothrix*-like on the basis of morphology, physiology, and immunological assays (Temara et al., 1993; Brigmon and de Ridder, 1998).

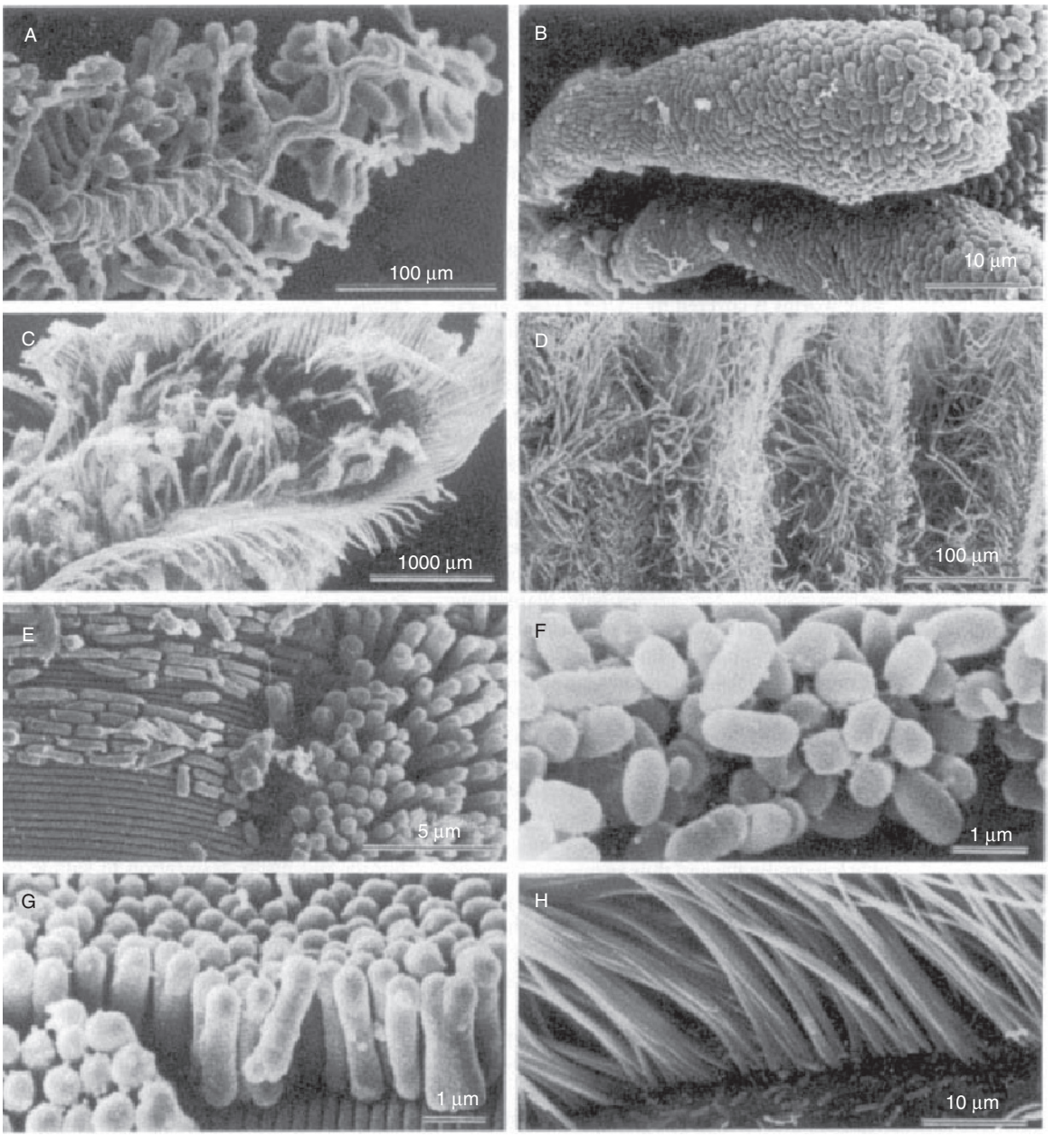

Fig. 4. Scanning electron micrographs showing the morphological diversity of ectosymbiotic bacteria on the colonial ciliate *Zoothamnium niveum* (A, B), the shrimp *Rimicaris exoculata* (C, D), and the nematodes within the subfamily Stilbonematinae (E-H). (A) Entire ciliate colony with zooids attached to a common stem, and (B) bacterial epigrowth on an individual zooid. (C) Shrimp appendage covered by dense arrays of filamentous bacteria, and (D) detail of the hair-like bacterial covering. Epigrowth on different species of nematodes showing (E) irregular epigrowth of two morphological types on *Robbea* sp., (F) coccoid bacteria forming multilayers on *Stibonema* sp., (G) upright standing, longitudinally dividing rods on *Laxus oneistus*, and (H) dense array of nonseptate filaments that can reach up to 100 mm in length on *Eubostrichus dianae*. From Polz et al. (2000), with permission.

vestimentiferan tubeworms; Figs. 2, 3, and 8). In intracellular endosymbioses the symbionts are housed within specialized host cells called "bacteriocytes" and are contained within a host-derived membrane bound vacuole (Cavanaugh, 1983; Cavanaugh, 1994; Fisher, 1990). Host morphology clearly suggests a nutritional benefit from these intimate associations. Indeed, as in the giant vent tubeworms, the digestive system of many endosymbiont-containing marine invertebrates is either reduced (e.g., in coastal solemyid protobranchs) or absent altogether (e.g., in oligochaetes and vestimentiferan and pogonophoran tubeworms), consistent with

host dependence on the symbiont for part or all of its nutrition.

All of the members of the tubeworm family Siboglinidae examined to date, including the vestimentiferan and the smaller pogonophoran tubeworms, contain intracellular symbionts. Most of these symbionts are inferred to be chemoautotrophic, but methanotrophs have been found in one host species (*Siboglinum poseidoni*; Schmaljohann and Flügel, 1987). The vent tubeworm *Riftia pachyptila* and other vestimentiferan and pogonophoran tubeworm species possess a unique morphological adaptation to accommodate their symbionts. Tubeworm bacteria reside within a lobular and highly vascularized organ (the trophosome) that occupies most of the tubeworm trunk and functions specifically to house bacteria (Cavanaugh, 1981; Felbeck, 1981a; Figs. 3 and 9). The symbiosis is obligate for these worms, as they are mouthless and gutless as adults and depend on their internal bacteria for their nutrition.

Chemosynthetic symbioses are widespread within the Mollusca and have been detected in five bivalve and two gastropod families (Table 1). In mollusk symbioses the bacteria occur only in the gills; bacteria have been found within gill epithelial cells of solemyid protobranchs (Cavanaugh, 1983; Krueger et al., 1996b; Figs.

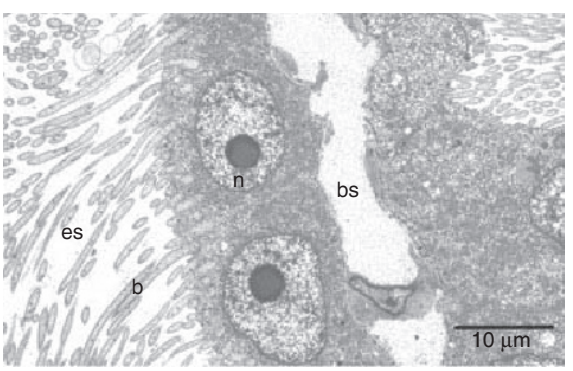

Fig. 5. *Lepetodrilus fucensis*. Transverse section of gill tissue from the hydrothermal vent limpet showing episymbiotic filamentous bacteria partially embedded in the host epithelium. b, bacteria; es, extracellular space; n, nucleus; bs, blood space. Courtesy of Amanda Bates.

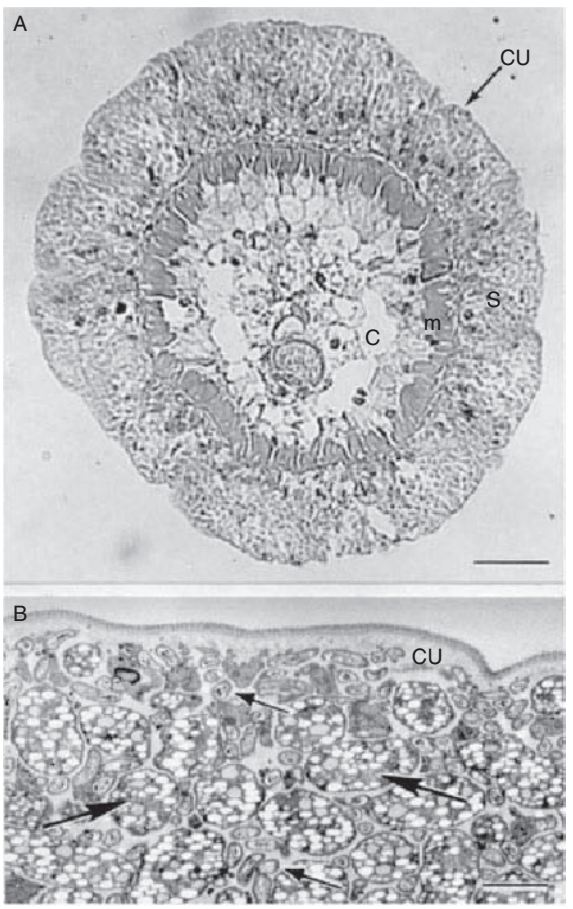

Fig. 7. *Inanidrilus leukodermatus*. (A) Light micrograph of a cross section of an oligochaete worm. (B) Transmission electron micrograph of symbiont-containing region just below the cuticle. Note smaller and larger symbiont morphotypes (smaller and larger arrows, respectively). c, coelomic cavity; m, muscle tissue; s, symbiont-containing region between cuticle and epidermis; cu, cuticle. Scale bars: A, 20 µm; B, 2 µm. From Dubilier et al. (1995), with permission.

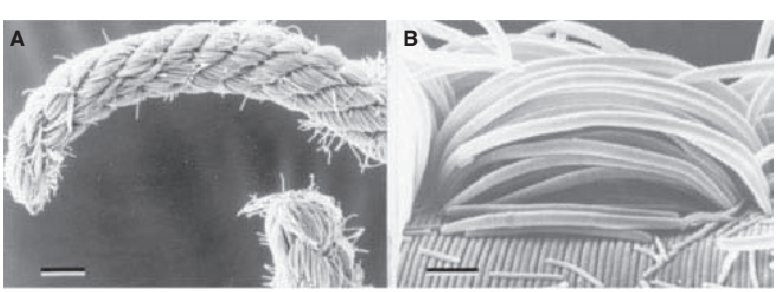

Fig. 6. *Eubostrichus* cf. *parasitiferus*. Scanning electron micrographs showing the symbiotic bacteria on the surface of the nematode. (A) Anterior (bottom) and posterior (top) end with symbionts arranged in a characteristic helix. (B) Higher magnification. Bacteria are attached with both ends to the worm's cuticle. Note the increasing length of the cells from proximal to distal along the worm's surface. Scale bars: A, 20 µm; B, 2 µm. From Polz et al. (1992), with permission.

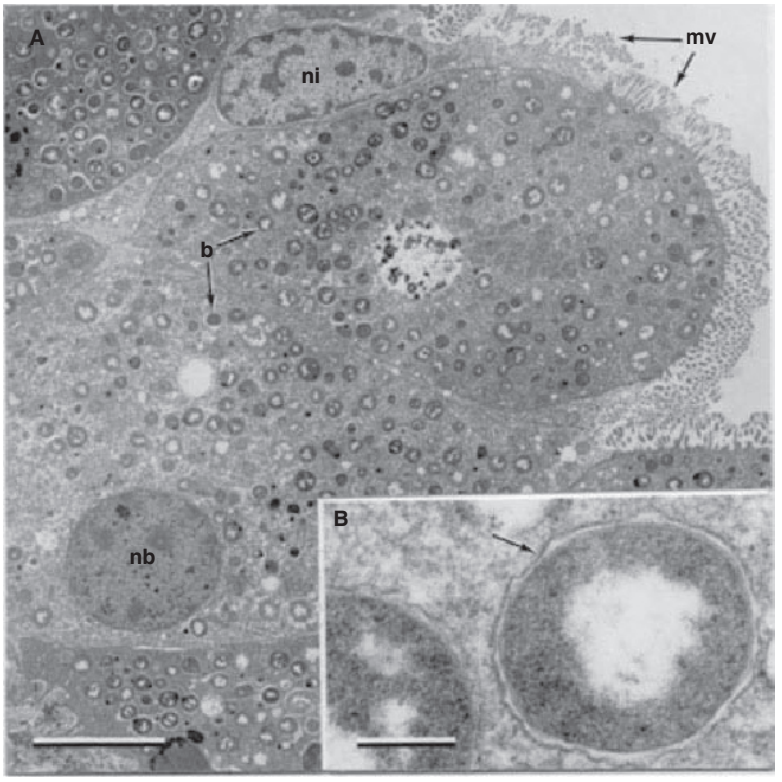

Fig. 8. *Calyptogena magnifica* Boss and Turner (21°N East Pacific Rise). (A) Transmission electron micrograph of slightly oblique transverse section of gill filament, showing coccoid-shaped bacteria within gill bacteriocyte and intercalary cells lacking symbionts; b: bacteria; mv: microvilli (of both cell-types); nb: nucleus of bacteriocyte; ni: nucleus of intercalary cell. (B) Same, higher magnification, transverse section of coccoid-shaped symbionts, showing cell ultrastructure typical of Gram-negative bacteria and peribacterial membrane (arrow). Scale bars: A, 5 μm; B, 0.25 μm. From Cavanaugh (1985), with permission.

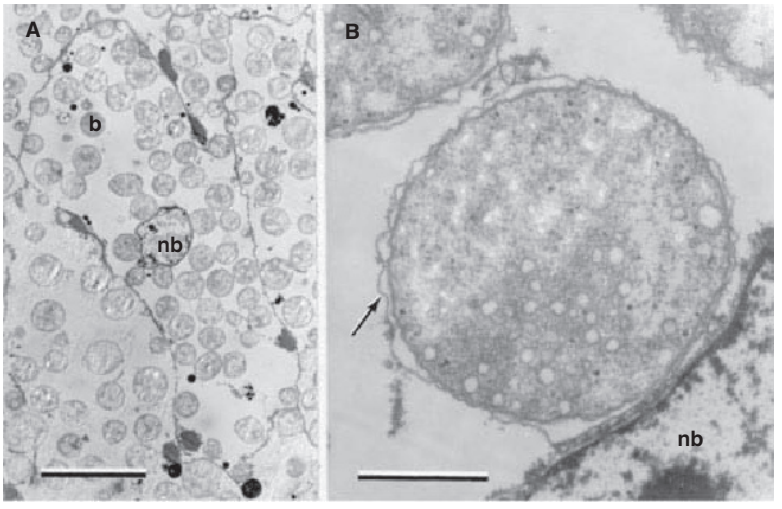

Fig. 9. Escarpia spicata Jones (San Clemente Fault). (A) Transmission electron micrograph, portion of trophosome lobule showing numerous coccoid- to ovoid-shaped bacterial symbionts, some of which appear intracellular. (B) Same, higher magnification, showing bacterial cell envelope (resembling that of Gram-negative bacteria) and intracytoplasmic membrane-bound vesicles; arrow: peribacterial membrane; b: bacteria; nb: nucleus of bacteriocyte. Scale bars: A, 10 μm; B, 1 μm. From Cavanaugh (1985), with permission.

10–13), lucinid clams (Cavanaugh, 1983; Felbeck, 1983a), thyasirid clams (Felbeck et al., 1981b; Cavanaugh, 1983; Arp et al., 1984), vesicomyid clams (Boss and Turner, 1980; Rau, 1981; Arp et al., 1984; Fig. 8), mytilid mussels (Fiala-Médioni, 1984; Le Pennec and Hily, 1984; Figs. 2 and 14), and provannid gastropods (Stein et al., 1988; Windoffer and Giere, 1997). Within certain mollusk families (e.g., Solemyidae, Lucinidae and Thyasiridae), all species examined form symbioses with chemoautotrophic bacteria. In other families, such as the Mytilidae, chemoautotrophic symbionts have been detected only in members of the subfamily Bathymodiolinae, which are found exclusively in the deep-sea. Further, dual symbioses involving both methanotrophs and chemoautotrophs are restricted to species of deep-sea bathymodioline mussels collected from methane seeps and hydrothermal vents (Cavanaugh, 1994; Nelson and Fisher, 1995a; Van Dover, 2000; Fig. 14).

Chemosynthetic bacteria also occur as episymbionts on marine invertebrates (Table 1; Fig. 4). These symbionts include the Epsilonproteo-

Fig. 10. *Solemya* sp. (right hand) collected from deep-sea vent sites (2380 m depth) along the subduction zone off Oregon, and *Solemya velum* (left hand) collected from subtidal reducing sediments (<1 m depth, mean low tide) of Massachusetts eelgrass beds. Photo courtesy of Dr. Ruth D. Turner.

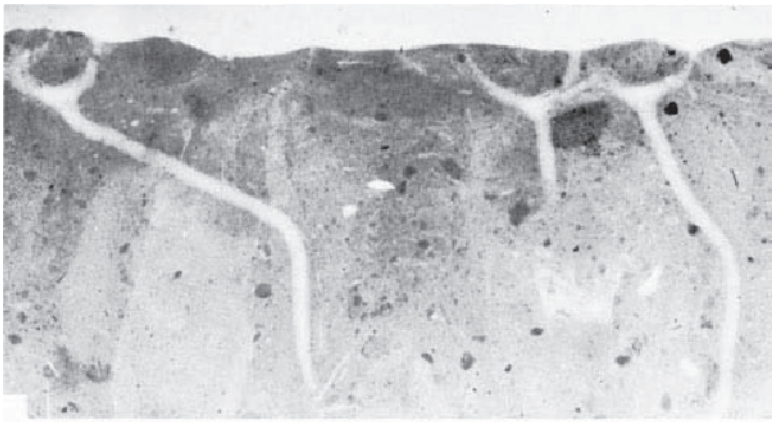

PLATE 3. SOLEMYA

Fig. 11. *Solemya velum*. Characteristic Y-shaped burrows dug by the coastal protobranch clam to bridge the oxic-anoxic interface and access both reduced sulfur (from below) and oxygen (from above). From Stanley (1970), with permission.

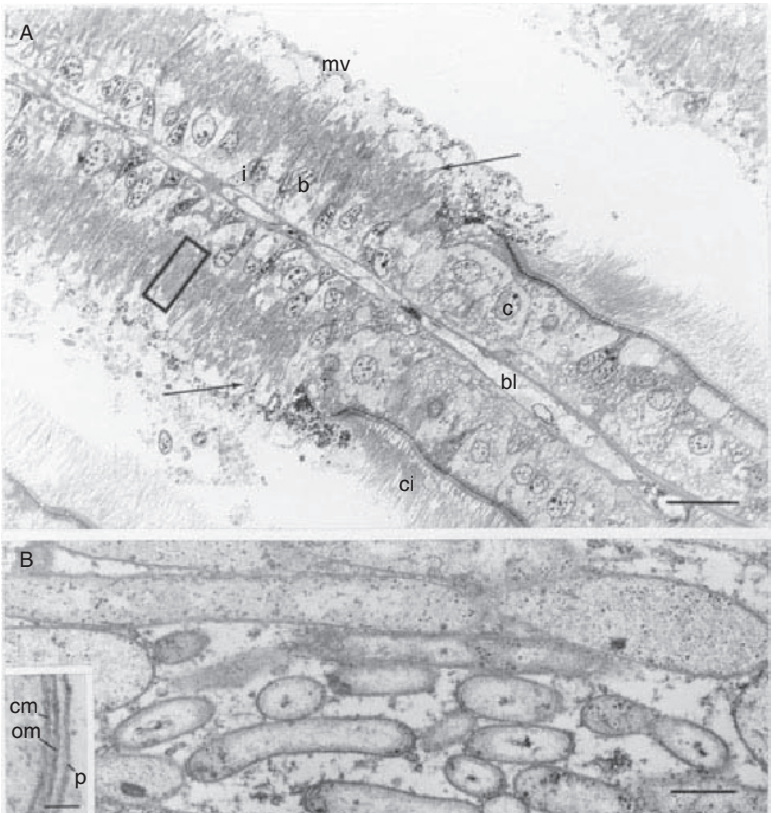

Fig. 12. Solemya borealis. (A) Transverse section of gill filaments showing intracellular rod-shaped bacteria (arrows, rectangle). Bacteriocytes are confined to the region proximal to the ciliated edge of the gill and are flanked by symbiont-free intercalary cells that appear to comprise the microvillar surface of the gill filament. Light micrograph. b: bacteriocyte nucleus; c: ciliated cell nucleus; i: intercalary cell nucleus; bl: blood space; ci: cilia; mv: microvilli. (B) Higher magnification of symbionts showing cell ultrastructure typical of Gram-negative. Inset: Detail of symbiont cell envelope and peribacterial membrane. p: peribacterial membrane; cm: cell membrane; om: outer membrane. Scale bars: A, 20 µm; B, 1 µm; inset, 0.05 µm. From Conway et al. (1992b), with permission.

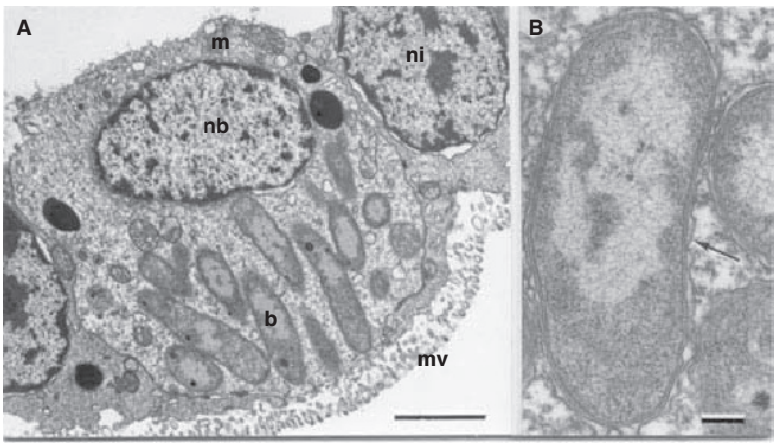

Fig. 13. *Solemya velum*. (A) Transmission electron micrograph, transverse section of gill filament, showing rod-shaped bacteria within gill bacteriocyte and intercalary cells lacking symbionts; b: bacteria; mv: microvilli; nb: nucleus of bacteriocyte; ni: nucleus of intercalary cell. (B) Same, higher magnification, transverse section of rod-shaped bacterium, showing cell ultrastructure typical of Gram-negative bacteria and peribacterial membrane (arrows). Scale bars: A, 3 μm; B, 0.2 μm. From Cavanaugh (1985), with permission.

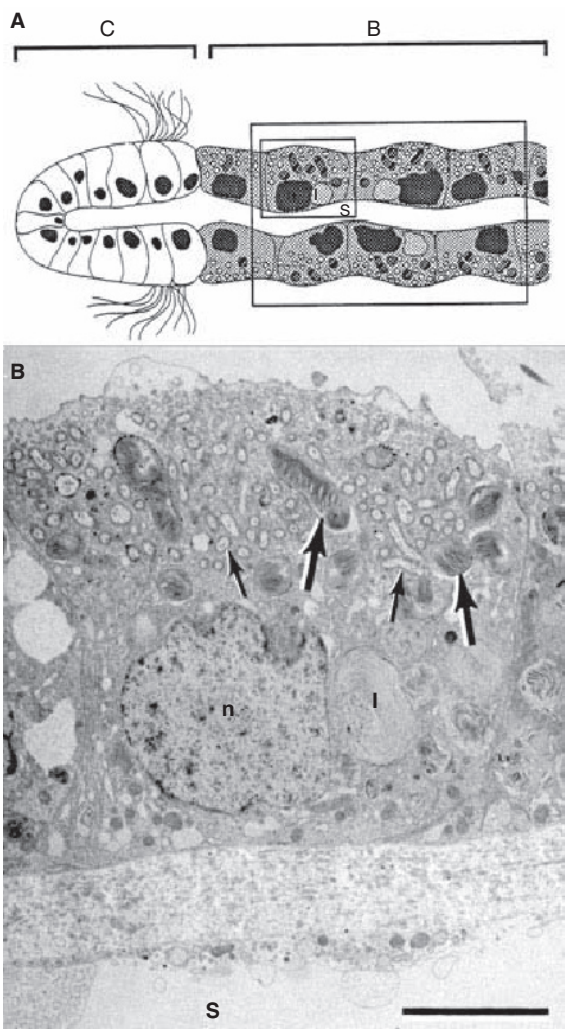

Fig. 14. *Bathymodiolus puteoserpentis*. Transverse section of Mid-Atlantic Ridge (MAR) mytilid gill filament, showing symbiont-containing gill epithelial cells (bacteriocytes). (A) Diagram of gill filament. Bacteriocytes are confined to the region proximal to the ciliated border of the gill. Small box shows positions of Figs. 14B. B: symbiont-containing bacteriocyte region; and C: symbiont-free ciliated region. (B) Transmission electron micrograph. Large and small symbionts (large and small arrows, respectively) are located in the apical region of the cells, while nuclei and lysosomal residual bodies occupy the region closest to the blood sinus. Note centrally stacked intracytoplasmic membranes in large symbionts. 1, Lysosomal residual body; n, bacteriocyte nucleus; and s, blood sinus. Scale bar: 5 μm. From Distel et al. (1995), with permission.

bacteria that cover the cuticle of *Rimicaris* shrimp, dominant members of the metazoan fauna at vents on the Mid-Atlantic Ridge (MAR; Polz et al., 1998) and the Central Indian Ridge (CIR; Van Dover et al., 2001; Van Dover, 2002a), and the surfaces of alvinellid polychaetes (Desbruyères et al., 1985; Cary et al., 1997). Chemosynthetic episymbionts also associate with nematodes (Weiser, 1959; Ott et al., 1991; Polz et al., 1992; Polz et al., 1994) and ciliates

(e.g., Fenchel and Finlay, 1989; Bauer-Nebelsick et al., 1996). In addition, methanotrophic episymbionts have been found living on a deep-sea sponge (Vacelet et al., 1995; Vacelet et al., 1996). Vent limpet-bacteria associations seem to be an intermediate between epi- and endosymbioses; bacteria exist partially embedded in the limpet gill epidermis and may be endocytosed or fed on by the host (de Burgh and Singla, 1984; Bates et al., 2004; Fig. 5). Some epibiont communities, like those residing on the *Rimicaris* shrimp and the nematode *Laxus* sp., are dominated by a single phylotype (Polz et al., 1994; Polz and Cavanaugh, 1995), while others are quite diverse (Polz et al., 1999; Campbell et al., 2003). But given that morphological plasticity often belies the phylogenetic identity of symbionts (Polz et al., 1999; Giere and Krieger, 2001), symbiont diversity estimates are only appropriate when putative symbiont phylotypes are confirmed using hybridization methods (e.g., FISH).

Symbiont Diversity

Morphology and Ultrastructure

Symbiont morphology varies among functional types (chemoautotroph vs. methanotroph), among phylotypes within the same functional group, and among individuals in a population of a single phylotype. The symbionts all have a Gram-negative cell envelope but range from small (ca. 0.25 μm diameter) coccoid endosymbionts within mussel gills (Cavanaugh, 1985; Dubilier et al., 1998) to large (>10 μm length) rod-shaped and filamentous episymbionts on vent shrimp (Hentschel et al., 1993b; Polz and Cavanaugh, 1995; Fig. 4). Some bathymodioline mussels host two metabolically distinct symbionts: small (<0.5 μm) chemoautotrophs and larger (1.5–2.0 μm) methanotrophic bacteria possessing stacked intracytoplasmic membranes, which are typical of Type I methanotrophs (e.g., Childress et al., 1986; Cavanaugh et al., 1987; Cavanaugh et al., 1992; Fiala-Médioni et al., 2002; Pimenov et al., 2002; Fig. 14). Morphological diversity can also occur throughout monospecific populations within a single host animal. For example, populations of sulfur-oxidizing chemoautotrophic symbionts in the tubeworm *Riftia pachyptila* contain distinct morphotypes that vary in abundance depending on location within the trophosome lobule (Bright et al., 2000); small, rod-shaped bacteria occur primarily in the innermost zone of the lobule nearest the host's axial blood vessel, while small and large cocci (1.6–10.7 μm diameter) occupy zones nearer the periphery of the trophosome (Bright et al., 2000). Such variability may relate to differences in the lifecycle stage and metabolism among symbiont cells (Bright et al., 2000).

Symbiont Phylogeny

While chemoautotrophic symbionts have consistently evaded culture, the suite of cellular and molecular methods used to characterize these bacteria has revealed startling evolutionary trends. Investigators have successfully sequenced 16S rRNA genes from symbiont-containing tissue and subsequently confirmed the symbiont origin of these sequences via hybridization with symbiont-specific probes. In contrast to the wide diversity of host taxa involved in these symbioses, chemosynthetic symbionts cluster primarily within a single bacterial division, the Gammaproteobacteria, on the basis of 16S rRNA gene sequences (Distel and Cavanaugh, 1994; Dubilier et al., 1999; McKiness, 2004). Such analyses have also shown that most host species typically form a relationship with a unique symbiont phylotype. But this clearly is not always the case. For example, while strain level variation may occur, vestimentiferan tubeworms belonging to the genera *Riftia*, *Tevnia*, and *Oasisia* appear to share a single, or very similar, symbiont phylotype based on 16S rRNA gene sequences (Feldman et al., 1997; Laue and Nelson, 1997; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003), as do some species of tropical lucinid clams (Durand and Gros, 1996a; Durand et al., 1996b).

Recently, McKiness (2004) reported phylogenetic analyses of 16S rRNA gene sequences from chemosynthetic symbionts within the Gammaproteobacteria. This study represented the most comprehensive analysis of chemoautotrophic symbionts to date. It included 39 symbiont sequences and over 30 sequences from free-living bacteria representatives of chemoautotrophs, methanotrophs, and marine bacterioplankton. Here, an expanded phylogenetic analysis is presented that includes the Epsilonproteobacteria symbionts of shrimp and alvinellid worms (Fig. 1). This consensus tree illustrates the strong level of resolution afforded by the 16S rRNA gene and shows that almost all of the chemosynthetic symbionts for which sequence data are available cluster into two main clades. The first clade includes symbionts of lucinid and thyasirid clams, solemyid protobranchs, tubeworms, nematodes, and oligochaetes, and the second clade includes the mytilid mussel and vesicomyid clam symbionts.

Though the first clade as a whole has relatively low bootstrap support, smaller clusters within the first clade are strongly supported. For example, the monophyletic cluster of nematode and oligochaete symbionts has 100% bootstrap sup-

port. Similarly, the vestimentiferan tubeworm symbiont clade also has high bootstrap support, corroborating prior evidence that these worms share a single or very similar symbiont phylotype, which is consistent with environmental transmission of symbionts (Feldman et al., 1997; Laue and Nelson, 1997; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003; see the section Ecology and Evolution in this Chapter). In contrast, the clam symbionts exhibit a more complicated relationship. The lucinid clam symbionts form a paraphyletic group; some are sister to the tubeworm symbionts while others group with thyasirid and solemyid symbionts. The solemyid symbionts show similarly complicated relationships, as they are polyphyletic and scattered throughout the first clade. *Solemya velum* and *S. occidentalis* symbionts cluster with the nematode and oligochaete symbionts, while *S. terraeregina* and *S. pusilla* symbionts cluster with lucinid and thyasirid clam symbionts. Thus, this disjointed distribution does not suggest cospeciation between host taxa and symbionts in this first clade and indicates that there were multiple initiations of symbiosis within the solemyid and lucinid clams.

The position of the *S. reidi* symbiont within this first main group is curious; this symbiont falls at the base of this first main group, clustering with an intracellular pathogen, *Coxiella burnetti*, and an environmental clone from a Japanese cold seep, Gamma JTB254 (discussed further below). This symbiont sequence has held a basal position in other analyses (see Bayesian analysis in McKiness, 2004) and provokes questions concerning the nature of symbiosis in protobranch bivalves. As additional sequences become available, it will be necessary to reassess the position of the *S. reidi* symbiont with respect to other chemoautotrophic symbionts.

The second clade of symbionts, which includes the mytilid mussel and vesicomyid clam symbionts from vents and cold seeps, shows 100% bootstrap support (support for the mussel and vesicomyid clades being 70% and 99%, respectively). The symbiont from the Central Indian Ridge mussel, *Bathymodiolus* aff. *brevior*, falls at the base of the vesicomyid clam symbionts, with 82% bootstrap support. In contrast to the first symbiont clade (Fig. 1, top), evidence suggests that an ancestral symbiont initiated symbioses with both the vesicomyid clams and the bathymodioline mussels and predated the split between these bivalve lineages. On the basis of 16S rRNA gene sequence data, the divergence of the clam and mussel symbionts has been dated to 125–300 million years ago (Mya). This is corroborated by the fossil record, which dates the bathymodioline mussel hosts to 150 Mya and the vesicomyid clams to 100 Mya (Distel, 1998).

The symbionts of the thyasirid clam *Maorhithyas hadalis* occupy unique positions in this phylogenetic framework. On the basis of 16S rRNA sequence data and in situ hybridization, Fujiwara et al. (2001) described two different symbionts within this clam. One of the symbionts shows evolutionary relatedness to the bathymodioline mussel and vesicomyid clam symbionts, occurring basal to the clade containing these bacteria, and the other symbiont clusters with the free-living *Thiomicrospira* spp. This free-living "symbiont" phylotype, however, may be a contaminant. Difficulties with in situ hybridization have precluded attempts to describe the microdistribution of the two symbiont types (Fujiwara and Uematsu, 2002), bringing into question the phylogenetic identity of the clam symbionts.

The phylogenetic positions of the methanotrophic endosymbionts and the filamentous epibionts are shown also in Fig. 1. The methanotrophic symbionts characterized to date all belong to the Gammaproteobacteria, forming a clade with 100% bootstrap support. The sister group of this clade consists of free-living Type I methanotrophs (*Methylococcus*, *Methylobacter* and *Methylomonas* spp.). Given their monophyly, the mussel symbionts apparently arose from a common ancestor. But the question of whether these symbionts subsequently cospeciated with their hosts remains unanswered.

The episymbionts in this analysis include the sulfur-oxidizing Epsilonproteobacteria found on the Mid-Atlantic Ridge shrimp *Rimicaris exoculata* and the eastern Pacific polychaete *Alvinella pompejana.* Interestingly, the shrimp epibiont clusters with the polychaete epibionts despite the fact that *R. exoculata* occurs on the Mid-Atlantic Ridge while alvinellid polychaetes inhabit vents in the eastern Pacific Ocean.

Free-living microorganisms can potentially provide insight into the ancestral form of endosymbionts. For instance, the evolution of insect endosymbionts (e.g., *Wolbachia* and *Buchnera* spp.) is commonly studied by comparative analyses with free-living, closely related microbes (Wernegreen, 2002; Moran, 2003). But until recently, the chemoautotrophic symbiont clades have not included any free-living bacteria. Figure 1 includes two species of bacteria that are not chemoautotrophic symbionts (*Coxiella burnetti* and *Achromatium oxaliferum*) and an environmental clone (JTB254). All three of these sequences fall within the first symbiont clade. *Coxiella burnetii* is an intracellular pathogenic bacterium (Woldehiwet, 2004) and the Gamma JTB254 clone was recovered from a deep-sea cold seep in the Japan Trench (Li et al., 1999). They both fall in a cluster with the *S. reidi* symbiont. Both *C. burnetii* and the *S. reidi* symbionts are able to maintain an intracellular existence in

eukaryotic hosts. *Coxiella burnetii*, however, is capable of growth in animal cell lines (e.g., Woldehiwet, 2004) and pathogenically infects a wide range of hosts (Niemczuk and Kondracki, 2004; Watanabe, 2004; Woldehiwet, 2004). In addition, *A. oxaliferum*, a freshwater sulfur-oxidizing bacterium, falls out with the nematode and oligochaete symbionts clade. *Achromatium oxaliferum* occurs in freshwater sediments along the redox zone where it has access to sulfide and oxygen (Head et al., 1996; Glockner et al., 1999; Gray et al., 1999). As cultivation methods improve and sequences are added to the 16 rRNA gene database, other free-living bacteria that are closely related to symbionts will likely be identified. Indeed, recent studies incorporating 16S rRNA gene sequences from unidentified environmental clones into phylogenetic analyses of free-living and symbiotic bacteria suggest that chemosynthetic symbionts may in fact resolve into three distinct clades (N. Dubilier, personal communication; Duperron et al., 2004). Free-living relatives of chemosynthetic symbionts should reveal much about the ecological and evolutionary constraints on the symbiont as well as about the potential for gene loss during the transition from the free-living to the symbiotic state.

Symbiont Characterization

Enzyme Activities

Researchers routinely demonstrate chemoautotrophy or methanotrophy in symbionts by the activity or presence of diagnostic enzymes. Indeed, given the inability to culture chemoautotrophic symbionts, detection of such enzymes is often the only evidence used to infer symbiont metabolism. This characterization often involves physiological assays using tissue or purified protein extract, immunodetection, or PCR-based gene probing. Such studies were initially conducted on the tubeworm *Riftia pachyptila*. For example, Felbeck (1981a) assayed tubeworm trophosome tissue for the activities of key enzymes of the Calvin cycle, the CO_2-fixing enzyme, ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO), and phosphoribulose kinase (PRK) as well as enzymes associated with the oxidation of reduced inorganic sulfur compounds. The activity or presence of RubisCO has subsequently been used to diagnose symbiont autotrophy in a diversity of host species, including all of the chemoautotroph-harboring invertebrates listed in Table 1 (excluding alvinellid polychaetes; see below): shallow water solemyid and lucinid bivalves, vent and seep tubeworms and bivalves (including mussels hosting both methanotrophic and chemoautotrophic symbionts), nematodes, oligochetes, shrimp, sea urchins, and ciliates (Felbeck et al., 1981b; Cavanaugh, 1983; Cavanaugh et al., 1988; Polz et al., 1992; Johnson et al., 1994; Nelson et al., 1995b; Bauer-Nebelsick et al., 1996; Krieger et al., 2000; Elsaied et al., 2002; Fiala-Médioni et al., 2002). While RubisCO has not been detected in the alvinellid epibionts, genes encoding citrate lyase, a key enzyme of the reductive tricarboxylic acid (TCA) cycle, recently have been detected via analyses of symbiont DNA sequences, suggesting that the Epsilonproteobacteria symbionts of alvinellid worms fix carbon via this pathway (Campbell et al., 2003).

Enzymes involved in chemosynthetic energy generation have also been used to characterize these symbionts. Although the sulfur metabolism enzymes are not unique to sulfur oxidation, certain enzymes such as ATP sulfurylase, when detected in high activities, have been used to infer sulfur-based chemolithotrophy (Felbeck, 1981a; Fisher et al., 1993b; Laue and Nelson, 1994). As methane monoxygenase, the enzyme that catalyzes the first step in the oxidation of methane in aerobic methanotrophs, is notoriously labile (Prior and Dalton, 1985; Cavanaugh, 1993), methanol dehydrogenase (MeDH), the enzyme that catalyzes the second oxidation step (i.e., methanol to formaldehyde) and is known to occur only in methylotrophs, has been used extensively to diagnose methanotrophy. MeDH has been detected in gill extracts of mussels hosting methanotrophs or both methanotrophs and thioautotrophs (Cavanaugh et al., 1992; Fisher et al., 1993b; Robinson et al., 1998; Fiala-Médioni et al., 2002; Pimenov et al., 2002; Barry et al., 2002) and in a deep-sea sponge (Vacelet et al., 1996). Such enzymatic evidence strongly suggests methanotrophy, particularly when coupled with ultrastructural observations showing symbionts with the complex intracytoplasmic membranes that are characteristic of Type I methanotrophs.

Stable Isotope Signatures

Carbon Isotopes In addition to enzymology, stable isotope data provided some of the first evidence in support of chemoautotrophy in marine invertebrate-bacteria symbioses (e.g., Rau and Hedges, 1979; Spiro et al., 1986) and continue to be useful in assessing symbiont metabolism and tracking energy and carbon transfer in chemosynthetic symbioses (Colaco et al., 2002; Levin and Michener, 2002; Van Dover, 2002a; Robinson et al., 2003; Scott et al., 2004). Because enzymes involved in distinct carbon fixation pathways discriminate differently against the use of the heavier carbon isotope (^{13}C), the stable

carbon isotope ratio comparing ^{13}C to ^{12}C ($\delta^{13}C$) can be used to help distinguish different autotrophic metabolisms. For example, whereas $\delta^{13}C$ values of marine phytoplankton typically vary between µ18‰ and µ28‰ (Fry and Sherr, 1984; Gearing et al., 1984; Goericke et al., 1994), carbon derived chemosynthetically at vents is either considerably lighter (enriched in ^{12}C), with $\delta^{13}C$ values from µ27‰ to µ35‰, or heavier (depleted in ^{12}C), with values from µ9‰ to µ16‰ (Childress and Fisher, 1992; Robinson and Cavanaugh, 1995; Robinson et al., 2003). Depending on the source of methane, symbioses between mussels and methanotrophic bacteria may be even more depleted in ^{13}C, with $\delta^{13}C$ ranging from µ37‰ to µ78‰ (Cavanaugh, 1993; Nelson and Fisher, 1995a; Barry et al., 2002).

Because consumers generally retain the carbon isotopic signature of their food (i.e., "you are what you eat"; DeNiro and Epstein, 1979), comparisons between $\delta^{13}C$ signatures of symbiont-containing host tissue and symbiont-free host tissue can be used to study the transfer of symbiont-derived carbon to the host. For example, $\delta^{13}C$ values (µ30.8‰ to µ35.8‰) in symbiont-containing gill tissue from the western Pacific vent mussel *Bathymodiolus brevior* were significantly lower than values from symbiont-free foot tissue, suggesting that *B. brevior* supplements its diet via filter feeding on photosynthetically derived carbon (Dubilier et al., 1998). In contrast, other studies show a high dependence on symbiont carbon by host species, including coastal solemyid protobranchs (Fisher and Childress, 1986; Conway and Capuzzo, 1991), thyasirid clams (Dando and Spiro, 1993; Fiala-Médioni et al., 1993), and vestimentiferan tubeworms (Kennicutt et al., 1992), as well as suggest differences in the contribution of methanotrophs and chemoautotrophs to host carbon in mussels containing dual symbioses (Cavanaugh, 1993; Trask and Van Dover, 1999; Fiala-Médioni et al., 2002; Yamanaka et al., 2003).

Stable carbon isotope signatures have also been used to detect chemosynthetic symbioses in fossil bivalves of the clam family Lucinidae, whose extant members all host chemosynthetic symbionts. CoBabe (1991), by determining the $\delta^{13}C$ values of organic matrix material extracted from lucinid fossils dating to ca. 120,000 ya, showed that fossilized lucinid (*Epilucina* sp.) shells ($\delta^{13}C$ = µ25‰) were about 5‰ lighter than values from other bivalves collected in the same deposit (Pt. Loma, CA). Also, the organic matter of lucinid fossils was similar to that from modern samples, implying that the fossil organic matrix did not decay or change significantly over time. These results, along with strong evidence showing that shell $\delta^{13}C$ values are reasonable proxies for tissue values in extant species, suggest that the fossil lucinid hosted chemosynthetic symbionts ca. 120,000 ya (CoBabe, 1991). Thus, stable carbon isotope analysis may be an effective tool for tracing the evolution of chemosynthetic symbioses in the fossil record.

One of the main factors affecting stable carbon isotope signatures of chemoautotroph-invertebrate symbioses appears to be the form of RubisCO used by the symbionts. While $\delta^{13}C$ values for vent bivalves hosting sulfide-oxidizing symbionts cluster between µ27‰ and µ35‰ and resemble values for free-living chemoautotrophic bacteria, values for vent tubeworms, shrimp episymbionts, and many free living bacterial mats at vents are significantly heavier, ranging from µ9‰ to µ16‰ (Childress and Fisher, 1992; Van Dover and Fry, 1994; Robinson and Cavanaugh, 1995; Cavanaugh and Robinson, 1996; Robinson et al., 2003). The difference between these groups relates to the form of RubisCO used to fix CO_2 by the symbionts, with form I RubisCO occurring in most members of the isotopically lighter group and form II in all members of the heavier tubeworm group (Robinson and Cavanaugh, 1995). Corroborating this hypothesis, Robinson et al. (2003) showed that the kinetic isotope effect (ε value), the relative rate of $^{13}CO_2$ to $^{13}CO_2$ fixation ($^{12}k/^{13}k$) and a measure of discrimination against ^{13}C by the purified RubisCO enzyme in vitro, is significantly lower for form II RubisCO from *Riftia pachyptila* symbionts (ε = 19.5‰) than for the form I enzyme (ε = 22–30‰). Such variation may have arisen from evolution under differing concentrations of CO_2 and O_2 (Robinson et al., 2003).

But isotopic discrimination by RubisCO does not fully account for ^{13}C-enrichment in these symbioses. For example, to explain the discrepancy in *R. pachyptila* biomass $\delta^{13}C$ values, Scott (2003) used a mass balance model to show that steep gradients in [CO_2] among symbiont, host, and environmental pools may drive ^{13}C enrichment. RubisCO, which occurs in the symbiont cytoplasm, preferentially fixes $^{12}CO_2$, leaving $^{13}CO_2$ behind. If fixation is rapid, CO_2 equilibration between the isotopically lighter host cytoplasm and the isotopically heavier symbiont cytoplasm cannot occur, causing RubisCO to draw from a more enriched $^{13}CO_2$ pool and accounting for the relatively heavy $\delta^{13}C$ of tubeworm biomass. Further, stable carbon isotope values are also affected by the $\delta^{13}C$ of the environmental carbon pool (Fisher, 1995; Colaco et al., 2002). Scott et al. (2004) demonstrated that the "light" $\delta^{13}C$ values of the symbionts of the coastal protobranch clam *Solemya velum* are explained not only by the kinetic isotope effect of symbiont form I RubisCO (ε = 24.5‰) but also by the $\delta^{13}C$ value of the CO_2 in the sediment.

Similarly, the source of methane production can significantly impact the isotopic signature of methanotroph symbioses (Fisher, 1995; MacAvoy et al., 2002). The $\delta^{13}C$ of methane varies considerably depending on whether it is produced thermogenically ($\delta^{13}C$ of CH_4 >μ45‰) or biologically by methanogens ($\delta^{13}C$ of CH_4 <μ60‰; Lilley et al., 1993; Fisher, 1995), and this variability is reflected in the $\delta^{13}C$ values of chemosynthetic symbioses (Fisher, 1995). Therefore, in instances where the $\delta^{13}C$ of the source methane is unknown, conclusions about the contribution of methanotrophic symbionts to host $\delta^{13}C$ values should be interpreted with caution (Fisher, 1995). In addition, interpreting $\delta^{13}C$ signatures may be especially problematic for dual symbioses in which both methanotrophic and thioautotrophic symbionts co-occur in the same host cell. In these symbioses the $\delta^{13}C$ values of the symbionts and the host reflect a mix of methanotrophic and thioautotrophic metabolism (Fisher, 1995). These signatures are potentially confounded in instances when the thioautotrophic symbionts use the CO_2 respired by the methanotrophs, resulting in a second discrimination against an already light pool of CO_2 and an anomalously light tissue $\delta^{13}C$ value (Fisher, 1993a).

Thus, while $\delta^{13}C$ values often provide the first evidence that chemoautotrophic or methanotrophic symbioses occur in certain animal species, researchers must recognize that $\delta^{13}C$ is inherently responsive to physical, environmental and enzymatic factors. Stable carbon isotope signatures therefore should not be used apart from other corroborating evidence (e.g., physiological and enzyme activity assays and genetic characterization) to identify carbon fixation pathways or methane oxidation in chemosynthetic symbioses (Fisher, 1995; Scott, 2003; Scott et al., 2004).

Sulfur and Nitrogen Isotopes In addition to carbon isotopes, stable isotopes of sulfur and nitrogen are also used to study sources and metabolism of these elements in symbioses. The extent to which different sources of reduced sulfur—geothermal production in vent fluid or microbial sulfate reduction in bottom sediment—support thioautotrophic metabolism has been inferred from the $\delta^{34}S$ value of biological samples. Such analyses revealed hydrothermally derived sulfide as the dominant sulfide source for deep-sea vent symbioses (Fry et al., 1983; Yamanaka et al., 2003). In contrast, symbiotic bacteria within a shallow water vestimentiferan tubeworm, *Lamellibrachia satsuma* (Miura et al., 2002), and the protobranch, *Solemya velum* (Conway et al., 1989), rely predominantly on sulfide derived from microbial sulfate reduction.

Similarly, $\delta^{15}N$ values, because they vary predictably and largely between producer and consumer trophic levels (increase of ca. 3.4 per level), are particularly useful markers for studying aquatic food web interactions (Minagawa and Wada, 1984). In general, $\delta^{15}N$ values of chemoautotrophic organisms are significantly lighter (<0‰; Van Dover and Fry, 1994) than values for photosynthetic organisms (>6‰; see Michener and Schell [1994] and Fisher [1995]). Researchers have used this discrepancy and the predictable trophic level fractionation of ^{15}N to show host reliance on symbiont-derived organic matter in a number of symbioses including the coastal clams *Solemya velum* and *S. borealis* (Conway et al., 1989; Conway et al., 1992b) and in vent mussels from the Mid-Atlantic Ridge (MAR) and the Galapagos Rift (Fisher et al., 1988; Trask and Van Dover, 1999). In addition, $\delta^{15}N$ values have been used extensively in conjunction with $\delta^{13}C$ values to show the flow of chemosynthetically derived organic matter through vent food webs, including those on the MAR (Vereshchaka et al., 2000; Colaco et al., 2002), the Central Indian Ridge (Van Dover, 2002a), and the Galapagos Rift (Fisher et al., 1994). As with $\delta^{13}C$ data, $\delta^{15}N$ values vary considerably among sites; $\delta^{15}N$ may depend in part on the $\delta^{15}N$ of the dissolved inorganic nitrogen (DIN) pool, the proportions and $\delta^{15}N$ values of different components (NH_4^+, $NO_3^{\mu 2}$, NO_2^{μ}, and urea) in the DIN pool (Waser et al., 1998; Colaco et al., 2002), the uptake kinetics of different DIN assimilation pathways (Waser et al., 1998; Krueger, 1996a), and, as shown for vent shrimp (Vereshchaka et al., 2000) and mussels (Trask and Van Dover, 1999), the ontogenetic stage of the host. Therefore, as noted above with stable carbon isotopes, in the absence of additional enzymatic, genetic and environmental data, caution must be used when comparing $\delta^{15}N$ values from different habitats and species.

Ecophysiology

Symbioses between chemosynthetic bacteria and marine invertebrates must acquire all of the substrates necessary for chemosynthetic metabolism: reduced sulfur or methane, oxygen, dissolved inorganic carbon (DIC, as CO_2 or CH_4), and other nutrients (e.g., nitrogen and phosphorus) for use in biosynthesis. In particular, to support energy generation, these symbioses must obtain substrates from both oxic and anoxic environments. To meet these demands, the host-symbiont association relies on specialized biochemistry, physiology and behavior. These adaptations are best studied in thioau-

totrophic endosymbioses and are discussed primarily within this context below.

Spanning the Oxic-Anoxic Interface

Access to both oxygen and reduced chemicals is necessary for aerobic respiration by chemosynthetic symbionts. Specifically, thioautotrophs shuttle electrons from reduced sulfur (e.g., sulfide) to a terminal electron acceptor during oxidative phosphorylation, generating a proton gradient that drives ATP synthesis. Though some thioautotrophic symbionts (such as those in the tubeworm *Riftia pachyptila* [Hentschel and Felbeck, 1993a] and the clam *Lucinoma aequizonata* [Hentschel et al., 1993b]) may use nitrate as an electron acceptor during periods of anoxia, most thioautotrophic symbionts typically use molecular oxygen for respiration. Similarly, methanotrophs must obtain oxygen for respiration as well as methane for both energy generation (via methane oxidation) and carbon assimilation (Anthony, 1982).

This dual requirement for oxygen and reduced compounds poses unique problems for thioautotrophs and methanotrophs. First, these organisms must obtain energy substrates from mutually exclusive environments—oxygen is absent or at very low levels in the anoxic zones from which sulfide or methane is typically obtained. Second, sulfide, the predominant energy source for thioautotrophy, spontaneously reacts with oxygen to form less-reduced sulfur compounds (S^0, $S_2O_3^{\mu 2}$, or $SO_4^{\mu 2}$; Zhang and Millero, 1993), thereby decreasing the availability of substrates for thioautotrophy. Though such abiotic oxidation may be several orders of magnitude slower than biological sulfide oxidation (Millero et al., 1987; Johnson et al., 1988), thioautotrophic symbioses must still compete with oxygen for free sulfide. Also, in habitats containing both sulfide and methane, abiotic oxidation of sulfide may limit the oxygen available for methanotrophy. These limitations force free-living thioautotrophs and methanotrophs into microaerophilic zones at the interface, or chemocline, between oxic (e.g., water column) and anoxic (e.g., vent fluid and sediment pore water) habitats. Such free-living bacteria demonstrate unique mechanisms to support life at the oxic-anoxic interface; these adaptations may be behavioral (e.g., tracking the chemocline via gliding by *Beggiatoa*), anatomical (e.g., keeping cells in the chemocline via "veil" formation by *Thiovulum* or creation of a filamentous sulfur matrix by *Arcobacter*), biochemical (e.g., internal or external sulfur deposition that serves as an electron source or sink when sulfide or oxygen is limiting, as by *Beggiatoa* and *Arcobacter*), or developmental (e.g., resting stage formation by methanotrophs; Table 2 and references therein).

Table 2. Adaptations of thioautotrophs and methanotrophs for life at oxic-anoxic interfaces.[a]

Adaptation	Example
Attachment	*Thiothrix*
Motility, chemotaxis	*Beggiatoa Thioploca*
Elemental sulfur deposition	*Beggiatoa Thiothrix*
Nitrate and sulfur storage	*Thiomargarita Thioploca*
Create own interface	*Thiovulum*
Filamentous sulfur production	*Arcobacter* sp.
Resting cysts	Methanotrophs
Associate with eukaryote	Thioautotroph and Methanotroph symbionts

[a]From Anthony (1982), Jørgensen and Postgate (1982), Cavanaugh (1985), Schulz et al. (1999), and Wirsen et al. (2002).

Symbiosis thus may be viewed as an adaptation to simultaneously obtain sulfide (or methane) and oxygen from anoxic-oxic interfaces, allowing thioautotroph or methanotroph symbionts, via association with a eukaryotic host, to circumvent many of the problems of sulfide acquisition (Cavanaugh, 1985). Similarly to free-living sulfur bacteria, thioautotrophic symbioses use specialized behavioral, anatomical or physiological mechanisms, either to spatially or temporally bridge sulfidic and oxic zones or to simultaneously sequester sulfide and oxygen (Cavanaugh, 1994; Fisher, 1996; Polz et al., 2000). For instance, the cold seep vestimentiferan tubeworm *Lamellibrachia* cf. *luymesi* acquires oxygen via its anterior plume while extending a posterior section of its tube (the root) deep into the sediment to acquire sulfide (Julian et al., 1999; Freytag et al., 2001). Similar burrowing tactics occur in some species of symbiont-containing thyasirid clams, which possess a superextensile foot (up to 30 times the length of the shell) that burrows into the sediment to access hydrogen sulfide (Dufour and Felbeck, 2003), and in protobranchs of the genus *Solemya*, which dig Y-shaped burrows in reducing sediments to allow simultaneous pumping of oxygenated water from above and sulfide-rich pore water from below (Stanley, 1970; Cavanaugh, 1983; Fig. 11). Also, shrimp, nematodes and oligochaetes migrate vertically along the oxygen-sulfide gradient or between separate oxic and anoxic zones, thereby enabling their symbionts to simultaneously access both energy substrates or to store reduced sulfur compounds for later oxidation (Polz et al., 2000).

The vent tubeworm *Riftia pachyptila* possesses a remarkable biochemical adaptation to simultaneously acquire sulfide and oxygen. *R. pachyptila* produces coelomic and vascular hemoglobins that, in contrast to most invertebrate and verte-

brate hemoglobins, can bind oxygen in the presence of sulfide (Arp et al., 1985; Arp et al., 1987; Childress et al., 1991; Zal et al., 1996). *R. pachyptila* appears to preferentially take up HS^{μ} from the surrounding fluid, despite a large H_2S gradient from tubeworm blood to the environment (Goffredi et al., 1997a). The HS^{μ} diffuses across the plume of the worm (Goffredi et al., 1997a) and then binds reversibly and independently of O_2 at two free cysteine residues, each located on a distinct globin type (Zal et al., 1997; Zal et al., 1998; Bailly et al., 2002). These residues are well conserved in both symbiont-containing and symbiont-free annelids from sulfidic environments but are absent in annelids from sulfide-free habitats (Bailly et al., 2002; Bailly et al., 2003). Bailly et al. (2003) suggest that the sulfide binding function may have been lost via positive selection, if the sulfide-binding cysteine residues react disadvantageously with other blood components in the absence of sulfide.

Extracellular hemoglobins that simultaneously bind sulfide and oxygen are absent in most other marine invertebrates that host sulfide-oxidizing symbionts (Weber and Vinogradov, 2001); such organisms have evolved other mechanisms for regulating sulfide toxicity and delivery. For instance, the vesicomyid clam *Calyptogena magnifica* synthesizes a di-globular, non-heme molecule that readily binds free sulfide within the blood serum, perhaps via zinc residues (Arp et al., 1984; Zal et al., 2000). Also, several thioautotroph-containing species, including the vent mussel *Bathymodiolus thermophilus* and the coastal clam *Solemya velum*, appear to mediate detoxification in part by storage of sulfur in amino acids (e.g., taurine and thiotaurine; Conway and Capuzzo, 1992a; Pruski et al., 2000a; Joyner et al., 2003; Pruski and Fiala-Médioni, 2003). Indeed, thiotaurine may be used effectively as a biomarker of thioautotrophic symbioses (Pruski et al., 2000b).

Other host organisms, including some bivalve mollusks, apparently avoid sulfide toxicity via mitochondrial oxidation of sulfide. Powell and Somero (1986) first demonstrated mitochondrial sulfide oxidation in the coastal protobranch *S. reidi*. The authors showed that mitochondria isolated from the gill and foot of *S. reidi* exhibit ADP-stimulated oxygen uptake and ATP synthesis following the addition of sulfide. On the basis of the effects of cytochrome and reduced nicotinamide adenine dinucleotide (NADH) oxidase inhibitors, electrons from sulfide oxidation appear to enter the respiratory chain at cytochrome *c* in *S. reidi* mitochondria (Powell and Somero, 1986). Further characterization of this system using ^{35}S showed that sulfide is oxidized exclusively to thiosulfate (O'Brien and Vetter, 1990), a nontoxic intermediate that can function as the energy source in symbiotic carbon fixation. Subsequently, researchers have demonstrated mitochondrial sulfide oxidation across a wide range of organisms, including polychaete worms, clams, fishes and chickens (Grieshaber and Volkel, 1998; Yong and Searcy, 2001). These data lend credence to the hypothesis that mitochondria evolved from sulfide-oxidizing endosymbiotic bacteria (Searcy, 1992).

Readers should consult several additional reviews (e.g., Cavanaugh, 1994; Fisher, 1996; Polz et al., 2000) for a more extensive discussion of the remarkable adaptations used by chemoautotrophic symbioses to sequester both oxygen and reduced chemicals across oxic-anoxic zones.

Carbon Uptake and Transport

In addition to oxygen and reduced sulfur compounds, thioautotrophic symbionts utilizing the Calvin cycle require CO_2 for autotrophic carbon fixation. Acquisition of CO_2 is not trivial given that relative concentrations of the three distinct chemical species (CO_2, HCO_3^{μ} and $CO_3^{\mu 2}$) in the dissolved inorganic carbon (DIC) pool can vary considerably depending on pH (pK_a of 6.4 for $CO_2 : HCO_3^{\mu}$ at 25°C; see the section Habitat Chemistry in this Chapter). In general, the majority of DIC in seawater (pH ~ 8.0) is HCO_3^{μ}. But at vents the typically lower pH of the mixed vent fluid and ambient bottom water generates higher concentrations of CO_2, giving organisms that use the Calvin cycle a distinct advantage.

The tubeworm *Riftia pachyptila* provides an interesting model in which to study the uptake and transport of DIC. Goffredi et al. (1997b) demonstrated that for *R. pachyptila*, pH plays an important role in DIC uptake. The acidity of diffuse vent fluid (pH ca. 6) around tubeworms ensures that CO_2 (pK_a of 6.1 at in situ temperature and pressure of ca. 10°C and 101.3 kPa; Dickson and Millero, 1987) is the dominant DIC form in the vent environment. This contrasts with the vascular fluid of the worm, which has an alkaline pH of 7.1–7.5, apparently because of the action of H^+-ATPases (Goffredi et al., 1999; Goffredi and Childress, 2001; Girguis et al., 2002). The alkaline pH inside *Riftia* results in rapid conversion of CO_2 to HCO_3^{μ}, which, because of its negative charge, cannot diffuse out of the worm; this in effect creates a bicarbonate "trap" (Childress et al., 1993). Thus, a gradient of higher external [CO_2] to lower internal [CO_2] develops across the tubeworm plume and drives diffusion of DIC into the blood (Childress et al., 1993; Goffredi et al., 1997b; Scott, 2003). Following diffusion into the plume, DIC (as CO_2 and HCO_3^{μ}) is transported by the vascular system to the symbiont-containing trophosome. Here, carbonic anhydrase, the enzyme that reversibly con-

verts CO_2 into HCO_3^{μ} in both prokaryotes and eukaryotes, may play a role in converting HCO_3^{μ} into CO_2, the DIC species used by RubisCO (Kochevar and Childress, 1996; De Cian et al., 2003a; De Cian et al., 2003b). As discussed above for *Riftia*, DIC incorporation into symbiont biomass occurs via CO_2 fixation by a form II RubisCO of the Calvin-Benson cycle. Rapid CO_2 fixation rates create steep internal [CO_2] gradients between symbiont and host cytoplasm that may, in combination with the relatively low discrimination of form II RubisCO against ^{13}C, result in a ^{13}C-enriched signature of symbiont and host biomass (Robinson et al., 2003; Scott, 2003).

In chemosynthetic endosymbioses the host benefits by obtaining part or all of its nutrition from the symbiont, via two potential transfer mechanisms: the host may assimilate autotrophically fixed carbon that has been released by the symbiont and translocated to host cells in the form of soluble organic molecules, or the host may directly digest bacterial cells. Radiotracer analysis and microscopy have proven particularly useful in studying host nutrition. For example, Fisher and Childress (1986) showed a rapid (within hours) appearance of radiolabeled carbon in the symbiont-free tissues of the host clam *Solemya reidi* following exposure to ^{14}C-labeled bicarbonate, suggesting release of fixed carbon by the symbiont population. In contrast, a slow (1–5 days) transfer of labeled organic carbon from methanotroph-containing tissue to symbiont-free tissue of a seep mussel exposed to ^{14}C-labeled methane was inferred to be due to initial $^{14}CH_4$ incorporation by the symbionts with host digestion of symbionts occurring later (Fisher and Childress, 1992). Electron microscopy showing symbionts being degraded in the basal region of bacteriocytes in other methane-based and dual chemoautotroph-methanotroph mussel symbioses supports this interpretation (Cavanaugh et al., 1992; Barry et al., 2002), as does the detection of lysosomal enzymes in the gills of the vent bivalves *Calyptogena magnifica* and *Bathymodiolus thermophilus* (Fiala-Médioni et al., 1994; Boetius and Felbeck, 1995) and the shallow water clam *Lucinoma aequizonata* (Boetius and Felbeck, 1995).

In the *R. pachyptila* tubeworm symbiosis, the transfer of carbon from symbiont to host appears to occur via both translocation and digestion (Bright et al., 2000). Felbeck (1985) and Felbeck and Turner (1995) documented a rapid (within seconds) appearance of labeled succinate and malate in trophosome tissue and in vascular and coelomic blood following exposure of whole worms (in pressure vessels) and plumes to ^{14}C-bicarbonate. Subsequently, Felbeck and Jarchow (1998) showed that succinate, malate, and several other organic acids and sugars were excreted by purified suspensions of *R. pachyptila* symbionts, suggesting that these simple organic compounds might be important intermediates in the transfer of fixed carbon from symbionts to host. Corroborating these data, Bright et al. (2000), using pulse labeling analysis, showed that the bulk of organic carbon assimilated into *R. pachyptila* tissue is first released by metabolically active bacteria at the center of a trophosome lobule. However, these authors also showed that a smaller fraction of host carbon is obtained by digestion of bacterial cells at the lobule periphery (Bright et al., 2000). This evidence for digestion is supported by prior studies showing degenerative stages of bacteria within the *R. pachyptila* trophosome (Bosch and Grassé, 1984; Hand, 1987). In addition, relatively high lysozyme activity in *Riftia* tissue further suggests that digestion of symbionts plays a role in tubeworm nutrition (Boetius and Felbeck, 1995).

Nitrogen

The partners in a symbiosis must also acquire all of the other macro- and micronutrients, particularly nitrogen and phosphorus, for use in the biosynthesis of organic compounds. Currently, very little is known about how various forms (inorganic and organic) of phosphorus are transferred to and among different pools within chemoautotrophic endosymbioses. Most studies have focused on nitrogen metabolism, using a combination of enzyme characterizations and physiological experiments to elucidate nitrogen assimilation pathways. Nitrate ($NO_3^{\mu 2}$), which is abundant at vents (in situ concentrations of ~40 μM; Johnson et al., 1988), appears to be the predominant nitrogen source for vent symbioses. For example, Lee et al. (1999) demonstrated the activity of nitrate reductase, a bacterial enzyme involved in converting nitrate to ammonia for either assimilatory or respiratory purposes, in the vent tubeworms *Riftia pachyptila* and *Tevnia jerichonana* and the mussel *Bathymodiolus thermophilus*. In addition, the ammonia assimilation enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were detected in these symbioses, and almost all GS activity in symbiont-containing tissue was shown to be due to enzyme produced by the bacterial symbiont and not the host (Lee et al., 1999). Supporting these data, physiological experiments on *R. pachyptila* kept in pressurized chambers showed that the symbiont population reduces nitrate to ammonia not for respiratory purposes but for incorporation into both symbiont and host biomass (Girguis et al., 2000). However, in *R. pachyptila,* high GS activity also occured in symbiont-free branchial plume tissue, suggesting

that the host may also be involved in assimilation of ammonia from the vent environment (Minic et al., 2001). But further enzymatic characterization of *Riftia* tissues demonstrated that the tubeworm depends on its symbionts for the de novo synthesis of pyrimidine nucleotides (Minic et al., 2001) as well as for the biosynthesis of polyamines (Minic and Herve, 2003), suggesting that the trophosome is a primary site for nitrogen assimilation and metabolism.

In contrast, in the thioautotrophic symbiosis involving the shallow-water clam *Solemya reidi*, inorganic nitrogen is readily assimilated in the form of ammonia (Lee and Childress, 1994), which is abundant in the shallow water, nutrient-rich habitats of the clam (e.g., sewage outfalls). Ammonia incorporation rates are highest in the symbiont-containing gill tissue, and the sulfur-containing amino acid taurine appears to be a major end product of ammonia assimilation (Lee et al., 1997). The mechanisms by which chemosynthetic symbionts, particularly those contained within the cells of invertebrate hosts (such as *Solemya* and *Riftia*), acquire all of the other macro- and micronutrients for biosynthesis have yet to be characterized.

Ecology and Evolution

History

Prior to the use of molecular techniques, researchers considered vent taxa to be relic species. These organisms, whose strange morphologies suggest a primitive state, purportedly survived past extinction events due to the relative isolation of vents from the photic zone (McArthur and Tunnicliffe, 1998). This perception of vents as ancient ecosystems is supported by the fossil record, which shows that over 80% of vent species are found only at vent sites (Tunnicliffe, 1991; Tunnicliffe, 1992; Little et al., 1997; Little and Vrijenhoek, 2003) and that the oldest vent site dates to the Silurian (~430 Mya; Little et al., 2004). But the fossil record for vents is relatively poor. There are only 19 known fossilized vent sites on the planet, perhaps because calcium carbonate structures dissolve relatively quickly in vent fluids (Hunt, 1992; Kennish and Lutz, 1999). Also, studying vent fauna evolution based on the morphological characters of fossils is problematic if much of the specimen has degraded or if the preserved character is plastic or isomorphic. In particular, vestimentiferan tubeworms are known for the phenotypic plasticity of their tubes (Southward et al., 1995; Black et al., 1998).

In contrast, molecular evidence suggests that vent taxa evolved more recently (22–150 Mya; later-Mesozoic and Cenozoic) and suggests an alternative hypothesis to vent taxa as living relics: vents were recently populated from shallow seeps or whale falls (Van Dover et al., 2002b; Hurtado, 2002). Indeed, the communities most similar to those of vents occur at seeps. Compared to the spatially and temporally patchy distribution of vent fossils (with most being concentrated in the Silurian and Devonian rocks of the Ural mountains), seep fossils are ubiquitous (Little and Vrijenhoek, 2003). At least 50, and perhaps as many as 200, fossilized seep sites dating from the Devonian to the Pleistocene have been uncovered. These specimens are much better preserved than most vent fossils and include extant vent taxa not yet uncovered at fossil vent sites (e.g., vesicomyids, thyasirids, mytilids and solemyids; Little and Vrijenhoek, 2003). This greater diversity supports the seeps-to-vents hypothesis. However, opponents argue that the vent fossil record has been greatly affected by high calcium carbonate dissolution rates (Little and Vrijenhoek, 2003).

While the discrepancy between the evolutionary histories suggested by the fossil and molecular evidence needs to be resolved, it must also be stressed that these data are not evidence for chemosynthetic symbioses. In a unique study, CoBabe (1991) was able to deduce a chemosynthetic symbiosis by analyzing the organic matrix from fossil lucinid shells using stable carbon isotopes. This result is encouraging and suggests that both the age of these organisms and their symbiosis can be addressed using current methods.

Organism Interactions

In the relatively featureless and nutrient poor deep sea, vent and seep environments are ecological oases (Laubier, 1989). Initially, free-living chemoautotrophic bacteria were hypothesized to provide the bulk of primary production in these communities (Lonsdale, 1977). Indeed, at some vent sites, suspended bacteria or bacteria in surface-attached mats are a large food source for higher trophic levels (Humes and Lutz, 1994; Van Dover, 2000). But the dominant strategy for the major vent and seep fauna is symbiosis with chemoautotrophic bacteria (Cavanaugh, 1994), and these symbioses significantly influence the ecology of the nonsymbiotic community. Not only are chemosynthetic symbioses a major and stable source of organic carbon (Sarrazin and Juniper, 1999), but as biogenic structures, they also provide living space for a diversity of species in an otherwise two-dimensional landscape of basalt or sediment (Bergquist et al., 2003). For example, the tubes of chemosynthetic vestimentiferans support mussels, sponges and limpets,

many of which host their own chemosynthetic symbionts (Yamamoto et al., 2002; Bergquist et al., 2003; Bates et al., 2004).

Vent symbioses may also significantly impact the free-living bacterial community by providing increased surface area for attachment. Free-living bacteria that cluster phylogenetically with known chemoautotrophic and heterotrophic groups have been isolated from tubeworm surfaces (Lopez-Garcia et al., 2002; Yamamoto et al., 2002). On the Mid-Atlantic Ridge, a single phylotype of shrimp episymbionts, which appear to be transmitted among hosts via the environment, represented over 60% of the free-living bacteria (Polz and Cavanaugh, 1995). This suggests that the host inoculates inanimate surfaces continuously, increasing the probability of symbiont attachment relative to the free-living community (Polz and Cavanaugh, 1995). Such environmental inoculation may also occur in tubeworm and lucinid clam symbioses, in which the symbionts also appear to be transmitted environmentally (Durand and Gros, 1996a; Durand et al., 1996b; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003).

Transmission Strategies and Effects on Symbiosis

The transmission strategy of a symbiosis reveals much about the evolutionary dynamics between host and symbiont. Symbiont transmission can occur environmentally (through a free-living population of symbiotic bacteria), horizontally (between contemporary organisms sharing the same habitat), or vertically (from parent to offspring). Vertically transmitted endosymbionts are effectively disconnected from their free-living counterparts. These symbionts experience elevated rates of mutation and fixation of slightly deleterious alleles because of genetic drift (Wernegreen, 2002). For the most part, these evolutionary effects are due to a vastly different selective regime inside the host and a severely depreciated population size (Ohta, 1973); endosymbionts undergo a population bottleneck upon host colonization and another upon transmission (Mira and Moran, 2002). But the asexuality and lack of recombination in endosymbionts exacerbate these genetic problems through what is known as "Muller's rachet" (Muller, 1964; Moran, 1996). In Muller's ratchet, wildtype recombinants cannot be introduced into the endosymbiont population (Moran and Baumann, 1994; Dale et al., 2003); genetic drift therefore occurs quickly, and the population cannot recover after fixation of deleterious alleles.

In contrast, symbiont populations that are environmentally transmitted are effectively larger and more genetically heterogeneous than populations transmitted vertically. Comparisons of 16S rRNA gene evolution between free-living bacteria, in which significant recombination occurs (Dykhuizen and Green, 1991; Levin and Bergstrom, 2000), and symbiotic chemosynthetic bacteria revealed unexpected differences in rates of evolution depending on mode of transmission (Peek et al., 1998). While chemoautotrophic, maternally transmitted endosymbionts did exhibit rapid evolutionary rates, consistent with their small population sizes, environmentally transmitted symbionts evolved more slowly than their free-living counterparts (Peek et al., 1998). The authors suggest that this slower rate of evolution could be caused by purifying selection in a large population. These results, however, were based on one gene across many lineages; a true genomic analysis of evolution in chemosynthetic endosymbionts is necessary to extend these findings.

Because chemosynthetic symbionts have yet to be cultured and their hosts are difficult to maintain in the laboratory, the transmission strategy of a symbiosis has been inferred by phylogenetic analysis or PCR-based detection of bacteria in host reproductive tissues or gametes. If the symbionts are maternally transmitted and the symbioses stable, congruence of host and symbiont phylogenies should occur (e.g., Chen et al., 1999; Thao et al., 2000; Degnan et al., 2004) and bacterial symbionts should be found in ovaries or oviducts of the host. Using these techniques, vertical transmission has been proposed for the solemyid protobranchs (Cary, 1994; Krueger et al., 1996b) and vesicomyid clams (Endow and Ohta, 1990; Cary and Giovannoni, 1993a; Peek et al., 1998; Hurtado et al., 2003). Interestingly, although bacteria have been detected via PCR in the gonads of female hosts, this does not necessarily imply direct bacterial endocytic localization in host eggs. Indeed, in *Solemya reidi*, the internal contents of oocytes do not contain bacteria, and instead the transmission mechanism is thought to occur via ingestion; the larvae ingest the bacteria, which are then engulfed by hemocytes in the larval perivisceral cavity and transported to the developing gill (Gustafson and Reid, 1988). The oligochaetes also exhibit an interesting mechanism of vertical transmission. During oviposition, the eggs appear to be infected with the symbiotic bacteria via the adult's genital pad (Giere and Langheld, 1987). During the development of the larvae, many of the bacteria exist intracellularly, but as the animal matures, the symbionts take their primarily extracellular form.

However, of the putatively vertically transmitted symbioses, only associations involving vesicomyid clams show phylogenetic congruence

between host and symbiont (Peek et al., 1998; Hurtado et al., 2003). Cospeciation does not appear to have occurred in the solemyid protobranchs (Durand et al., 1996b; Krueger and Cavanaugh, 1997) or in the mytilid mussels (McKiness, 2004). When evaluating phylogenetic congruence, however, other factors that influence a phylogenetic reconstruction, such as geographic constraints, must be taken into account. Also, robust phylogenies with adequate taxa sampling for both host and symbiont are necessary; incomplete phylogenies may be hindering analyses of the solemyid and mytilid symbioses.

Lack of PCR-based evidence and phylogenetic incongruence has been used to infer an environmental mode of transmission for several of the chemosynthetic symbioses. For instance, the lucinid clams exhibit environmental transmission (Durand and Gros, 1996a; Gros et al., 1996; Gros et al., 1998; Gros et al., 2003a; Gros et al., 2003b). Researchers have even been able to exchange symbionts between lucinid species without affecting the development of the juvenile animal (Gros et al., 2003a). In addition, vent tubeworms appear to acquire their symbionts from the environment (Distel and Cavanaugh, 1994; Feldman et al., 1997; Laue and Nelson, 1997; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003), as evidenced in part by the presence of functional genes for sensing and responding to the environment as well as a flagellin gene in the *Riftia* symbiont (Hughes et al., 1997; Hughes et al., 1998; Millikan et al., 1999). Indeed the 16S rRNA phylotype has been detected in vent environments via both PCR and in situ hybridization, suggesting the vent tubeworm symbionts are environmentally transmitted (Harmer et al., 2004). While environmental transmission of tubeworm symbionts seems to be a potentially risky strategy, given the stochastic nature of environmental transmission and the complete dependence of the adult tubeworms on their symbionts for nutrition, detection of "wild" symbionts in conjunction with the phylogenetic evidence supports environmental transmission in this species.

The mechanism of transmission for the mytilid mussels remains largely unresolved; on the basis of varying evidence, researchers have suggested both vertical and environmental transmission. Vertical transmission in *Bathymodiolus thermophilus*, the thioautotroph-hosting mussel, was suggested in 1993, but evidence supporting this report is not yet published (Cary and Giovannoni, 1993a). In contrast, a recent study based on genetic and ultrastructural data of the chemoautotrophic symbionts of *B. azoricus*, a MAR mussel hosting both thioautotrophs and methanotrophs, indicated environmental acquisition of the chemoautotrophic symbionts (Won et al., 2003a; DeChaine et al., 2004). In addition, McKiness (2004) provided the first assessment of cospeciation between symbiont and host in *Bathymodiolus* mussels, analyzing molecular data for both methanotrophic and chemoautotrophic symbionts and testing phylogenetic congruence with the hosts. The results showed weak support for vertical transmission of the chemoautotrophic symbionts but provided no evidence for vertical transmission of the methanotrophs.

Biogeography and Population Genetics

The view of hydrothermal vents as deep-sea islands frames questions of vent biogeography and population genetics. Compared to the relatively uniform and stable environment of the abyssal deep sea, hydrothermal vents are ephemeral, dynamic and geographically fragmented. A chain of vents along a mid-ocean ridge resembles a chain of islands in an archipelago. However, genetic data for many vent species do not cleanly fit an "island" or "stepping-stone" model of biogeography (Vrijenhoek et al., 1998). Some host taxa do exhibit a decline in gene flow with increasing distance between sites (Black et al., 1994), as a stepping-stone model would predict (Kimura and Weiss, 1964), while others show a more widespread gene flux (Karl et al., 1996) or appear to encounter barriers to dispersal other than distance (Black et al., 1998). These differences should be resolved with a greater understanding of the major variables affecting vent biogeography, including larval development and dispersal, symbiont distribution, oceanic flow, and past and current bathymetry. This section focuses predominantly on host biogeography because research on the population genetics and biogeography of bacterial symbionts is lacking. Understanding host population dynamics, however, does provide valuable insight into the distribution of the chemosynthetic symbionts to which most vent fauna are tightly linked.

Vent habitats are highly ephemeral and sensitive to variations in tectonic activity, hydrothermal inputs, and geologic events. Consequently, the persistence of vent organisms, which are predominantly sessile as adults, depends on successful larval dispersal to new sites. The dispersal strategy of larvae can significantly impact the biogeography of the adult organism (Lalou and Brichet, 1982; Fustec et al., 1987). On the basis of laboratory studies and comparisons with shallow water species, researchers infer that some vent larvae are planktotrophic while others are lecithotrophic (Lutz et al., 1980; Turner et al., 1985; Young et al., 1996; Marsh et al., 2001). Although both forms are pelagic, planktotrophic larvae are positively buoyant and feed in the

water column while lecithotrophic larvae are negatively buoyant and nonfeeding (Poulin et al., 2001). Larvae of the large vesicomyid clam *Calyptogena magnifica* typify a planktotrophic dispersal strategy successfully exploiting the vent plume to carry them many kilometers (Pradillon et al., 2001; Mullineaux et al., 2002). Although planktotrophic larvae risk being carried off the ridge axis by cross currents, *C. magnifica* apparently encounters no significant barriers to dispersal across the equator on the East Pacific Rise (EPR; Karl et al., 1996). Conversely, lecithotrophic larvae are less affected by cross currents but, because they are non-feeding, have relatively short larval stages and therefore limited time for dispersal. For example, in laboratory studies, larval *Riftia pachyptila* exhibit a lecithotrophic strategy, surviving a maximum of 38 days (Marsh et al., 2001). Assuming flow rates characteristic of EPR currents, this interval suggests a maximum dispersal distance of 100 km (Marsh et al., 2001); however, the in situ dispersal distance is unknown given that *Riftia* larvae have not been detected in the wild.

The geology and tectonic activity associated with mid-ocean ridges also impact the biogeography of vent organisms. For instance, Iceland, an active site of crust formation, rises out of the ocean along the northern MAR, forming a barrier that prevents dispersal along the ridge axis (Tyler and Young, 2003). Given that Iceland interrupted the MAR approximately 55 Mya, the ridge axis north of Iceland constitutes one of the most isolated vent systems on the planet, perhaps representing a new biogeographic province (Bilyard and Carey, 1980; Dunton, 1992; Svavarsson et al., 1993). Similar dispersal barriers are seen among vent fields abutting the Azores Rise in the Atlantic (Tyler and Young, 2003) and also evident between the EPR and the Northeast Pacific vent fields (Tunnicliffe, 1988; Tunnicliffe and Fowler, 1996). These barriers are insurmountable and may provide the conditions for allopatric speciation of both host and symbiont.

Research on EPR bathymodiolid mussels and their symbionts provides a good example of how larval dispersal strategy, current regime, and bathymetry interact to structure biogeography (Lonsdale, 1977; Corliss et al., 1979). Except at Northern Pacific sites, which are separated from the EPR by the North America landmass, vent communities on the Pacific ridge axis appear relatively uniform. For example, the mussel *Bathymodiolus thermophilus*, which undergoes a planktotrophic larval stage, occurs over a distance of 4900 km (from 13°N to 32°S) on the EPR. Mussel populations along 13°N and 11°S are genetically indistinguishable, indicating no population subdivision (Craddock et al., 1995; Won et al., 2003). Deep ocean currents that flow primarily NNW and SSE along the axis (Marsh et al., 2001) may facilitate dispersal of larval *B. thermophilus*, contributing to the homogeneity observed along the EPR. However, there is some genetic structure in the EPR mytilid populations; the westward currents across the ridge axis at 15°N and the Easter Microplate are obstacles for planktotrophic larvae. At 15°N, a westward current flows across the ridge axis, partially isolating the 17°S population from the other northern populations. Further south, at the Easter Microplate, mussel populations are severely divergent (Won et al., 2003). Although morphologically indistinguishable, mussels north and south of the Microplate are genetically distinct (Won et al., 2003). The Easter Microplate therefore appears to be a significant topographic obstacle for larval dispersal. Such a feature can produce cross-axis currents, like those at 15°N, that may sweep bathymodiolid larvae (which are positively buoyant) off the ridge axis (Fujio and Imasato, 1991; Mullineaux et al., 1995). The degree to which such barriers also impact the genetic diversity and biogeography of chemosynthetic symbiont populations remains an open question.

Summary

Scientific understanding of chemosynthetic symbioses continues to expand. The spectacular discovery of hydrothermal vents highlighted the importance of chemosynthetic bacteria both in food webs and in symbioses with eukaryotes and provided the impetus to examine less exotic environments for such associations. As other oxic-anoxic environments (e.g., freshwater) and the invertebrates and protists that inhabit them are explored, new symbioses will undoubtedly be discovered. Further, chemosynthetic bacteria that use other sources of energy (e.g., hydrogen) may be found in similar associations.

Current studies of these fascinating symbioses involve a range of experimental and diagnostic tools, including physiological assays in specialized growth chambers, enzyme characterizations, immunodetections, and stable isotope analyses. Increasingly, molecular techniques, such as PCR-based gene probing, FISH, and 16 rRNA phylogenetic analysis, are used to complement traditional methods. These studies provide valuable insight into the population dynamics, evolutionary history, and carbon and nutrient metabolism of symbionts. In addition, projects are currently underway to sequence the genomes of some of the chemosynthetic symbionts described in this review (e.g., symbionts of *Riftia pachyptila* and *Solemya velum*). Genomic analysis, in conjunction with new technologies to manipulate symbioses under in situ conditions

(e.g., via vascular catheters; Felbeck et al., 2004) and to sample the physical environment (e.g., electrochemical sampling; Luther et al., 2001), will contribute significantly to our understanding of symbiont biology. Scientists are now poised to reveal how interactions with the host and the abiotic environment impact symbiotic chemosynthetic bacteria over both ecological and evolutionary timescales.

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Literature Cited

Alt, J. C. 1995. Subseafloor processes in mid-ocean ridge hydrothermal systems. *In:* S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, and R. E. Thomson (Eds.) Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions. American Geophysical Union. Geophysical Monograph 91.

Anderson, A., J. Childress, and J. Favuzzi. 1987. Net uptake of CO_2 driven by sulfide and thiosulfite oxidation in the bacterial symbiont-containing clam Solemya reidi. J. Exp. Biol. 133:1–131.

Anthony, C. 1982. The Biochemistry of Methylotrophs. Academic Press. London, UK.

Arp, A. J., J. J. Childress, and C. R. J. Fisher. 1984. Metabolic and blood gas transport characteristics of the hydrothermal vent bivalve Calyptogena magnifica. Physiol. Zool. 57:648–662.

Arp, A. J., J. J. Childress, and C. R. Fisher. 1985. Blood gas transport in Riftia pachyptila. Bull. Biol. Soc. Washington 6:289–300.

Arp, A. J., J. J. Childress, and R. D. Vetter. 1987. The sulphide-binding protein in the blood of the vestimentiferan tube-worm, Riftia Pachyptila, is the extracellular haemoglobin. J. Exp. Biol. 128:139–158.

Bailly, X., D. Jollivet, S. Vanin, J. Deutsch, F. Zal, F. Lallier, and A. Toulmond. 2002. Evolution of the sulfide-binding function within the globin multigenic family of the deep-sea hydrothermal vent tubeworm Riftia pachyptila. Molec. Biol. Evol. 19:1421–1433.

Bailly, X., R. Leroy, S. Carney, O. Collin, F. Zal, A. Toulmond, and D. Jollivet. 2003. The loss of the hemoglobin H_2S-binding function in annelids from sulfide-free habitats reveals molecular adaptation driven by Darwinian positive selection. Proc. Natl. Acad. Sci. USA 100:5885–5890.

Barry, J. P., K. R. Buck, R. K. Kochevar, D. C. Nelson, Y. Fujiwara, S. K. Goffredi, and J. Hashimoto. 2002. Methane-based symbiosis in a mussel, Bathymodiolus platifrons, from cold seeps in Sagami Bay, Japan. Invertebr. Biol. 121:47–54.

Bauer-Nebelsick, M., C. F. Bardele, and J. A. Ott. 1996. Electron microscopic studies on Zoothamnium niveum (Hemprich & Ehrenberg, 1831) Ehrenberg 1838 (Oligohymenophora, Peritrichida), a ciliate with ectosymbiotic, chemoautotrophic bacteria. Eur. J. Protistol. 32:202–215.

Bennett, B. A., C. R. Smith, B. Glaser, and H. L. Maybaum. 1994. Faunal community structure of a chemoautotrophic assemblage on whale bones in the deep northeast Pacific Ocean. Mar. Ecol. Progr. Ser. 108:205–223.

Bergquist, D. C., T. Ward, E. E. Cordes, T. McNelis, S. Howlett, R. Kosoff, S. Hourdez, R. Carney, and C. R. Fisher. 2003. Community structure of vestimentiferan-generated habitat islands from Gulf of Mexico cold seeps. J. Exp. Mar. Biol. Ecol. 289:197–222.

Bilyard, G. R., and A. G. Carey. 1980. Zoogeography of Western Beaufort Sea Polychaeta (Annelida). Sarsia 65:19–25.

Black, M. B., R. A. Lutz, and R. C. Vrifenhoek. 1994. Gene flow among vestimentiferan tube worm (Riftia pachyptila) populations from hydrothermal vents of the Eastern Pacific. Mar. Biol. 120:33–39.

Black, M. B., A. Trivedi, P. A. Y. Maas, R. A. Lutz, and R. C. Vrijenhoek. 1998. Population genetics and biogeography of vestimentiferan tube worms. Deep Sea Res. Pt. II Top. Stud. Oceanogr. 45:365–382.

Boetius, A., and H. Felbeck. 1995. Digestive enzymes in marine-invertebrates from hydrothermal vents and other reducing environments. Mar. Biol. 122:105–113.

Bosch, C., and P. P. Grassé. 1984. Cycle partiel des bactéries chimioautotrophes symbiotiques et eurs rapports avec les bacteriocytes chez Riftia pachyptila Jones (Pogonophore Vestimentifére). II: L'evolution des bactéries symbotiques et des bacteriocytes. Comptes Rendus L'Acad. Sci. Ser. III: Sciences de la Vie—Life Sciences 299:413–319.

Boss, K. J., and R. D. Turner. 1980. The giant white clam from the Galapagos Rift, Calyptogena magnifica species novum. Malacologia 20:161–194.

Bright, M., H. Keckeis, and C. R. Fisher. 2000. An autoradiographic examination of carbon fixation, transfer and utilization in the Riftia pachyptila symbiosis. Mar. Biol. 136:621–632.

Brigmon, R. L., and C. De Ridder. 1998. Symbiotic relationship of Thiothrix spp. with an echinoderm. Appl. Environ. Microbiol. 64:3491–3495.

Brooks, J. M., M. C. Kennicutt 2nd, C. R. Fisher, S. A. Macko, K. Cole, J. J. Childress, R. R. Bidigare, and R. D. Vetter. 1987. Deep-sea hydrocarbon seep communities: Evidence for energy and nutritional carbon sources. Science 238:1138–1142.

Buck, K. R., J. P. Barry, and A. G. B. Simpson. 2000. Monterey Bay cold seep biota: Euglenozoa with chemoautotrophic bacterial epibionts. Eur. J. Protistol. 36:117–126.

Campbell, B. J., J. L. Stein, and S. C. Cary. 2003. Evidence of chemolithoautotrophy in the bacterial community associated with Alvinella pompejana, a hydrothermal vent polychaete. Appl. Environ. Microbiol. 69:5070–5078.

Cary, S. C., and S. J. Giovannoni. 1993a. Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. Proc. Natl. Acad. Sci. USA 90:5695–5699.

Cary, S. C., W. Warren, E. Anderson, and S. J. Giovannoni. 1993b. Identification and localization of bacterial endosymbionts in hydrothermal vent taxa with symbiont-specific polymerase chain reaction amplification and in situ hybridization techniques. Molec. Mar. Biol. Biotechnol. 2:51–62.

Cary, S. C. 1994. Vertical transmission of a chemoautotrophic aymbiont in the protobranch bivalve, Solemya reidi. Molec. Mar. Biol. Biotechnol. 3:121–130.

Cary, S. C., M. T. Cottrell, J. L. Stein, F. Camacho, and D. Desbruyères. 1997. Molecular identification and localization of filamentous symbiotic bacteria associated with the hydrothermal vent annelid Alvinella pompejana. Appl. Environ. Microbiol. 63:1124–1130.

Cavanaugh, C. M., S. L. Gardiner, M. L. Jones, H. W. Jannasch, and J. B. Waterbury. 1981. Prokaryotic cells in the hydrothermal vent tube worm Riftia pachyptila Jones: Possible chemoautotrophic symbionts. Science 213:340–342.

Cavanaugh, C. M. 1983. Symbiotic chemoautotrophic bacteria in marine invertebrates from sulfide-rich habitats. Nature 302:58–61.

Cavanaugh, C. M. 1985. Symbioses of chemoautotrophic bacteria and marine invertebrates from hydrothermal vents and reducing sediments. Biol. Soc. Wash. Bull. 6:373–388.

Cavanaugh, C. M., P. R. Levering, J. S. Maki, R. Mitchell, and M. E. Lidstrom. 1987. Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325:346–348.

Cavanaugh, C. M., M. S. Abbott, and M. Veenhuis. 1988. Immunochemical localization of ribulose-1,5-bisphosphate carboxylase in the symbiont-containing gills of Solemya velum (Bivalvia:Mollusca). Proc. Natl. Acad. Sci. USA 85:7786–7789.

Cavanaugh, C. M., C. Wirsen, and H. J. Jannasch. 1992. Evidence for methylotrophic symbionts in a hydrothermal vent mussel (Bivalvia:Mytilidae) from the Mid-Atlantic Ridge. Appl. Environ. Microbiol. 58:3799–3803.

Cavanaugh, C. M. 1993. Methanotroph-invertebrate symbioses in the marine environment: Ultrastructural, biochemical, and molecular studies. *In:* J. C. Murrell and D. P. Kelly (Eds.) Microbial Growth on C1 Compounds. Intercept.

Cavanaugh, C. M. 1994. Microbial symbiosis: Patterns of diversity in the marine environment. Am. Zool. 34:79–89.

Cavanaugh, C. M., and J. J. Robinson. 1996. CO_2 fixation in chemoautotroph-invertebrate symbioses: Expression of Form I and Form II RuBisCO. *In:* M. E. Lidstrom and F. R. Tabita (Eds.) Microbial Growth on C1 Compounds. Kluwer. Dordrecht, The Netherlands.

Chen, X. A., S. Li, and S. Aksoy. 1999. Concordant evolution of a symbiont with its host insect species: Molecular phylogeny of genus Glossina and its bacteriome-associated endosymbiont, Wigglesworthia glossinidia. J. Molec. Evol. 48:49–58.

Childress, J. J., C. R. Fisher, J. M. Brooks, M. C. Kennicutt 2nd, R. Bidigare, and A. E. Anderson. 1986. A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: Mussels fueled by gas. Science 233:1306–1308.

Childress, J. J., C. R. Fisher, J. A. Favuzzi, R. E. Kochevar, N. K. Sanders, and A. M. Alayse. 1991. Sulfide-driven autotrophic balance in the bacterial symbiont-containing hydrothermal vent tubeworm, Riftia pachyptila Jones. Biol. Bull. 180:135–153.

Childress, J. J., and C. R. Fisher. 1992. The biology of hydrothermal vent animals: Physiology, biochemistry, and autotrophic symbioses. Oceanogr. Mar. Biol. Ann. Rev. 30:337–441.

Childress, J. J., R. W. Lee, N. K. Sanders, H. Felbeck, D. R. Oros, A. Toulmond, D. Desbruyères, M. C. Kennicutt, and J. Brooks. 1993. Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental pCO_2. Nature 362:147–169.

CoBabe, E. A. 1991. Lucinid Bivalve Evolution and the Detection of Chemosymbiosis in the Fossil Record [PhD thesis]. Harvard University. Cambridge, MA.

Colaco, A., F. Dehairs, and D. Desbruyères. 2002. Nutritional relations of deep-sea hydrothermal fields at the Mid-Atlantic Ridge: A stable isotope approach. Deep Sea Res. Pt. I Oceanogr. Res. Pap. 49:395–412.

Conway, N., J. Capuzzo, and B. Fry. 1989. The role of endosymbiotic bacteria in the nutrition of Solemya velum: Evidence from a stable isotope analysis of endosymbionts and hosts. Limnol. Oceanogr. 34:249–255.

Conway, N., and J. M. Capuzzo. 1991. Incorporation and utilization of bacterial lipids in the Solemya velum symbiosis. Mar. Biol. 108:277–292.

Conway, N. M., and J. E. M. Capuzzo. 1992a. High taurine levels in the Solemya velum symbiosis. Comp. Biochem. Physiol. B Biochem. Molec. Biol. 102:175–185.

Conway, N., M. C. Kennicutt, and C. L. Van Dover. 1992b. Stable isotopes in the study of marine chemosynthesis-based ecosystems. *In:* K. Lajtha and R. Michener (Eds.) Stable Isotopes in Ecology. Blackwell. Oxford, UK.

Corliss, J. B., J. Dymond, L. I. Gordon, J. M. Edmond, R. P. V. Herzen, R. D. Ballard, K. Green, D. Williams, A. Bainbridge, K. Crane, and T. H. Van Andel. 1979. Submarine thermal springs on the Galapagos Rift. Science 203:1073–1083.

Craddock, C., W. R. Hoeh, R. A. Lutz, and R. C. Vrijenhoek. 1995. Extensive gene flow among mytilid (Bathymodiolis thermophilus) populations from hydrothermal vents of the Eastern Pacific. Mar. Biol. 124:137–146.

Dale, C., B. Wang, N. Moran, and H. Ochman. 2003. Loss of DNA recombinational repair enzymes in the initial stages of genome degeneration. Molec. Biol. Evol. 20:1188–1194.

Dando, P. R., and A. J. Southward. 1986. Chemoautotrophy in the bivalve molluscs of the genus Thyasira. J. Mar. Biol. Assoc. UK 66:915–929.

Dando, P. R., and B. Spiro. 1993. Varying nutritional dependence of the thyasirid bivalves Thyasira sarsi and T. equalis on chemoautotrophic symbiotic bacteria, demonstrated by isotope ratios of tissue carbon and shell carbonate. Mar. Ecol. Progr. Ser. 92:151–158.

De Bary, A. 1879. Die Erscheinung der Symbiose. Verlag von Karl J. Trubner. Strasburg, Germany.

de Burgh, M. E., and C. L. Singla. 1984. Bacterial colonization and endocytosis on the gill of a new limpet species from a hydrothermal vent. Mar. Biol. 84:1–6.

de Burgh, M. E., S. K. Juniper, and C. L. Singla. 1989. Bacterial symbiosis in northeast Pacific vestimentifera—a TEM study. Mar. Biol. 101:97–105.

De Cian, M. C., A. C. Andersen, X. Bailly, and F. H. Lallier. 2003a. Expression and localization of carbonic anhydrase and ATPases in the symbiotic tubeworm Riftia pachyptila. J. Exp. Biol. 206:399–409.

De Cian, M. C., X. Bailly, J. Morales, J. M. Strub, A. Van Dorsselaer, and F. H. Lallier. 2003b. Characterization of carbonic anhydrases from Riftia pachyptila, a symbiotic invertebrate from deep-sea hydrothermal vents. Prot. Struct. Funct. Genet. 51:327–339.

Degnan, P. H., A. B. Lazarus, C. D. Brock, and J. J. Wernegreen. 2004. Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: Cospeciation of Camponotus species and their endosymbionts, Candidatus blochmannia. System. Biol. 53: 95–110.

DeNiro, M. J., and S. Epstein. 1979. Relationship between the oxygen isotope ratios of terrestrial plant cellulose, carbon dioxide, and water. Science 204:51–53.

Desbruyères, D., F. Gaill, L. Laubier, D. Prieur, and G. H. Rau. 1983. Unusual nutrition of the "Pompeii worm" Alvinella pompejana (polychaetous annelid) from a hydrothermal vent environment: SEM, TEM, ^{13}C and ^{15}N evidence. Mar. Biol. 75:201–205.

Desbruyères, D., F. Gaill, L. Laubier, and Y. Fouquet. 1985. Polychaetous annelids from hydrothermal vent ecosystems: An ecological overview. Biol. Soc. Wash. Bull. 6:103–116.

Dickson, A. G., and F. J. Millero. 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res. Pt. A Oceanogr. Res. Pap. 34:1733–1743.

Di Meo, C. A., A. E. Wilbur, W. E. Holben, R. A. Feldman, R. C. Vrijenhoek, and S. C. Cary. 2000. Genetic variation among endosymbionts of widely distributed vestimentiferan tubeworms. Appl. Environ. Microbiol. 66:651–658.

Distel, D. L., and C. M. Cavanaugh. 1994. Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbioses in marine bivalves. J. Bacteriol. 176:1932–1938.

Distel, D. L., H. K. W. Lee, and C. M. Cavanaugh. 1995. Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel. Proc. Natl. Acad. Sci. USA 92:9598–9602.

Distel, D. 1998. Evolution of chemoautotrophic endosymbioses in bivalves. BioScience 48:277–286.

Dubilier, N., O. Giere, and M. K. Grieshaber. 1995. Morphological and ecophysiological adaptations of the marine oligochaete Tubificoides benedii to sulfidic sediments. Am. Zool. 35:163–173.

Dubilier, N., R. Windoffer, and O. Giere. 1998. Ultrastructure and stable carbon isotope composition of the hydrothermal vent mussels Bathymodiolus brevior and B. sp. affinis brevior from the North Fiji Basin, western Pacific. Mar. Ecol. Progr. Ser. 165:187–193.

Dubilier, N., R. Amann, C. Erseus, G. Muyzer, S. Y. Park, O. Giere, and C. M. Cavanaugh. 1999. Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochete Olavius loisae (Annelida). Mar. Ecol. Progr. Ser. 178:271–280.

Dubilier, N., C. Mulders, T. Ferdelman, D. de Beer, A. Pernthaler, M. Klein, M. Wagner, C. Erseus, F. Thiermann, J. Krieger, O. Giere, and R. Amann. 2001. Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. Nature 411:298–302.

Dufour, S. C., and H. Felbeck. 2003. Sulphide mining by the superextensile foot of symbiotic thyasirid bivalves. Nature 426:65–67.

Dunton, K. 1992. Arctic biogeography: The paradox of the marine benthic fauna and flora. Trends Ecol. Evol. 7:183–189.

Duperron, S., T. Nadalig, J. Caprais, M. Sibuet, A. Fiala-Medioni, R. Amann, and N. Dubilier. 2004. Dual symbiosis in a Bathymodiolus mussel from a methane seep on the Gabon continental margin (South East Atlantic): 16S rRNA phylogeny and distribution of the symbionts in the gills. Appl. Environ. Microbiol. 71(4): 1694–1700.

Durand, P., and O. Gros. 1996a. Bacterial host specificity of Lucinacea endosymbionts: Interspecific variation in 16S rRNA sequences. FEMS Microbiol. Lett. 140:193–198.

Durand, P., O. Gros, L. Frenkiel, and D. Prieur. 1996b. Phylogenetic characterization of sulfur-oxidizing bacterial endosymbionts in three tropical Lucinidae by 16S rDNA sequence analysis. Molec. Mar. Biol. Biotechnol. 5:37–42.

Dykhuizen, D. E., and L. Green. 1991. Recombination in Escherichia coli and the definition of biological species. J. Bacteriol. 173:7257–7268.

Elderfield, H., and A. Schultz. 1996. Mid-ocean ridge hydrothermal fluxes and the chemical composition of the ocean. Ann. Rev. Earth Planet. Sci. 24:191–224.

Elsaied, H., H. Kimura, and T. Naganuma. 2002. Molecular characterization and endosymbiotic localization of the gene encoding D-ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) form II in the deep-sea vestimentiferan trophosome. Microbiology 148:1947–1957.

Endow, K., and S. Ohta. 1989. The symbiotic relationship between bacteria and the mesogastropod snail, Alviniconcha hessleri, collected from hydrothermal vents of the Mariana back-arc basin. Bull. Jpn. Soc. Microb. Ecol. 3:73–82.

Endow, K., and S. Ohta. 1990. Occurrence of bacteria in the primary oocytes of vesicomyid clam Calyptogena soyoae. Mar. Ecol. Progr. Ser. 64:309–311.

Felbeck, H. 1981a. Chemoautotrophic potential of the hydrothermal vent tube worm, Riftia pachyptila Jones (Vestimentifera). Science 213:336–338.

Felbeck, H., J. J. Childress, and G. N. Somero. 1981b. Calvin-Benson cycle and sulfide oxidation enzymes in animals from sulfide-rich habitats. Nature 293:291–293.

Felbeck, H. 1983a. Sulfide oxidation and carbon fixation by the gutless clam Solemya reidi: An animal-bacteria symbiosis. J. Comp. Physiol. 152:3–11.

Felbeck, H., G. Liebezeit, R. Dawson, and O. Giere. 1983b. CO_2 fixation in tissues of marine oligochaetes (Phallodrilus leukodermatus and P. planus) containing symbiotic, chemoautotrophic bacteria. Mar. Biol. 75:187–191.

Felbeck, H. 1985. CO_2 fixation in the hydrothermal vent tube worm Riftia pachyptila (Jones). Physiol. Zool. 58:272–281.

Felbeck, H., and D. L. Distel. 1991. Prokaryotic symbionts of marine invertebrates. *In:* A. Balows A., H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY.

Felbeck, H., and P. J. Turner. 1995. CO_2 transport in catheterized hydrothermal vent tubeworms, Riftia pachyptila (Vestimentifera). J. Exp. Zool. 272:95–102.

Felbeck, H., and J. Jarchow. 1998. Carbon release from purified chemoautotrophic bacterial symbionts of the hydro-

thermal vent tubeworm Riftia pachyptila. Physiol. Zool. 71:294–302.

Felbeck, H., C. Arndt, U. Hentschel, and J. J. Childress. 2004. Experimental application of vascular and coelomic catheterization to identify vascular transport mechanisms for inorganic carbon in the vent tubeworm, Riftia pachyptila. Deep Sea Res. Pt. I Oceanogr. Res. Pap. 51:401–411.

Feldman, R., M. Black, C. Cary, R. Lutz, and R. Vrijenhoek. 1997. Molecular phylogenetics of bacterial endosymbionts and their vestimentiferan hosts. Molec. Mar. Biol. Biotechnol. 6:268–277.

Fenchel, T., and B. J. Finlay. 1989. Kentrophoros—a mouthless ciliate with a symbiotic kitchen garden. Ophelia 30:75–93.

Fiala-Médioni, A. 1984. Ultrastructural evidence of abundance of intracellular symbiotic bacteria in the gill of bivalve mollusks of deep hydrothermal vents. Comptes Rendus L'Acad. Sci. Ser. III Sciences de la Vie—Life Sciences 298:487–492.

Fiala-Médioni, A., J. Boulègue, S. Ohta, H. Felbeck, and A. Mariotti. 1993. Source of energy sustaining the Calyptogena populations from deep trenches in subduction zones off Japan. Deep Sea Res. 40:1241–1258.

Fiala-Médioni, A., J.-C. Michalski, J. Jollès, C. Alonso, and J. Montreuil. 1994. Lysosomic and lysozyme activities in the gill of bivalves from deep hydrothermal vents. Comptes Rendus L'Acad. Sci. Ser. III Sciences de la Vie—Life Sciences 317:239–244.

Fiala-Médioni, A., Z. P. McKiness, P. Dando, J. Boulegue, A. Mariotti, A. M. Alayse-Danet, J. J. Robinson, and C. M. Cavanaugh. 2002. Ultrastructural, biochemical, and immunological characterization of two populations of the mytilid mussel Bathymodiolus azoricus from the Mid-Atlantic Ridge: Evidence for a dual symbiosis. Mar. Biol. 141:1035–1043.

Fisher, C. R., and J. J. Childress. 1986. Translocation of fixed carbon from symbiotic bacteria to host tissues in the gutless bivalve, Solemya reidi. Mar. Biol. 93:59–68.

Fisher, C., J. Childress, A. Arp, J. Brooks, D. Distel, J. Favuzzi, H. Felbeck, R. Hessler, K. Johnson, M. Kennicut 2nd, S. Macko, A. Newton, M. Powell, G. Somero, and T. Soto. 1988. Microhabitat variation in the hydrothermal vent mussel, Bathymodiolus thermophilus, at the Rose Garden vent on the Galapagos Rift. Deep Sea Res. 35:1769–1791.

Fisher, C. R. 1990. Chemoautotrophic and methanotrophic symbioses in marine invertebrates. Rev. Aquat. Sci. 2:399–436.

Fisher, C. R., and J. J. Childress. 1992. Organic carbon transfer from methanotrophic symbionts to the host hydrocarbon seep mussel. Symbiosis 12:221–235.

Fisher, C. R. 1993a. Oxidation of methane by deep-sea mytilids in the Gulf of Mexico. *In:* R. S. Oremland (Ed.) Biogeochemistry of Global Change: Radiatively Active Trace Gases. Chapman and Hall. New York, NY.

Fisher, C. R., J. M. Brooks, J. S. Vodenichar, J. M. Zande, J. J. Childress, and R. A. Burke Jr. 1993b. The co-occurrence of methanotrophic and chemoautotrophic sulfur-oxidizing bacterial symbionts in a deep-sea mussel. Marine Ecology Pubblicazioni Della Stazione Zoologica Di Napoli I 14:277–289.

Fisher, C. R., J. J. Childress, S. A. Macko, and J. M. Brooks. 1994. Nutritional interactions in Galapagos Rift hydrothermal vent communities: Inferences from stable carbon and nitrogen isotope analyses. Mar. Ecol. Progr. Ser. 103:45–55.

Fisher, C. R. 1995. Toward an appreciation of hydrothermal vent animals: Their environment, physiological ecology, and tissue stable isotope values. *In:* Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions. American Geophysical Union.

Fisher, C. R. 1996. Ecophysiology of primary production at deep-sea vents and seeps. Biosystem. Ecol. Ser. 11:313–336.

Fox, M., S. K. Juniper, and H. Vali. 2002. Chemoautotrophy as a possible nutritional source in the hydrothermal vent limpet Lepetodrilus fucensis. Cahiers Biol. Mar. 43:371–376.

Freytag, J. K., P. R. Girguis, D. C. Bergquist, J. P. Andras, J. J. Childress, and C. R. Fisher. 2001. A paradox resolved: Sulfide acquisition by roots of seep tubeworms sustains net chemoautotrophy. Proc. Natl. Acad. Sci. USA 98:13408–13413.

Fry, B., H. Gest, and J. M. Hayes. 1983. Sulfur isotopic compositions of deep-sea hydrothermal vent animals. Nature 306:51–52.

Fry, B., and E. B. Sherr. 1984. $\delta^{13}C$ measurements as indicators of carbon flow in marine and freshwater ecosystems. Contrib. Mar. Sci. 27:13–47.

Fujio, S. Z., and N. Imasato. 1991. Diagnostic calculation for circulation and water mass movement in the deep Pacific. J. Geophys. Res. Oceans 96:759–774.

Fujiwara, Y., C. Kato, N. Masui, K. Fujikura, and S. Kojima. 2001. Dual symbiosis in the cold-seep thyasirid clam Maorithyas hadalis from the hadal zone in the Japan Trench, western Pacific. Mar. Ecol. Progr. Ser. 214:151–159.

Fujiwara, Y., and K. Uematsu. 2002. Microdistribution of two endosymbionts in gill tissue from a hadal thyasirid clam Maorithyas hadalis. Cahiers Biol. Mar. 43:341–343.

Fustec, A., D. Desbruyères, and S. K. Juniper. 1987. Deep-sea hydrothermal vent communities at 13°N on the East Pacific Rise: Microdistribution and temporal variations. Biol. Oceanogr. 4:121–164.

Gearing, J. N., P. J. Gearing, D. T. Rudnick, A. G. Requejo, and M. J. Hutchins. 1984. Isotopic variability of organic-carbon in a phytoplankton-based, temperate estuary. Geochim. Cosmochim. Acta 48:1089–1098.

Giere, O. 1981. The gutless marine oligochaete Phallodrilus leukodermatus: Structural studies on an aberrant tubificid associated with bacteria. Mar. Ecol. Progr. Ser. 5:353–357.

Giere, O. 1985. The gutless marine tubificid Phallodrilus planus, a flattened oligochaete with symbiotic bacteria. Zool. Scripta 14:279–286.

Giere, O., and C. Langheld. 1987. Structural organization, transfer and biological fate of endosymbiotic bacteria in gutless oligochaetes. Mar. Biol. 93:641–650.

Giere, O., and J. Krieger. 2001. A triple bacterial endosymbiosis in a gutless oligochaete (Annelida): Ultrastructural and immunocytochemical evidence. Invertebr. Biol. 120:41–49.

Girguis, P. R., R. W. Lee, N. Desaulniers, J. J. Childress, M. Pospesel, H. Felbeck, and F. Zal. 2000. Fate of nitrate acquired by the tubeworm Riftia pachyptila. Appl. Environ. Microbiol. 66:2783–2790.

Girguis, P. R., J. J. Childress, J. K. Freytag, K. Klose, and R. Stuber. 2002. Effects of metabolite uptake on proton-equivalent elimination by two species of deep-sea vestimentiferan tubeworm, Riftia pachyptila and Lamel-

librachia cf luymesi: Proton elimination is a necessary adaptation to sulfide-oxidizing chemoautotrophic symbionts. J. Exp. Biol. 205:3055–3066.

Glockner, F. O., H. D. Babenzien, J. Wulf, and R. Amann. 1999. Phylogeny and diversity of Achromatium oxaliferum. System. Appl. Microbiol. 22:28–38.

Goericke, R., J. P. Montoya, and B. Fry. 1994. Physiology of isotopic fractionation in algae and cyanobacteria. *In:* K. Lajtha and R. H. Michener (Eds.) Stable Isotopes in Ecology and Environmental Science. Blackwell. Oxford, UK.

Goffredi, S. K., J. J. Childress, N. T. Desaulniers, and F. H. Lallier. 1997a. Sulfide acquisition by the hydrothermal vent tubeworm Riftia pachyptila appears to be via uptake of HS^-, rather than H_2S. J. Exp. Biol. 200:2069–2616.

Goffredi, S. K., J. J. Childress, N. T. Desaulniers, R. W. Lee, F. H. Lallier, and D. Hammond. 1997b. Inorganic carbon acquisition by the hydrothermal vent tubeworm Riftia pachyptila depends upon high external pCO_2 and upon proton-equivalent ion transport by the worm. J. Exp. Biol. 200:883–896.

Goffredi, S. K., J. J. Childress, F. H. Lallier, and N. T. Desaulniers. 1999. The ionic composition of the hydrothermal vent tube worm Riftia pachyptila: Evidence for the elimination of SO_4^{2-} and H^+ and for a Cl^-/HCO^{3-} shift. Physiol. Biochem. Zool. 72:296–306.

Goffredi, S. K., and J. J. Childress. 2001. Activity and inhibitor sensitivity of ATPases in the hydrothermal vent tubeworm Riftia pachyptila: A comparative approach. Mar. Biol. 138:259–265.

Grassle, J. F. 1985. Hydrothermal vent animals: Distribution and biology. Science 229:713–717.

Gray, N. D., R. Howarth, A. Rowan, R. W. Pickup, J. G. Jones, and I. M. Head. 1999. Natural communities of Achromatium oxaliferum comprise genetically, morphologically, and ecologically distinct subpopulations. Appl. Environ. Microbiol. 65:5089–5099.

Grieshaber, M. K., and S. Volkel. 1998. Animal adaptations for tolerance and exploitation of poisonous sulfide. Ann. Rev. Physiol. 60:33–53.

Gros, O., A. Darrasse, P. Durand, L. Frenkiel, and M. Moueza. 1996. Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve Codakia orbicularis. Appl. Environ. Microbiol. 62:2324–2330.

Gros, O., P. De Wulf-Durand, L. Frenkiel, and M. Moueza. 1998. Putative environmental transmission of sulfur-oxidizing bacterial symbionts in tropical lucinid bivalves inhabiting various environments. FEMS Microbiol. Lett. 160:257–262.

Gros, O., M. Liberge, and H. Felbeck. 2003a. Interspecific infection of aposymbiotic juveniles of Codakia orbicularis by various tropical lucinid gill endosymbionts. Mar. Biol. 142:57–66.

Gros, O., M. Liberge, A. Heddi, C. Khatchadourian, and H. Felbeck. 2003b. Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat (Thalassia testudinum environment). Appl. Environ. Microbiol. 69:6264–6267.

Gustafson, R. G., and R. G. B. Reid. 1988. Association of bacteria with larvae of the gutless protobranch bivalve Solemya reidi (Cryptodonta, Solemyidae). Mar. Biol. 97:389–401.

Hand, S. C. 1987. Trophosome ultrastructure and the characterization of isolated bacteriocytes from invertebrate-sulfur bacteria symbioses. Biol. Bull. 173:260–276.

Head, I. M., N. D. Gray, K. J. Clarke, R. W. Pickup, and J. G. Jones. 1996. The phylogenetic position and ultrastructure of the uncultured bacterium Achromatium oxaliferum. Microbiol. SGM 142:2341–2354.

Hentschel, U., and H. Felbeck. 1993a. Nitrate respiration in the hydrothermal vent tubeworm Riftia pachyptila. Nature 366:338–340.

Hentschel, U., S. C. Cary, and H. Felbeck. 1993b. Nitrate respiration in chemoautotrophic symbionts of the bivalve Lucinoma aequizonata. Mar. Ecol. Progr. Ser. 94:35–41.

Herry, A., and M. Le Pennec. 1987. Endosymbiotic bacteria in the gills of the littoral bivalve molluscs Thyasira flexuosa (Thyasiridae) and Lucinella divaricata (Lucinidae). Symbiosis 4:25–36.

Hughes, D. S., H. Felbeck, and J. L. Stein. 1997. A histidine protein kinase homolog from the endosymbiont of the hydrothermal vent tubeworm Riftia pachyptila. Appl. Environ. Microbiol. 63:3494–3498.

Hughes, D. S., H. Felbeck, and J. L. Stein. 1998. Signal transduction and motility genes from the bacterial endosymbionts of Riftia pachyptila. Cahiers Biol. Mar. 39:305–308.

Humes, A. G., and R. A. Lutz. 1994. Aphotopontius acanthinus, new species (Copepoda, Siphonostomatoida), from deep-sea hydrothermal vents on the East Pacific Rise. J. Crustac. Biol. 14:337–345.

Hunt, S. 1992. Structure and composition of the shell of the archaeogastropod limpet Lepetodrilus elevatus-elevatus (Mclean, 1988). Malacologia 34:129–141.

Hurtado, L. A. 2002. Evolution and Biogeography of Hydrothermal Vent Organisms in the Eastern Pacific Ocean [PhD thesis]. Rutgers University. New Brunswick, NC.

Hurtado, L. A., M. Mateos, R. A. Lutz, and R. C. Vrijenhoek. 2003. Coupling of bacterial endosymbiont and host mitochondrial genomes in the hydrothermal vent clam Calyptogena magnifica. Appl. Environ. Microbiol. 69: 2058–2064.

Jacobs, D. K., and D. R. Lindberg. 1998. Oxygen and evolutionary patterns in the sea: Onshore/offshore trends and recent recruitment of deep-sea faunas. Proc. Natl. Acad. Sci. USA 95:9396–9401.

Johnson, K. S., J. J. Childress, R. R. Hessler, C. M. Sakamoto-Arnold, and C. L. Beehler. 1988. Chemical and biological interactions in the Rose Garden hydrothermal vent field, Galapagos spreading center. Deep Sea Res. 35: 1723–1744.

Johnson, M., M. Diouris, and M. Le Pennec. 1994. Endosymbiotic bacterial contribution in the carbon nutrition of Loripes lucinalis (Mollusca : Bivalvia). Symbiosis 17:1–13.

Jones, M. L. 1981. Riftia pachyptila Jones: Observations on the vestimentiferan worm from the Galapagos Rift. Science 213:333–336.

Jones, J. L., and S. L. Gardiner. 1985. Light and scanning electron microscope studies of spermatogenesis in the vestimentiferan tube worm Riftia pachyptila (Pogonophora : Obturata). Trans. Am. Microscop. Soc. 104:1–18.

Jørgensen, B. B., and J. R. Postgate. 1982. Ecology of the bacteria of the sulfur cycle with special reference to anoxic oxic interface environments. Phil. Trans. Roy. Soc. Lond. Ser. B Biol. Sci. 298:543–561.

Joyner, J. L., S. M. Peyer, and R. W. Lee. 2003. Possible roles of sulfur-containing amino acids in a chemoautotrophic bacterium-mollusc symbiosis. Biol. Bull. 205:331–338.

Julian, D., F. Gaill, E. Wood, A. J. Arp, and C. R. Fisher. 1999. Roots as a site of hydrogen sulfide uptake in the hydrocarbon seep vestimentiferan Lamellibrachia sp. J. Exp. Biol. 202:2245–2257.

Karl, S. A., S. Schutz, D. Desbruyères, R. Lutz, and R. C. Vrijenhoek. 1996. Molecular analysis of gene flow in the hydrothermal vent clam (Calyptogena magnifica). Molec. Mar. Biol. Biotechnol. 5:193–202.

Kennicutt, M. C., R. A. Burke, I. R. MacDonald, J. M. Brooks, G. J. Denoux, and S. A. Macko. 1992. Stable isotope partitioning in seep and vent organisms: Chemical and ecological significance. Chem. Geol. 101:293–310.

Kennish, M. J., and R. A. Lutz. 1999. Calcium carbonate dissolution rates in deep-sea bivalve shells on the East Pacific Rise at 21°N: Results of an 8-year in situ experiment. Palaeogeogr. Palaeoclimatol. Palaeoecol. 154: 293–299.

Kimura, M., and G. H. Weiss. 1964. The stepping stone model of population structure and the decrease of genetic correlation with distance. Genetics 49:561–576.

Kochevar, R. E., and J. J. Childress. 1996. Carbonic anhydrase in deep-sea chemoautotrophic symbioses. Mar. Biol. 125:375–383.

Krieger, J., O. Giere, and N. Dubilier. 2000. Localization of RubisCO and sulfur in endosymbiotic bacteria of the gutless marine oligochaete Inanidrilus leukodermatus (Annelida). Mar. Biol. 137:239–244.

Krueger, D. M. 1996a. Ecology and Evolution of Solemya spp., Marine Bivalves Living in Symbiosis with Chemoautotrophic Bacteria [PhD thesis]. Harvard University. Cambridge, MA.

Krueger, D. M., N. Dubilier, and C. M. Cavanaugh. 1996b. Chemoautotrophic symbiosis in the tropical solemyid Solemya occidentalis (Bivalvia : Protobranchia): Ultrastructural and phylogenetic analysis. Mar. Biol. 126:55–64.

Krueger, D. M., and C. M. Cavanaugh. 1997. Phylogenetic diversity of bacterial symbionts of Solemya hosts based on comparative sequence analysis of 16S rRNA genes. Appl. Environ. Microbiol. 63:91–98.

Lalou, C., and E. Brichet. 1982. Ages and implications of East Pacific Rise sulfide deposits at 21°N. Nature 300:169–171.

Laubier, L. 1989. Ecosystèmes benthiques profonds et chimiosynthèse bactérienne: Sources hydrothermales et suintement froids [Deep benthic ecosystems and bacterial chemosynthesys: Hydrothermal wells and cold seepages]. *In:* M. Denis (Ed.) Oceanology: Today and in the Near Future.

Laue, B. E., and D. C. Nelson. 1994. Characterization of the gene encoding the autotrophic ATP sulfurylase from the bacterial endosymbiont of the hydrothermal vent tubeworm Riftia pachyptila. J. Bacteriol. 176:3723–3729.

Laue, B. E., and D. C. Nelson. 1997. Sulfur-oxidizing symbionts have not co-evolved with their hydrothermal vent tubeworm hosts: An RFLP analysis. Molec. Mar. Biol. Biotechnol. 6:180–188.

Lee, R., and J. Childress. 1994. Assimilation of inorganic nitrogen by marine invertebrates and their chemoautotrophic and methanotrophic symbionts. Appl. Environ. Microbiol. 60:1852–1858.

Lee, R. W., J. J. Childress, and N. T. Desaulniers. 1997. The effects of exposure to ammonia on ammonia and taurine pools of the symbiotic clam Solemya reidi. J. Exp. Biol. 200:2797–2805.

Lee, R. W., J. J. Robinson, and C. M. Cavanaugh. 1999. Pathways of inorganic nitrogen assimilation in chemoautotrophic bacteria-marine invertebrate symbioses: Expression of host and symbiont glutamine synthetase. J. Exp. Biol. 202:289–300.

Le Pennec, M., and A. Hily. 1984. Anatomy, structure and ultrastructure of the gill of a deep-sea hydrothermal vent mytilid. Oceanol. Acta 7:517–523.

Levin, B. R., and C. T. Bergstrom. 2000. Bacteria are different: Observations, interpretations, speculations, and opinions about the mechanisms of adaptive evolution in prokaryotes. Proc. Natl. Acad. Sci. USA 97:6981–6985.

Levin, L. A., and R. H. Michener. 2002. Isotopic evidence for chemosynthesis-based nutrition of macrobenthos: The lightness of being at Pacific methane seeps. Limnol. Oceanogr. 47:1336–1345.

Li, L., C. Kato, and K. Horikoshi. 1999. Microbial diversity in sediments collected from the deepest cold-seep area, the Japan Trench. Mar. Biotechnol. 1:391–400.

Lilley, M. D., D. A. Butterfield, E. J. Olson, J. E. Lupton, S. A. Macko, and R. E. McDuff. 1993. Anomalous CH_4 and NH^{4+} concentrations at an unsedimented mid-ocean-ridge hydrothermal system. Nature 364:45–47.

Little, C. T. S., R. J. Herrington, V. V. Maslennikov, N. J. Morris, and V. V. Zaykov. 1997. Silurian hydrothermal-vent community from the southern Urals, Russia. Nature 385:146–148.

Little, C. L. 2002. The fossil record of hydrothermal vent communities. Cahiers Biol. Mar. 43:313–316.

Little, C. T. S., and I. H. Thorseth. 2002. Hydrothermal vent microbial communities: A fossil perspective. Cahiers Biol. Mar. 43:317–319.

Little, C. T. S., and R. C. Vrijenhoek. 2003. Are hydrothermal vent animals living fossils? Trends Ecol. Evol. 18:582–588.

Little, C. T. S., T. Danelian, R. J. Herrington, and R. M. Haymon. 2004. Early Jurassic hydrothermal vent community from the Franciscan Complex, California. J. Paleontol. 78:542–559.

Lonsdale, P. 1977. Clustering of suspension-feeding macrobenthos near abyssal hydrothermal vents at oceanic spreading centers. Deep Sea Res. 24:857.

Lopez-Garcia, P., F. Gaill, and D. Moreira. 2002. Wide bacterial diversity associated with tubes of the vent worm Riftia pachyptila. Environ. Microbiol. 4:204–215.

Luther, G. W. I., T. F. Rozan, M. Taillefert, D. B. Nuzzio, C. Di Meo, T. M. Shank, R. A. Lutz, and S. C. Cary. 2001. Chemical speciation drives hydrothermal vent ecology. Nature 410:813–816.

Lutz, R. A., D. Jablonski, D. C. Rhoads, and R. D. Turner. 1980. Larval dispersal of a deep-sea hydrothermal vent bivalve from the Galapagos Rift. Mar. Biol. 57:127–133.

MacAvoy, S. E., S. A. Macko, and S. B. Joye. 2002. Fatty acid carbon isotope signatures in chemosynthetic mussels and tubeworms from Gulf of Mexico hydrocarbon seep communities. Chem. Geol. 185:1–8.

Marsh, A. G., L. S. Mullineaux, C. M. Young, and D. T. Manahan. 2001. Larval dispersal potential of the tubeworm Riftia pachyptila at deep-sea hydrothermal vents. Nature 411:77–80.

McArthur, A. G., and V. Tunnicliffe. 1998. Relics and antiquity revisited in the modern vent fauna. *In:* R. Mills and

K. Harrison (Eds.) Modern Ocean Floor Processes and the Geological Record. Geological Society of London. London, UK.

McKiness, Z. P. 2004. Evolution of Endosymbioses in Deep-sea Bathymodioline Mussels (Mollusca:Bivalvia) [PhD thesis]. Harvard University. Cambridge, MA.

McMullin, E. R., S. Hourdez, S. W. Schaeffer, and C. R. Fisher. 2003. Phylogeny and biogeography of deep-sea vestimentiferan tubeworms and their bacterial symbionts. Symbiosis 34:1–41.

Metivier, B., and R. Voncosel. 1993. Acharax alinae n sp., a giant solemyid (Mollusca, Bivalvia) from the Lau Basin. Comptes Rendus L'Acad. Sci. Ser. III Sciences de la Vie—Life Sciences 316:229–237.

Michener, R. H., and D. M. Schell. 1994. Stable isotope ratios as tracers in marine aquatic food webs. *In:* K. Lajtha and R. H. Michener (Eds.) Stable Isotopes in Ecology and Environmental Science. Blackwell. Oxford, UK.

Millero, F. J., T. Plese, and M. Fernandez. 1987. The dissociation of hydrogen-sulfide in seawater. Limnol. Oceanogr. 33:269–274.

Millikan, D. S., H. Felbeck, and J. L. Stein. 1999. Identification and characterization of a flagellin gene from the endosymbiont of the hydrothermal vent tubeworm Riftia pachyptila. Appl. Environ. Microbiol. 65:3129–3133.

Minagawa, M., and E. Wada. 1984. Stepwise enrichment of ^{15}N along food chains—further evidence and the relationship between delta ^{15}N and animal age. Geochim. Cosmochim. Acta 48:1135–1140.

Minic, Z., V. Simon, B. Penverne, F. Gaill, and G. Herve. 2001. Contribution of the bacterial endosymbiont to the biosynthesis of pyrimidine nucleotides in the deep-sea tubeworm Riftia pachyptila. J. Biol. Chem. 276:23777–23784.

Minic, Z., and G. Herve. 2003. Arginine metabolism in the deep-sea tube worm Riftia pachyptila and its bacterial endosymbiont. J. Biol. Chem. 278:40527–40533.

Mira, A., and N. A. Moran. 2002. Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. Microb. Ecol. 44:137–143.

Miura, T., M. Nedachi, and A. Hashimoto. 2002. Sulphur sources for chemoautotrophic nutrition of shallow water vestimentiferan tubeworms in Kagoshima Bay. J. Mar. Biol. Assoc. UK 82:537–540.

Moran, N., and P. Baumann. 1994. Phylogenetics of cytoplasmically inherited microorganisms of arthropods. Trends Ecol. Evol. 9:15–20.

Moran, N. A. 1996. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc. Natl. Acad. Sci. USA 93:2873–2878.

Moran, N. A. 2003. Tracing the evolution of gene loss in obligate bacterial symbionts. Curr. Opin. Microbiol. 6:512–518.

Muller, H. J. 1964. The relation of recombination to mutational advance. Mut. Res. 1:2–9.

Mullineaux, L. S., P. H. Wiebe, and E. T. Baker. 1995. Larvae of benthic invertebrates in hydrothermal vent plumes over Juan de Fuca Ridge. Mar. Biol. 122:585–596.

Mullineaux, L. S., K. G. Speer, A. M. Thurnherr, M. E. Maltrud, and A. Vangriesheim. 2002. Implications of cross-axis flow for larval dispersal along mid-ocean ridges. Cahiers Biol. Mar. 43:281–284.

Nelson, D., and C. Fisher. 1995a. Chemoautotrophic and methanotrophic endosymbiotic bacteria at deep-sea vents and seeps. *In:* D. Karl (Ed.) Microbiology of Deep-sea Hydrothermal Vents. CRC Press. Boca Raton, FL.

Nelson, D. C., K. D. Hagen, and D. B. Edwards. 1995b. The gill symbiont of the hydrothermal vent mussel Bathymodiolus thermophilus is a psychrophilic, chemoautotrophic, sulfur bacterium. Mar. Biol. 121: 487–495.

Nelson, K., and C. R. Fisher. 2000. Absence of cospeciation in deep-sea vestimentiferan tubeworms and their bacterial endosymbionts. Symbiosis 28:1–15.

Niemczuk, K., and M. Kondracki. 2004. Q fever as zoonosis. Medycyna Weterynaryjna 60:129–131.

O'Brien, J., and R. D. Vetter. 1990. Production of thiosulphate during sulphide oxidation by mitochondria of the symbiont-containing bivalve Solemya reidi. J. Exp. Biol. 149:133–148.

Ohta, T. 1973. Slightly deleterious mutant substitutions in evolution. Nature 246:96–98.

Ott, J. A., R. Novak, F. Schiemer, U. Hentschel, M. Nebelsick, and M. Polz. 1991. Tackling the sulfide gradient: A novel strategy involving marine nematodes and chemoautotrophic ectosymbionts. Marine Ecology Pubblicazioni Della Stazione Zoologica Di Napoli I 12:261–279.

Ott, J. A., M. Bright, and F. Schiemer. 1998. The ecology of a novel symbiosis between a marine peritrich ciliate and chemoautotrophic bacteria. Marine Ecology—Pubblicazioni Della Stazione Zoologica Di Napoli I 19:229–243.

Peek, A. S., R. C. Vrijenhoek, and B. S. Gaut. 1998. Accelerated evolutionary rate in sulfur-oxidizing endosymbiotic bacteria associated with the mode of symbiont transmission. Molec. Biol. Evol. 15:1514–1523.

Pimenov, N. V., M. G. Kalyuzhnaya, V. N. Khmelenina, L. L. Mityushina, and Y. A. Trotsenko. 2002. Utilization of methane and carbon dioxide by symbiotrophic bacteria in gills of mytilidae (Bathymodiolus) from the rainbow and Logachev hydrothermal fields on the Mid-Atlantic Ridge. Microbiology 71:587–594.

Podar, M., L. Mullineaux, H. R. Huang, P. S. Perlman, and M. L. Sogin. 2002. Bacterial group II introns in a deep-sea hydrothermal vent environment. Appl. Environ. Microbiol. 68:6392–6398.

Polz, M. F., H. Felbeck, R. Novak, M. Nebelsick, and J. A. Ott. 1992. Chemoautotrophic, sulfur-oxidizing symbiotic bacteria on marine nematodes: Morphological and biochemical characterization. Microb. Ecol. 24:313–329.

Polz, M. F., D. L. Distel, B. Zarda, R. Amann, H. Felbeck, J. A. Ott, and C. M. Cavanaugh. 1994. Phylogenetic analysis of a highly specific association between ectosymbiotic, sulfur-oxidizing bacteria and a marine nematode. Appl. Environ. Microbiol. 60:4461–4467.

Polz, M. F., and C. M. Cavanaugh. 1995. Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. Proc. Natl. Acad. Sci. USA 92:7232–7236.

Polz, M. F., J. J. Robinson, C. M. Cavanaugh, and C. L. Van Dover. 1998. Trophic ecology of massive shrimp aggregations at a Mid-Atlantic Ridge hydrothermal vent site. Limnol. Oceanogr. 43:1631–1638.

Polz, M. F., C. Harbison, and C. M. Cavanaugh. 1999. Diversity and heterogeneity of epibiotic bacterial communities on the marine nematode Eubostrichus dianae. Appl. Environ. Microbiol. 65:4271–4275.

Polz, M. F., J. A. Ott, M. Bright, and C. M. Cavanaugh. 2000. When bacteria hitch a ride. ASM News 66:531–539.

Poulin, E., S. von Boletzky, and J. P. Feral. 2001. Combined ecological factors permit classification of developmental

patterns in benthic marine invertebrates: A discussion note. J. Exp. Mar. Biol. Ecol. 257:109–115.

Powell, M. A., and G. N. Somero. 1986. Adaptations to sulfide by hydrothermal vent animals: Sites and mechanisms of detoxification and metabolism. Biol. Bull. 171:274–290.

Pradillon, F., B. Shillito, C. M. Young, and F. Gaill. 2001. Deep-sea ecology: Developmental arrest in vent worm embryos. Nature 413:698–699.

Prior, S. D., and H. Dalton. 1985. The effect of copper ions on membrane content and methane monooxygenase activity in methanol-grown cells of Methylococcus capsulatus (Bath). J. Gen. Microbiol. 131:155–163.

Pruski, A. M., A. Fiala-Médioni, C. R. Fisher, and J. C. Colomines. 2000a. Composition of free amino acids and related compounds in invertebrates with symbiotic bacteria at hydrocarbon seeps in the Gulf of Mexico. Mar. Biol. 136:411–420.

Pruski, A. M., A. Fiala-Médioni, R. Prodon, and J. C. Colomines. 2000b. Thiotaurine is a biomarker of sulfide-based symbiosis in deep-sea bivalves. Limnol. Oceanogr. 45:1860–1867.

Pruski, A. M., and A. Fiala-Médioni. 2003. Stimulatory effect of sulphide on thiotaurine synthesis in three hydrothermal vent species from the East Pacific Rise. J. Exp. Biol. 206:2923–2930.

Rau, G. A., and J. I. Hedges. 1979. Carbon-13 depletion in a hydrothermal vent mussel: Suggestion of a chemosynthetic food source. Science 203:648–649.

Rau, G. H. 1981. Hydrothermal vent clam and tube worm $^{13}C/^{12}C$: Further evidence of non photosynthetic food sources. Science 213:338–340.

Robinson, J. J., and C. M. Cavanaugh. 1995. Expressions of form I and form II RubisCO in chemoautotrophic symbioses: Implications for the interpretation of stable carbon isotope values. Limnol. Oceanogr. 40:1496–1502.

Robinson, J. J., M. F. Polz, A. Fiala-Medioni, and C. M. Cavanaugh. 1998. Physiological and immunological evidence for two distinct C-1-utilizing pathways in Bathymodiolus puteoserpentis (Bivalvia : Mytilidae), a dual endosymbiotic mussel from the Mid-Atlantic Ridge. Mar. Biol. 132:625–633.

Robinson, J. J., K. M. Scott, S. T. Swanson, M. H. O'Leary, K. Horken, and F. R. Tabita. 2003. Kinetic isotope effect and characterization of form II RubisCO from the chemoautotrophic endosymbionts of the hydrothermal vent tubeworm Riftia pachyptila. Limnol. Oceanogr. 48:48–54.

Rouxel, O., Y. Fouquet, and J. N. Ludden. 2004. Subsurface processes at the Lucky Strike hydrothermal field, Mid-Atlantic Ridge: Evidence from sulfur, selenium, and iron isotopes. Geochim. Cosmochim. Acta 68:2295–2311.

Sarrazin, J., and S. K. Juniper. 1999. Biological characteristics of a hydrothermal edifice mosaic community. Mar. Ecol. Progr. Ser. 185:1–19.

Schiemer, F., R. Novak, and J. Ott. 1990. Metabolic studies on thiobiotic free-living nematodes and their symbiotic microorganisms. Mar. Biol. 106:129–137.

Schmaljohann, R., and H. J. Flügel. 1987. Methane-oxidizing bacteria in pogonophora. Sarsia 72:91–98.

Schweimanns, M., and H. Felbeck. 1985. Significance of the occurrence of chemoautotrophic bacterial endosymbionts in lucinid clams from Bermuda. Mar. Ecol. Progr. Ser. 24:113–120.

Scott, K. M. 2003. A $d^{13}C$-based carbon flux model for the hydrothermal vent chemoautotrophic symbiosis Riftia pachyptila predicts sizeable CO_2 gradients at the host-symbiont interface. Environ. Microbiol. 5:424–432.

Scott, K. M., J. Schwedock, D. P. Schrag, and C. M. Cavanaugh. 2004. Influence of form IA RubisCO and environmental dissolved inorganic carbon on the delta13C of the clam-chemoautotroph symbiosis Solemya velum. Environ. Microbiol. 6(12):1210–1219.

Searcy, D. G. 1992. Origins of mitochondria and chloroplasts from sulfur-based symbioses. H. Hartman and K. Matsuno (Eds.) The Origin and Evolution of the Cell: World Scientific Press.

Sibuet, M., and K. Olu. 1998. Biogeography, biodiversity and fluid dependence of deep-sea cold-seep communities at active and passive margins. Deep Sea Res. II 45:517–567.

Smith, C. R., and A. R. Baco. 2003. Ecology of whale falls at the deep-sea floor. Oceanogr. Mar. Biol. 41:311–354.

Southward, A. J., E. C. Southward, P. R. Dando, G. H. Rau, H. Felbeck, and H. Flugel. 1981. Bacterial symbionts and low $^{13}C/^{12}C$ ratios in tissues of Pogonophora indicate unusual nutrition and metabolism. Nature 293:616–620.

Southward, E. C. 1982. Bacterial symbionts in Pogonophora. J. Mar. Biol. Assoc. UK 62:889–906.

Southward, A. J., and E. C. Southward. 1988. Pogonophora: Tube worms dependent on endosymbiotic bacteria. ISI Atlas of Science, Animal & Plant Sciences 1:203–207.

Southward, E. C., V. Tunnicliffe, and M. Black. 1995. Revision of the species of Ridgeia from North-East Pacific hydrothermal vents, with a redescription of Ridgeia piscesae Jones (Pogonophora : Obturata = Vestimentifera). Can. J. Zool. 73:282–295.

Spiro, B., P. B. Greenwood, A. J. Southward, and P. R. Dando. 1986. $^{13}C/^{12}C$ ratios in marine invertebrates from reducing sediments: Confirmation of nutritional importance of chemoautotrophic endosymbiotic bacteria. Mar. Ecol. Progr. Ser. 28:233–240.

Stanley, S. M. 1970. Shell form and life habits of the Bivalvia. Geol. Soc. Am. Mem. 125:119–121.

Stein, J., S. Cary, R. Hessler, S. Ohta, R. Vetter, J. Childress, and H. Felbeck. 1988. Chemoautotrophic symbiosis in a hydrothermal vent gastropod. Biol. Bull. 174:373–378.

Stumm, W., and J. J. Morgan. 1996. Aquatic Chemistry, 3rd ed. Wiley-Interscience.

Svavarsson, J., J. O. Stromberg, and T. Brattegard. 1993. The deep-sea asellote (Isopoda, Crustacea) fauna of the Northern Seas: Species composition, distributional patterns and origin. J. Biogeogr. 20:537–555.

Temara, A., C. de Ridder, J. G. Kuenen, and L. A. Robertson. 1993. Sulfide-oxidizing bacteria in the burrowing echinoid, Echinocardium cordatum (Echinodermata). Mar. Biol. 115:179–185.

Thao, M., N. Moran, P. Abbot, E. Brennan, D. Burckhardt, and P. Baumann. 2000. Cospeciation of psyllids and their primary prokaryotic endosymbionts. Appl. Environ. Microbiol. 66:2898–2905.

Trask, J. L., and C. L. Van Dover. 1999. Site-specific and ontogenetic variations in nutrition of mussels (Bathymodiolus sp.) from the Lucky Strike hydrothermal vent field, Mid-Atlantic Ridge. Limnol. Oceanogr. 44:334–343.

Tunnicliffe, V. 1988. Biogeography and evolution of hydrothermal-vent fauna in the eastern Pacific Ocean. Proc. Roy. Soc. Lond B 233:347–366.

Tunnicliffe, V. 1991. The biology of hydrothermal vents: Ecology and evolution. Oceanogr. Mar. Biol. 29:319–407.

Tunnicliffe, V., and C. Fowler. 1996. Influence of sea-floor spreading on the global hydrothermal vent fauna. Nature 379:531–533.

Turner, R. D., R. A. Lutz, and D. Jablonski. 1985. Modes of molluscan larval development at deep-sea hydrothermal vents. Biol. Soc. Wash. Bull. 6:167–184.

Tyler, P. A., and C. M. Young. 2003. Dispersal at hydrothermal vents: A summary of recent progress. Hydrobiologia 503:9–19.

Vacelet, J., N. Boury-Esnault, A. Fiala-Medioni, and C. R. Fisher. 1995. A methanotrophic carnivorous sponge. Nature 377:296.

Vacelet, J., A. Fiala-Médioni, C. R. Fisher, and N. Boury-Esnault. 1996. Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge. Mar. Ecol. Progr. Ser. 145:77–85.

Van Dover, C. L., B. Fry, J. F. Grassle, S. Humphris, and P. A. Rona. 1988. Feeding biology of the shrimp Rimicaris exoculata at hydrothermal vents on the Mid-Atlantic Ridge. Mar. Biol. 98:209–216.

Van Dover, C. L., and B. Fry. 1994. Microorganisms as food resources at deep-sea hydrothermal vents. Limnol. Oceanogr. 39:51–57.

Van Dover, C. 2000. The Ecology of Deep-sea Hydrothermal Vents. Princeton University Press. Princeton, NJ.

Van Dover, C. L., S. E. Humphris, D. Fornari, C. M. Cavanaugh, R. Collier, S. K. Goffredi, J. Hashimoto, M. Lilley, A. L. Reysenbach, T. M. Shank, K. L. Von Damm, A. Banta, R. M. Gallant, D. Götz, D. Green, J. Hall, T. L. Harmer, L. A. Hurtado, P. Johnson, Z. P. McKiness, C. Meredith, E. Olson, I. L. Pan, M. Turnipseed, Y. Won, C. R. Young 3rd, and R. C. Vrijenhoek. 2001. Biogeography and ecological setting of Indian Ocean hydrothermal vents. Science 294:818–822.

Van Dover, C. 2002a. Trophic relationships among invertebrates at the Kairei hydrothermal vent field (Central Indian Ridge). Mar. Biol. 141:761–772.

Van Dover, C. L., C. R. German, K. G. Speer, L. M. Parson, and R. C. Vrijenhoek. 2002b. Marine biology: Evolution and biogeography of deep-sea vent and seep invertebrates. Science 295:1253–1257.

Vereshchaka, A. L., G. M. Vinogradov, A. Y. Lein, S. Dalton, and F. Dehairs. 2000. Carbon and nitrogen isotopic composition of the fauna from the Broken Spur hydrothermal vent field. Mar. Biol. 136:11–17.

Vrijenhoek, R., T. Shank, and R. Lutz. 1998. Gene flow and dispersal in deep-sea hydrothermal vent animals. Cahiers Biol. Mar. 39:363–366.

Waser, N. A. D., P. J. Harrison, B. Nielsen, S. E. Calvert, and D. H. Turpin. 1998. Nitrogen isotope fractionation during the uptake and assimilation of nitrate, nitrite, ammonium, and urea by a marine diatom. Limnol. Oceanogr. 43:215–224.

Watanabe, A. 2004. Various clinical types of Q-fever disease. Int. Med. 43:12.

Weber, R. E., and S. N. Vinogradov. 2001. Nonvertebrate hemoglobins: Functions and molecular adaptations. Physiol. Rev. 81:569–628.

Weiser, W. 1959. Eine ungewöhnliche Assoziation zwischen Blaualagen und freilebenden marinen Nematoden. Österr. bot. Zeitschr. 106:81–87.

Wernegreen, J. J. 2002. Genome evolution in bacterial endosymbionts of insects. Nature Rev. Genet. 3:850–861.

Windoffer, R., and O. Giere. 1997. Symbiosis of the hydrothermal vent gastropod Ifremeria nautilei (Provannidae) with endobacteria: Structural analyses and ecological considerations. Biol. Bull. 193:381–392.

Wirsen, C. O., S. M. Sievert, C. M. Cavanaugh, S. J. Molyneaux, A. Ahmad, L. T. Taylor, E. F. DeLong, and C. D. Taylor. 2002. Characterization of an autotrophic sulfide-oxidizing marine Arcobacter sp. that produces filamentous sulfur. Appl. Environ. Microbiol. 68:316–325.

Woldehiwet, Z. 2004. Q fever (coxiellosis): Epidemiology and pathogenesis. Res. Vet. Sci. 77:93–100.

Won, Y. J., S. J. Hallam, G. D. O'Mullan, I. L. Pan, K. R. Buck, and R. C. Vrijenhoek. 2003a. Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus Bathymodiolus. Appl. Environ. Microbiol. 69:6785–6792.

Won, Y., C. R. Young, R. A. Lutz, and R. C. Vrijenhoek. 2003b. Dispersal barriers and isolation among deep-sea mussel populations (Mytilidae:Bathymodiolus) from eastern Pacific hydrothermal vents. Molec. Ecol. 12:169–184.

Yamamoto, H., K. Fujikura, A. Hiraishi, K. Kato, and Y. Maki. 2002. Phylogenetic characterization and biomass estimation of bacterial endosymbionts associated with invertebrates dwelling in chemosynthetic communities of hydrothermal vent and cold seep fields. Mar. Ecol. Progr. Ser. 245:61–67.

Yamanaka, T., C. Mizota, H. Satake, F. Kouzuma, T. Gamo, U. Tsunogai, T. Miwa, and K. Fujioka. 2003. Stable isotope evidence for a putative endosymbiont-based lithotrophic Bathymodiolus sp. mussel community atop a serpentine seamount. Geomicrobiol. J. 20:185–197.

Yong, R., and D. G. Searcy. 2001. Sulfide oxidation coupled to ATP synthesis in chicken liver mitochondria. Comp. Biochem. Physiol. Pt. B 129:129–137.

Young, C. M., E. Vazquez, A. Metaxas, and P. A. Tyler. 1996. Embryology of vestimentiferan tube worms from deep-sea methane/sulphide seeps. Nature 381:514–516.

Zal, F., F. H. Lallier, J. S. Wall, S. N. Vinogradov, and A. Toulmond. 1996. The multi-hemoglobin system of the hydrothermal vent tube worm Riftia pachyptila.1: Reexamination of the number and masses of its constituents. J. Biol. Chem. 271:8869–8874.

Zal, F., B. N. Green, F. H. Lallier, and A. Toulmond. 1997. Investigation by electrospray ionization mass spectrometry of the extracellular hemoglobin from the polychaete annelid Alvinella pompejana: An unusual hexagonal bilayer hemoglobin. Biochemistry 36:11777–11786.

Zal, F., E. Leize, F. H. Lallier, A. Toulmond, A. Van Dorsselaer, and J. J. Childress. 1998. S-sulfohemoglobin and disulfide exchange: The mechanisms of sulfide binding by Riftia pachyptila hemoglobins. Proc. Natl. Acad. Sci. USA 95:8997–9002.

Zal, F., E. Leize, D. R. Oros, S. Hourdez, A. Van Dorsselaer, and J. J. Childress. 2000. Haemoglobin structure and biochemical characteristics of the sulphide-binding component from the deep-sea clam Calyptogena magnifica. Cahiers Biol. Mar. 41:413–423.

Zhang, J. Z., and F. J. Millero. 1993. The products from the oxidation of H_2S in seawater. Geochim. Cosmochim. Acta 57:1705–1718.

PART 3

Biotechnology and Applied Microbiology

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CHAPTER 3.1

Organic Acid and Solvent Production Part I: Acetic, Lactic, Gluconic, Succinic and Polyhydroxyalkanoic Acids

PALMER ROGERS, JIANN-SHIN CHEN AND MARY JO ZIDWICK

In Memorium

Shortly before the completion of this chapter, Palmer Rogers passed away suddenly. Palmer was the one who motivated and shepherded us to the completion of this work. He had a lifelong dedication to education of students and helped them attain the satisfaction that achieving in-depth understanding of science through hard work can bring. Palmer was kind, creative, energetic and uncompromising in his scientific integrity and will be remembered with fondness by the many people whose lives he touched.

General Introduction

The objective of this chapter is to present the ways bacteria are effectively harnessed as biocatalysts to perform the synthesis of bulk organic acids and solvents. Prior to the development of the petroleum-based chemical industry, microbial fermentations of agricultural biomass were a major source of a number of useful bulk organic chemicals. Commercial chemical production often emerged from a much earlier food processing technology where grains, corns, milks and fruits were fermented to wines, beers, cheeses and vinegars. Beginning at the end of the 19th century and continuing to the present, specific bacterial strains were selected from nature to produce commercially needed bulk chemicals such as lactic acid, acetic acid, acetone and butanol and more recently gluconic acid and polyhydroxyalkanoates. With the advent of genetic engineering, bacterial strains are being altered for production of propanediols, butanediol and succinic acid at higher yields and productivity than are possible using natural strains.

Table 1 lists a selected number of organic acids and solvents (covered in detail in this chapter) produced by prokaryotes. The topical products are selected based on the following properties of the production process that are either well-known or under development:

1) Bacteria-catalyzed processes convert cheap biomass into the desired products at high yield and low cost.
2) Downstream separation and purification technologies have been applied successfully to the process.
3) The product, as a commodity chemical, has diverse applications and the promise of a strong future market.

Each section includes the following information about the product:

1) introduction and history of the process;
2) scientific background such as microbiological principles, physiology, biochemistry, genetics, and product chemistry;
3) commercial fermentation and bioprocess technologies, economics, and competitive processes;
4) research and development, such as approaches to strain improvement and new process technologies;
5) patent and regulatory issues;
6) prospects for the process; and
7) a reference list.

In today's world, the biotechnology industry, which includes the bacterial production of compounds listed in Table 1, accounts for a mere US $30 billion in chemical compounds produced by fermentation or other bioconversion processes. The total chemical market is greater than US $1,200 billion (Wilke, 1995), which includes all organic chemicals mostly produced from petroleum, natural gas, and coal feedstocks. Indeed, most of the organic acids and solvents listed in Table 1 are also synthesized today by chemical processes from these nonrenewable feedstocks. An exception is the polyhydroxyalkanoates, produced only by bacteria. However, cheap, petroleum-derived plastic polymers dominate the present-day market where the polyalkanoate products are designed to compete.

With this situation in mind, what are the needs for and advantages of developing processes for

Table 1. The major organic acids and solvents produced by prokaryotes.

Chapter section:	Products(s)
1.	Acetic acid
2.	Lactic acid
3.	Gluconic acid
4.	Succinic acid
5.	Polyhydroxyalkanoic acids
6.	Propionic and butyric acids
7.	Ethanol
8.	Acetone/isopropanol/butanol
9.	1,2- and 1–3-Propanediol
10.	2,3-Butanediol

producing fermentation-derived bulk organic acids and solvents? As the cost of nonrenewable feedstocks increases, the need for production of these high-volume organic chemicals from renewable alternative feedstocks is growing. For example, acetic acid, acetone and isopropanol are on the list of the top 50 organic chemicals produced worldwide (Wilke, 1995), and today they are largely products of chemical synthesis. However, there are active research efforts to develop new technologies to reduce costs in fermentation, bioconversion, and downstream processing—subjects to be covered in the following sections of this chapter. For producing essential organic acids and solvents, these efforts have the potential of adding a product stream based on renewable resources to existing chemical synthesis routes. In this chapter, the best example of a bulk process with this potential is the production of bioethanol from agricultural biomass and even municipal wastes. At present, however, the role of bacterial systems in bioethanol production is relatively minor.

Biosynthetic routes often have product-specific advantages over chemical synthesis, which are important for extending and adding value to some bulk products listed in Table 1. For example, optically active compounds, such as lactic acid, can be produced as either L- or D-lactic acid employing specific species of lactobacilli as biocatalysts, whereas chemical synthesis produces a racemic mixture of D, L-lactic acid. Specific properties of polylactide polymers and chemical derivatives of lactic acid differ importantly depending upon the chirality of the monomer (stereospecificity).

Biodegradability of final products such as plastic bottles and films has become an important environmental concern. Where biodegradability is a desirable property, polyhydroxyalkanoate- and polylactide-based plastics produced from bacterial processes are under development for replacement of poorly biodegradable polyvinyl, polyethylene, and other petroleum-based plastics.

As the world population increases beyond the 6-billion mark, urban, food processing and agricultural wastes become an overwhelming problem. However, microbiologists and bioprocess engineers have developed processes for utilizing wastes as alternative feedstocks. An example is the production of the deicer Ca-Mg-acetate from gasified municipal solid wastes or from waste cheese whey by bacterial fermentation covered in section 1 of this chapter. A second example is the planned facility for conversion of waste bagasse from sugar cane refineries into millions of gallons of ethanol by bacterial fermentation (McCoy, 1998). Thus, the future of bioethanol production from waste biomass may well depend upon development of bacterial fermentations.

Using genetically altered bacteria, chemical companies are developing single-step fermentation/conversion processes for carrying out multistep synthesis of bulk solvents or organic acids. Bacteria-catalyzed conversions of glucose into 1,2- or 1,3-propanediol are examples of processes presently in the experimental stage, which may be developed commercially in the future (Alpers, 1999).

Research into the bacterial production of 2,3-butanediol (summarized in a section of this chapter) also has been considerable. The future prospects of large-scale fermentative production of 2,3-butanediol as a fuel or as a major chemical feedstock will depend on the need to substitute for present petroleum-based products such as 1,3-butadiene and methyl ethyl ketone (Maddox, 1996).

Although the emphasis of this chapter is upon the development of bacteria-based fermentations to produce organic acids and solvents, we have pointed out the parallel role of fungal-based processes where relevant in some sections. For example, the yeast fermentation is of overwhelming importance in bioethanol production. Yet, the bacterial fermentation route has been developed to play important roles in the future utilization of major carbon sources. On the other hand, the lactic acid fermentation is dominated by bacterial processes, and yet a major commercial project employs a fungal fermentation (see Lactic Acid in this Chapter). Industrial production of gluconates and gluconic acid has been based on *Aspergillus niger* fermentations in the past. Yet much research and development predicts that fermentations using *Acetobacter methanolicus* and *Zymomonas mobilis* may become dominant in the future.

In the following sections, we try not only to indicate the major bacterial systems developed for the production of the specific organic acids or solvents but also to give a realistic assessment of the relative impact of competing processes.

Section 1: Acetic Acid

Introduction

Until the late 19th century, all acetic acid was derived from the classical process of sugar fermentation to ethyl alcohol followed by a second-stage microbial oxidation to acetic acid. The dilute solution of acetic acid produced by microbial metabolism is called "vinegar." Wine-souring leading to a variety of vinegars has been known as long as the practice of wine making, probably before 6000 BCE (Nickol, 1979). Early Babylonian writings (4000 BCE) report in great detail the production of vinegar and wine from date palms. Vinegar was used early in Babylonian, Greek and Roman culture for food flavoring, food preservation, pickling, for medicinal purposes such as wound healing, and as a popular drink for soldiers and peasants (Swings, 1992). The New Testament reports that Roman soldiers offered Jesus vinegar to drink during the crucifixion.

A number of natural sugar-containing juices or mashes that are first converted by fermentation to alcoholic solutions can serve as raw materials for vinegar production. The vinegar is named after the original raw material, such as wine vinegar, peach vinegar, apple cider vinegar, rice vinegar, malt vinegar, etc. Until about the early 1950s, most of the world supply of acetic acid was manufactured utilizing the microbial oxidation of raw alcoholic mixtures by a vinegar production process (Ghose and Bhadra, 1985). Perhaps the earliest vinegar manufacturing method was the "slow process," or "Orleans process," employed in the Orleans region of France even before the 17th century (Allgeier and Hildebrandt, 1960). Wooden casks were partly filled with a "good vinegar" as a source of inoculum followed by weekly additions of fresh wine. After five weeks some of the vinegar was replaced by more fresh wine. A thick bacterial mat develops on the surface, known as the "mother of vinegar." Air was supplied through holes in the cask above the surface of the mat. Thus a slow, continuous vinegar process was developed.

Vinegar was produced by the Orleans process or French method long before the discovery of the microbial basis for the oxidation of ethanol to acetic acid, and for that matter, the fermentation of sugars to alcohol. In 1732, Berman Boerhaave first suggested the biological nature of the "mother of vinegar;" then Persoon, in 1822, reported evidence of bacteria in this surface mat and named it "Mycoderma" (Ghose and Bhadra, 1985). Kutzing (1837) found that small microscopic organisms in the mother inoculum were necessary for the conversion of alcohol to acetic acid. Pasteur (1868) discovered that the cause of deterioration of wines was due to bacterial contamination during and after the yeast fermentation process, resulting in conversion of alcohol to acetic acid. Almost simultaneously, he made the observation that during vinegar manufacture similar bacteria, which he called "*Mycoderma aceti*," were responsible for production of the acetic acid by oxidation of ethanol. Subsequently, Pasteur showed that partial sterilization or "Pasteurization" of wine at 55°C prevented the spoilage process, and his discovery made him a hero of the French wine industry (Dubos, 1988).

The "quick process" or "German process" was invented in the early 1800s. Wine is added at the top of and allowed to trickle through a generator that consists of a tank packed with beechwood shavings. Bacterial cells grow attached to the beechwood shavings and catalyze the conversion of alcohol to acetic acid. Air is blown through holes from the bottom of the tank. In 1929 the "Frings process" was introduced as a major improvement of the quick process (Fig. 1). The Frings generator included both forced aerations and temperature control features. This trickle bed process yielded 98% conversion of a 12% ethanol solution to acetic acid in 5 days. It was also low cost and easy to control.

Hromatke and Ebner introduced the submerged reactor with an aerator fixed at the bottom of the reactor for oxidation of ethanol to acetic acid by *Acetobacter* species (Hromatke and Ebner, 1949). Today the Frings Acetator® is the most common reactor for the production of all types of vinegar by the submerged fermentation method (Ebner et al., 1996). The submerged cultivation reactor (Fig. 2) has the following advantages over the trickling bed reactor for commercial production:

1) alcohol oxidation to acetic acid is 30 times faster;
2) a smaller total reactor volume is required to achieve a given level of productivity;
3) between 5–8% higher yields are obtained; and
4) the process is easily automated. It was discovered that Acetobacter is very sensitive to short periods of low O_2 levels in submerged-culture conditions. Therefore, the success of this process depends upon continuous high rates of aeration of the broth (Agreda and Zoeller, 1993).

Most production of acetic acid from dilute sugar solutions is carried out by the two-step vinegar process, using the submerged reactor technology; however, during this decade, a one-step microbial fermentation of sugar to acetic acid production has been under development (Cheryan et al., 1997). This fermentation is carried out by acetogenic *Clostridium* species and has two major advantages over the two-step vin-

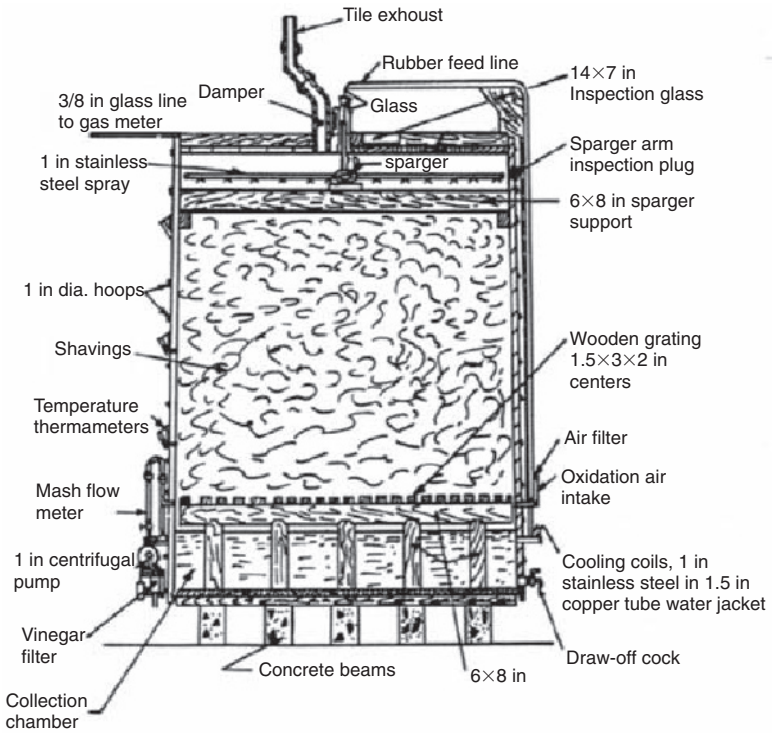

Fig. 1. The Frings generator. A typical reactor used in the old method of vinegar production by the "quick process" or the "German process." From Ghose and Bhadra (1985), with permission.

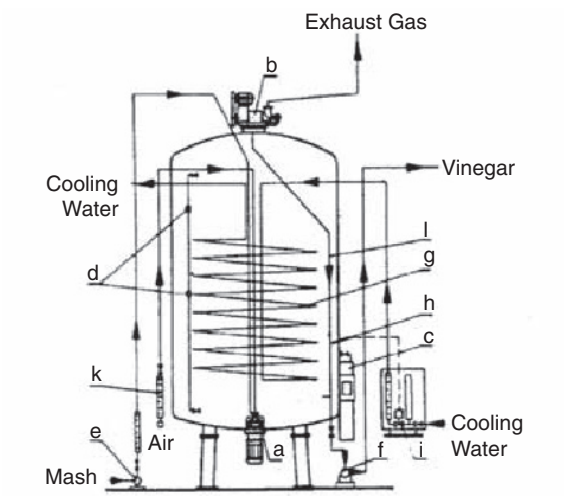

Fig. 2. Schematic drawing of the Frings Acetator: a., aerator; b., defoamer; c., alkograph; d., level control switch; e., mash pump; f., vinegar pump; g., cooler; h., thermometer for temperature control; i., cooling water valve; k., airflow meter; l., return pipe. From Ebner and Follman (1983), with permission.

egar process. First, the fermentation is anaerobic, which saves energy required for aeration during the process; secondly, the single bacterium converts sugar directly and almost stoichiometrically to acetic acid, with a theoretical yield of almost 100%, which reduces raw material costs. This bioprocess, when developed, may become competitive with chemically produced acetic acid for specific uses, such as calcium magnesium acetate for deicing roadways (Cheryan et al., 1997).

A third biological source of acetic acid is the distillation of wood by a process that was developed in the late 19th century (Agreda and Zoeller, 1993). It probably provided an additional source of acetic acid up to the early 20th century.

The first commercial plant for the chemical production of acetic acid came on line about 1916. Clearly this was the beginning of the expanding market for acetic acid as an important commodity chemical in industry (Agreda and Zoeller, 1993). Chemical synthesis of acetic acid is dependent upon petrochemicals from nonrenewable crude oil resources. There are three major processes in use today: oxidation of acetylene-derived acetaldehyde; catalytic butane oxidation; and the carbonylation of methanol (the Monsanto process; Agreda and Zoeller, 1993). Production by the Monsanto process provides the major source of glacial acetic acid used in industry worldwide. In the United States, chemical synthesis of acetic acid was reported as 2.34×10^6 tons/yr in 1995 (Kirschner, 1996), which demonstrates the importance of acetic acid as a commodity chemical in industry.

Scientific Background

THE AEROBIC PROCESS A two-step microbial process yields a dilute 5–12% solution of acetic

acid (vinegar) from a crude carbohydrate-containing mash. The first step is the production of ethanol from sugars by an anaerobic fermentation, usually by the yeast *Saccharomyces cerevisiae* or alternatively by a bacterium such as *Zymomonas mobilis*:

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2CH_3CH_2OH \quad (1)$$

The second step is the oxidative conversion of ethanol to acetic acid carried out by an *Acetobacter* strain. These bacteria catalyze an interesting incomplete oxidation where characteristically their capacity to oxidize ethanol and lactate far exceeds the oxidation of acetic acid to CO_2. During the incomplete oxidation of ethanol, the reducing equivalents are transferred to oxygen:

$$2CH_3CH_2OH + 2O_2 \rightarrow 2CH_3COOH + 2H_2O \quad (2)$$

Surprisingly, the microbiology of the modern vinegar fermentation is not entirely worked out as to the exact taxonomy of the participating *Acetobacter* species. It is still state of the art to start a new fermentation process with a "seed vinegar" which is a microbiologically undefined sample drawn from a running Frings-acetator (Ebner et al., 1996). For example, ten strains of *Acetobacter* were isolated from a series of running industrial acetators in southern Germany. Using 16S rRNA oligonucleotide probes to hybridize with DNA from these strains, four strains were identified as members of the *Acetobacter europeus/xylineum* species; six strains could not be identified as to species (Sokollek et al., 1998).

Table 2 lists some of the identified species of *Acetobacter* that have been isolated from various vinegar production processes. Phenotypic differentiation of *Acetobacter* from the closely related genera *Gluconobacter* and *Frateuria* is presented by Swings (1992).

But regardless of how these *Acetobacter* strains are classified, the vinegar producers' first interest is to use a strain of bacteria that produces a high concentration of acetic acid (12–14%). Also the strain chosen must tolerate a high acetic acid concentration; it should require small amounts of nutrients; it should not overoxidize the acetic acid formed; and it should maintain a high productivity (2.5–4% every 24 h; Ebner and Follmann, 1983). There are two reasons for the lack of defined pure starter cultures of preserved strains that embody the desired qualities for commercial acetic acid production. First, the successful preservation and cultivation of isolated production strains has been described only recently (reviewed by Sokollek and Hammes, 1997). Secondly, high frequencies of spontaneous mutations in the acetic acid bacteria cause deficiencies in alcohol oxidation, lowered resistance to acetic acid and loss of other important physiological properties of production strains (Beppu, 1993). IS elements have been identified as being the cause for some of these mutational events in *Acetobacter* species (Iversen et al., 1994; Takemura et al., 1991; Kondo and Horinouchi, 1997). To avoid the possible selection of mutants in preparation of starter cultures, a suggested procedure has been developed (Sokollek et al., 1998). Basically, the pure cultures are isolated on agar plates. A colony is selected, grown and tested in a pilot acetator to verify high productivity, maximum yield and tolerance to acetic acid. An aliquot of the test acetator is prepared and frozen or lyophilized as a starter culture (Sokollek and Hammes, 1997). After recovery, these cultures were found to be reliable inocula for starting fermentation in pilot acetators.

Table 2. Acetobacter used in vinegar production.

Organism	Properties	References
A. aceti ATCC 15973, 23746 IFO 3284	Vinegar production Japan	Sievers et al., 1994a
A. pasteurianus LMG 1635 (formerly *A. peroxydaus*) IFO 3188 and strains	Vinegar production Japan	Sievers et al., 1994a
A. europaeus DSMZ strains LTH strains	Industrial acetators Germany	Sievers et al., 1992
A. lovaniensis Strains SKU 1108, 1112	Growth and acetic acid produced at 37–40°C	Saeki et al., 1997

Abbreviations: LMG, Laboratory of Microbiology, University of Ghent, Belgium; DSMZ, Deutche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany; IFO, Institute for Fermentation, Osaka, Japan; LTH, Inst. fur Lebensmitteltechnologie, Universitat Hobenheim, Stuttgart, Germany; and SKU, Faculty of Science, Kasetsart University, Bangkok, Thailand.

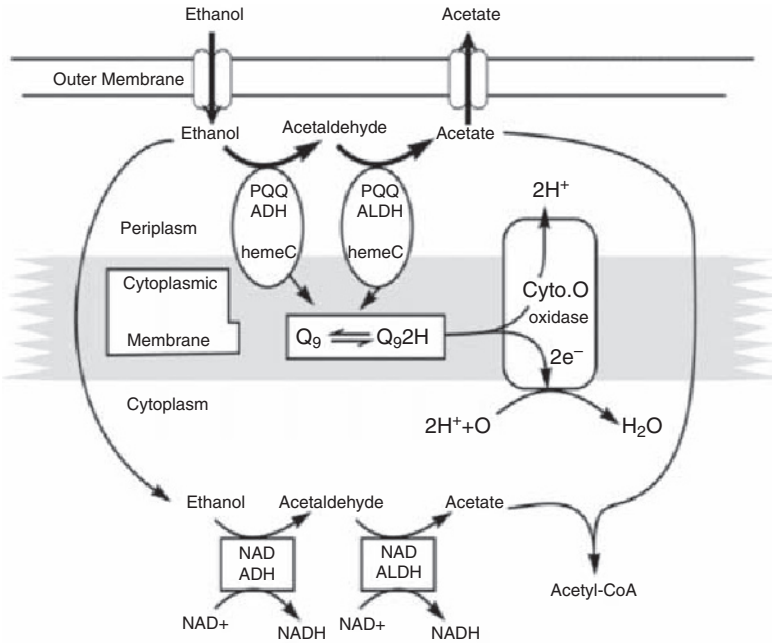

Fig. 3. Alcohol oxidizing systems in *Acetobacter* sp. See text for descriptions. Redrawn from Matsushita et al. (1994), with permission.

Oxygen deprivation during acetic acid production by *Acetobacter* in a submerged-culture reactor causes rapid loss in productivity. Muraoka et al. (1982) showed that when a submerged reactor with *Acetobacter aceti* reached 6% acetic acid, blocking aeration for 10 s completely inhibited further acid production. Loss of production was correlated with observed damage to bacterial cells shown by morphologic changes such as irregularly shaped cells. Yet, at less than 4% acetic acid in the reactor, oxygen limitation for 12 min had little effect on cell appearance or on acid production when aeration was started again. Thus, the sensitivity to oxygen depletion is related to sensitivity of the *Acetobacter* cells to accumulated acetic acid. Further, when all ethanol is used up in an active running acetic acid reactor, the acetic acid bacteria will be irreversibly damaged if addition of fresh ethanol is delayed (Ebner and Follmann, 1983). Mesa et al. (1996) modeled the death rate of *A. aceti* due to lack of O_2 in submerged culture as a function of acetic acid concentration. Also they included data showing the protective effect of increasing concentrations of ethanol.

Apparently, Acetobacter is protected from the toxic effects of high concentration of acetic acid by its own ethanol-oxidizing system, through maintenance of pH homeostasis. As the acetic acid concentration increases, the pH outside approaches 2–4, whereas the pH inside the cells must be maintained at pH 6.5–7. Thus for survival a ΔpH of 4–2.5 must be maintained very much as for other acidophiles (White, 1995).

The biochemistry and proposed bioenergetics of the alcohol-oxidizing systems together with the alcohol-oxidizing respiratory chains have been elucidated (Matsushita et al., 1994).

The acetic acid bacteria produce acetic acid from ethanol by two enzyme-catalyzed reactions of membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). In addition, some acetic acid bacteria can oxidize various sugars and sugar alcohols. These reactions are all "incomplete oxidations" because the oxidation products are usually accumulated in large amounts in the bacterial beers. Both ADH and ALDH have pyrroloquinoline quinone (PQQ) bound as a prosthetic group and are linked to the respiratory chain in the cytoplasmic membrane (Fig. 3). A second set of NAD(P)$^+$-dependent ADHs and ALDHs is found in the cytoplasm of acetic acid bacteria. These latter two enzymes have much lower specific activities than those of the PQQ-enzymes and are not involved in acetic acid production (see Fig. 3).

The quinoprotein dehydrogenases, ADH-PQQ and ALDH-PQQ, are tightly bound to the periplasmic side of the cytoplasmic membrane. Electrons from alcohol and aldehyde are donated to ubiquinone (Q_9) embedded in the membrane. The ubiquinol oxidase, either cytochrome O (Cyto.O) or cytochrome *a*, transfers electrons from Q_9 and generates an electrochemical proton gradient by reduction of oxygen with $2H^+$ or by pumping H^+ into the periplasmic space (Fig. 3). The major role of this alcohol respiratory system is to maintain homeostasis by consuming cytoplasmic protons and thus balancing the DpH. This permits the *Acetobacter* to grow slowly and survive on ethanol and acetic acid in the presence of high acetic acid concentrations.

The observed low biomass yield compared to the high rate of O_2 uptake is supported by experiments with ^{14}C-ethanol utilization by *A. rancens* (Mori and Terui, 1972). The O_2 uptake and ethanol oxidation were in agreement; but the growth yield was only 10% of that expected compared to other bacteria. Biomass yield was studied with *A. pasteurianus* using an ethanol-limited chemostat with about the same result (Luttik et al., 1997). These workers also measured proton translocation coupled to O_2 uptake for acetate oxidation by the same ethanol-limited cells and found that a very low proton translocation stoichiometry might explain the low biomass yield. Apparently production strains of *Acetobacter* are selected for very low rates of acetate oxidation. But how acetate oxidation is regulated is still incompletely understood (Saeki et al., 1997a).

The Anaerobic Process During studies with the anaerobic acetogenic clostridia, it was recognized that these bacteria can ferment sugars such as glucose, fructose and xylose almost exclusively to acetic acid by the following reactions:

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (3)$$

or

$$2C_5H_{10}O_5 \rightarrow 5CH_3COOH \quad (4)$$

In 1940, Wieringa described *Clostridium aceticum*, the first acetogenic anaerobic spore-forming bacterium (Adamse, 1980). It was discovered to grow on hydrogen and carbon dioxide, forming acetic acid (Braun et al., 1981), according to the following equation:

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$$
$$\Delta G^\circ = -107\,kJ \quad (5)$$

These organisms have been called "homoacetogens" because on most substrates they produce very little if any organic product, other than acetic acid. Alternatively, Drake (1994) prefers the name "acetogens," defined as "obligately anaerobic bacteria that can use the acetyl-CoA pathway as: 1) their predominant mechanism for the reductive synthesis of acetyl-CoA from CO_2, 2) their major terminal electron-accepting, energy-conserving process, and 3) their mechanism for the synthesis of cell carbon from CO_2." Acetogen (as defined above) distinguishes a process by which acetyl-CoA rather than acetate is formed as the final product. Also these bacteria often can produce products other than acetate under certain conditions using alternate pathways (Misoph and Drake, 1996). Acetogen (as defined above) also distinguishes these bacteria from the ethanol oxidizers, *Acetobacter* sp. In 1986, the acetogens were represented by 14 different species within five genera, three of which are thermophilic (Ljungdahl, 1986). By 1994, there were 40 species classified and 19 unclassified isolates reported (Drake, 1994). A selected list is presented in Table 3.

In addition to growth on H_2 and CO_2, some acetogens also grow on other one-carbon compounds, such as formate, carbon monoxide and methanol (equations 6–10):

Table 3. Characteristics of some acetogens.

Organism	Optimal growth temperature (°C)	Growth substrates Organic	One-carbon	References
Clostridium aceticum	30	Fructose, pyruvate, ethanol and malate	CO_2/H_2, HCOOH	Adamse, 1980
C. formicoaceticum	37	Fructose, gluconate, galacturonate, glucuronate, lactate and glycerol	CH_3OH/CO_2	Andreesen et al., 1970
C. thermoaceticum	60	Glucose, fructose, xylose and pyruvate	CO_2/H_2, HCOOH, CO, CH_3OH/CO_2	Fontaine et al., 1942
C. thermoautotrophicum	60	Fructose, glucose, glycerate and galactose	CO_2/H_2, HCOOH, CO, CH_3OH/CO_2	Wiegel et al., 1981
Acetobacterium woodii	30	Glucose, fructose, pyruvate, glycerol, and 1,2 propanediol	CO_2/H_2, HCOOH, CO, CH_3OH/CO_2	Balch et al., 1977
Acetogenium kivui	66	Glucose, fructose and pyruvate	CO_2/H_2, HCOOH	Leigh et al., 1981
Sporomusa sphaeroides	34	Ethanol, butanol, and *N*-methyl compounds	CO_2/H_2, HCOOH, CH_3OH/CO_2	Möller et al., 1984
Peptostreptococcus productus	37	Glucose, fructose, xylose, pyruvate and glycerol	CO_2/H_2, CO	Lorowitz and Bryant, 1984

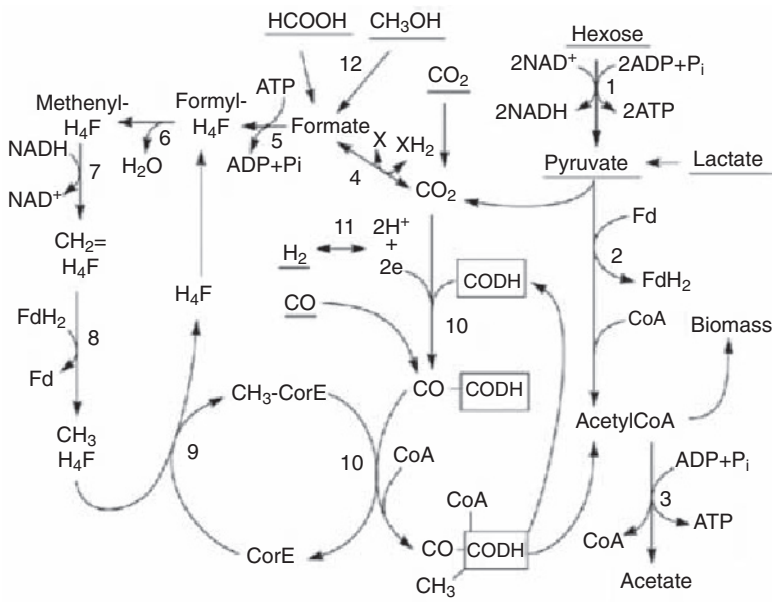

Fig. 4. Biochemical pathway used by acetogens for fermantation of sugars and utilization of one-carbon precursors for biomass and acetate production: 1, hexose oxidation to pyruvate by the Embden-Meyerhof pathway; 2, pyruvate-ferredoxin oxidoreductase; 3, phosphotransacetylase and acetate kinase; 4, formate dehydrogenase; 5, formate tetrahydrofolate synthetase; 6, methenyl tetrahydrofolate cyclohydrolase; 7, methylenetetrahydrofolate dehydrogenase; 8, methylenetetrahydrofolate reductase; 9, methyl transferase; 10, carbon monoxide dehydrogenase (CODH); 11, hydrogenase(s); 12, methanol dehydrogenase and formaldehyde dehydrogenase; Cor-E, corrinoid protein; and H_4F, tetrahydrofolate. Underlined compounds are substrates used by various acetogens. The Wood/Ljungdahl Pathway consists of reactions 4 through 10.

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (6)$$

$$4HCOOH \rightarrow CH_3COOH + 2CO_2 + 2H_2O \quad (7)$$

$$4CH_3OH + 2CO_2 \rightarrow 3CH_3COOH + 2H_2O \quad (8)$$

$$2CO + 2H_2 \rightarrow CH_3COOH \quad (9)$$

$$2HCOOH + 2H_2 \rightarrow CH_3COOH + 2H_2O \quad (10)$$

Thus, most of this versatile group of anaerobic organisms can grow on one-carbon compounds as both a sole carbon and energy source (equations 6–8) or by utilizing energy from the oxidation of H_2 (equations 5, 9 and 10). Acetic acid is the primary product during growth on selected sugars, alcohols, organic acids or one-carbon compounds.

It is immediately obvious that the fermentation carried out by this group of bacteria (Table 3) has three advantages over the aerobic process for acetic acid production using *Acetobacter*. First, this is an anaerobic process, so the energy and equipment costs for providing large amounts of oxygen during the fermentation are unnecessary. Second, the theoretical yield of the anaerobic process is 3 moles of acetic acid per mole of glucose used, or 100%. The theoretical yield of the aerobic process is only 67% acetic acid per g of glucose because one-third of the carbon is lost as CO_2 (see equation 2). Third, the ability of some of these bacteria to utilize CO_2 and other one-carbon precursors for growth and acetic acid production permits their use in biological conversion of synthesis gases produced from waste biomass to acetic acid. However, Busche (1991) concluded that the higher acetate concentration tolerated by *Acetobacter suboxydans* used in the aerobic process might outcompete the advantage of the one-step process with the high yield of three acetates per glucose by the anaerobic acetogens.

The biochemical pathway that permits the fermentation of glucose to three moles of acetate by these bacteria includes the enzyme-catalyzed reactions also essential for the fixation of CO_2 and other one-carbon precursors into cell material. But acetogens do not use the Calvin cycle for CO_2 fixation because the enzyme ribulosediphosphate carboxylase is not present in their cytoplasm. Rather, acetyl CoA is the first intermediate formed during CO_2 or CO fixation by homoacetogenic bacteria. The pathway (now established for both the acetate fermentation and growth on CO_2/H_2) is a combination of the Embden-Meyerhof pathway and the "Wood/Ljungdahl pathway" diagrammed in Fig. 4 (Ljungdahl, 1986; Drake, 1994).

Fermentation of hexose yields two pyruvates, and pyruvate is further oxidized to 2 acetyl-CoA and 2 CO_2 by the enzyme pyruvate:ferredoxin oxidoreductase. Then the 2 acetyl-CoAs are converted to 2 acetates. The 4 moles ATP per hexose produced are used for further metabolism. The oxidation-reduction balance is achieved by reduction of both CO_2 molecules to a third acetyl CoA and finally to acetate by the Wood/Ljungdahl pathway. The NADH and reduced ferredoxin generated from the oxidized forms during fermentation of hexose are the source of reducing power. One CO_2 is reduced to 5-methyltetrahydrofolate. The first reaction in this pathway (reaction 4, Fig. 4) is the formation of formate catalyzed by an unusual tungsten-

selenoprotein, formate dehydrogenase (Yamamoto et al., 1983). For *C. thermoaceticum*, the electron donor is NADPH. The methyl group of 5-methyltetrahydrofolate is transferred through a corrinoid-protein intermediate (CH_3-CorE) to carbon monoxide dehydrogenase (CODH) for the final condensation reaction with coenzyme A and CO to form acetyl CoA (reactions 9 and 10, Fig. 4). Also, CODH has been purified and characterized from a number of anaerobic bacteria such as *A. woodi* and *C. thermoaceticum* (Ragsdale, 1994). The enzyme from acetogens contains 6 Ni, 3 Zn and a number of FeS centers. Thus, the primary functions of the CODH of acetogenic bacteria are the reduction of CO_2 to a nickel-bound CO and the synthesis of acetyl CoA. Because the former function is the first step in the synthesis function, it has been suggested that the name of the enzyme should be "methyl-CO-CoA condensing enzyme" or just "acetyl CoA synthase" rather than CODH (Ljungdahl, 1986).

During autotrophic growth with CO_2/H_2 (equation 5) or with reduced one-carbon compounds such as CO, HCOOH, or CH_3OH (equations 6–8), energy must be derived from H_2 via hydrogenase or alternatively by an enzymatic transfer of electrons from the one-carbon compounds to electron transport proteins. *Clostridium thermoaceticum* contains membrane-bound cytochrome *b*, menaquinone, as well as two rubredoxins and two ferredoxins. *Clostridium thermoaceticum* also has an H^+-translocating ATPase, indicating that these anaerobes must utilize a proton-electrochemical gradient ($D\mu_{H^+}$) created by an electron transport system to produce ATP (Ljungdahl, 1994). This mechanism is essential for ATP synthesis during autotrophic growth inasmuch as the only other source of ATP is acetyl CoA to acetate.

Some acetogens, such as *A. woodi*, develop a sodium-electrochemical gradient ($D\mu_{Na^+}$) coupled to acetogenesis from growth with $H_2 + CO_2$ rather than a $D\mu_{H^+}$ formed by *C. thermoaceticum* (Müller and Gottschalk, 1994). Experiments with inverted vesicles suggested that the sodium-pump is a function of one of the membrane-bound enzymes, methylene-H_4F reductase or the methlytransferase (see Fig. 4, reactions 8 and 9). That ATP synthesis is driven by a membrane-bound, Na^+-translocating ATP synthase was demonstrated in *A. woodii*. Sodium ions also play a major role in the acetogens *A. kivuii* and *P. productus* during acetogenesis with H_2 and CO_2. Homoacetogens growing with sugars can produce adequate ATP from their conversion to pyruvate and then to acetate through acetyl CoA (Fig. 4, reactions 1 and 3). However, the observation of high growth yields of some homoacetogens when fermenting sugars together with the observed formation of some hydrogen gas suggests that an electron transport-induced proton-gradient may produce additional ATP under fermentation conditions as well.

When acetogens grow on one-carbon compounds using the Wood/Ljungdahl pathway, all cellular carbon compounds must be produced from acetyl CoA (Fig. 4). The incorporation of acetyl CoA carbon into lipids, amino acids, nucleotides, carbohydrates and thus all biopolymers has been demonstrated for *C. thermoaceticum* and *A. woodii* (Eden and Fuchs, 1982; Eden and Fuchs, 1983). Similar pathways are found for other strict anaerobes such as the sulfur-reducing bacteria growing on acetate and the methanogenic Archaea growing on CO_2 and H_2 or on acetate (Thauer, 1989).

The maintenance of pH homeostasis by acetogens during accumulation of acetic acid in the medium has not been carefully studied. The undissociated acetic acid in the medium freely diffuses into the cells where it dissociates into acetate and H^+ dependent on the cytoplasmic pH (~pH 6.0). When the H^+ concentration inside the cell is greater than or equal to the H^+ concentration outside in the medium, then the cell membrane potential is neutralized or reversed. How the acetogen cells deal with this situation is unknown; however, they are tolerant to only about 2.0–3.0 g of acetic acid per liter during batch fermentation. Experiments demonstrate that continuous control of the pH (6.6–6.9) during batch or fed-batch fermentation increases the yield of acetate to 46–56 g per liter (reviewed by Wiegel, 1994).

The production of acetic acid by acetogens is coupled to growth of their biomass. When growth is rapid, production of acetic acid increases, and when growth is limited or slowed, the production of acetic acid decreases. The nutritional requirements for maximum acetate yield and productivity by *C. thermoaceticum* growing on glucose are reviewed by Cheryan et al. (1997). The optimum concentrations of the metal iron, cobalt, molybdenum, selenite, zinc and nickel have been determined for maximum growth and acetate production by this bacterium. Cheap sources for the organic nitrogen, which are required for growth, have been studied for low-cost media development.

The availability of acetogens that are thermophilic (Table 3) certainly adds versatility when considering an organism for a production process (Wiegel, 1994). Costs of cooling a large volume of medium following sterilization and dissipation of heat generated during mixing and stirring, as well as the risk of contamination by foreign bacteria, are much reduced when operating a commercial-level fermentation system at the higher temperatures of 60–70°C. Thus, the thermophilic species, *C. thermoaceticum*, *C. ther-*

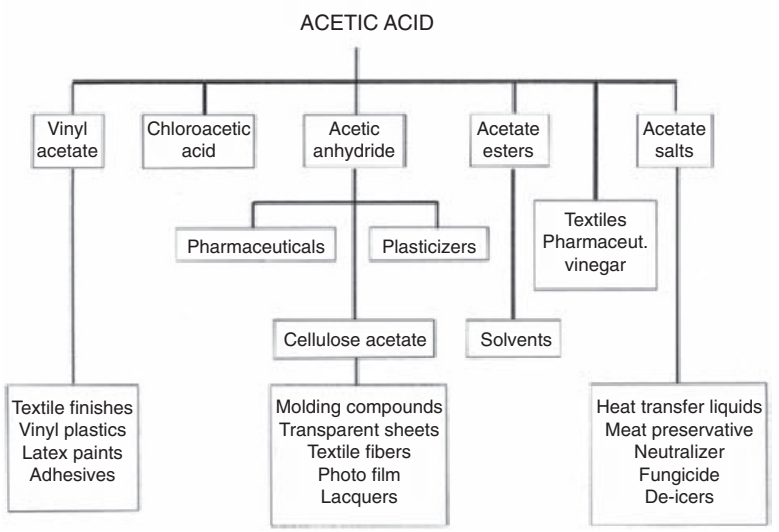

Fig. 5. Uses of acetic acid. Major commodity chemicals derived directly from acetic acid and commercial products in today's market.

moautotrophicum and *Acetogenicum kivii*, are attractive candidates for new process development.

Commercial Applications

INDUSTRIAL ACETIC ACID There are two major areas of commercial application of acetic acid today: food-grade vinegar, which is largely the product of bacterial oxidative conversion of diluted purified alcohol or alcoholic mashes from various fruits and grains, and chemically synthesized industrial acetic acid, 62% of which is produced by carbonylation of methanol and the rest by oxidation of *n*-butane. Chemically synthesized acetic acid is a commodity chemical that has become a major feedstock for the United States and worldwide chemical industry. Figure 5 displays the major chemicals derived from acetic acid and their commercial applications.

Petrochemically produced acetic acid reached a level of 4.68×10^9 lbs/yr in the United States by 1995 and was ranked 35th in abundance of all chemicals produced (Kirschner, 1996). Worldwide production in 1998 was estimated at 11.9×10^9 lbs/yr (Layman, 1998).

Bioderived acetic acid does not compete economically with acetic acid produced by chemical synthesis. This is because glacial acetic acid is the preferred starting material for most synthetic routes leading to other products. The cost of petroleum derived 100% acetic acid by the methanol carbonylation process (Monsanto) is from \$0.15 to \$0.35/lb, whereas the cost of 100% acetic acid from the *Acetobacter* aerobic oxidation of ethanol is estimated at \$0.35 to \$0.45/lb (quoted by Cheryan, 1999). The chemical processes have the advantages of initially high acetic acid concentrations (35–45%) and high production rates. The disadvantages are high cost of catalysts and dependence on nonrenewable petroleum for raw materials.

Industrial acetic acid could be synthesized by either of two microbial processes: 1) from sugars in the two-step vinegar process via ethanol using *Acetobacter* species, or 2) from sugars or syngas (CO, H_2, and CO_2) by the anaerobic fermentation process using *Clostridium thermoaceticum* or related organisms (see "Scientific Background" in this Chapter). These bioprocess routes have the advantage of using a variety of renewable raw materials, which presently are more expensive than petroleum-based resources. The major disadvantage is the cost of recovery of low concentrations of acetic acid (4–12%) from the fermentation beers, which are mostly water. A great deal of research and development effort is underway to improve the technology of product recovery and improve bacterial strains. These experimental efforts and improvements in the two microbial processes and separation processes are reviewed in the next section (see "Areas of Research and Development" in this Chapter).

VINEGAR ACETIC ACID A submerged fermentation is the most common technique used today for the production of all kinds of vinegar. The Frings Acetator® is the best-designed equipment capable of automated operation (Ebner et al., 1996). It is mainly used for production of alcohol vinegar but also can be used for conversion of alcoholic mashes of fruits and grains. By 1993, more than 600 acetator units were in operation worldwide with a total vinegar production of about 135×10^9 liters per yr (Ebner et al., 1996).

Figure 2 is a diagram of the Frings Acetator. The Frings Alkograph® is an automatic detector

that measures the amount of ethanol in the acetator. The Alkograph® automatically activates the vinegar discharge pump when the level of ethanol approaches zero. A level switch in the acetator stops this pump and starts the mash pump, which refills the fermenter slowly with fresh mash back to the original volume (Ebner and Follmann, 1983). New biosensors and modern membranes have permitted development of devices, such as the Frings Alkosens®, that measure alcohol levels without time delay (Ebner et al., 1996).

To produce vinegar with from 12–15% acetic acid in the acetator, Ebner (1985) developed a semicontinuous process. Each fermentation cycle (about 24–40 hours depending on the conditions) starts with 7–10% acetic acid and about 5% ethanol. When the alcohol concentration drops to between 0.05 and 0.3%, a volume of the fermentation beer is rapidly discharged. The acetator is recharged slowly with a volume of new mash containing 0–2% acetic acid and 12–15% ethanol, until the starting conditions are again reached. Then a new cycle begins again.

Ebner et al. (1996) reported that since 1994 the single-stage process could be modified to produce vinegar up to 19% acetic acid. Also a two-stage process has been developed to produce vinegar with 18.5% acetic acid (Ebner and Enenkel, 1978). Since 1993, vinegar of more than 20% acetic acid was produced by this process, and an automatic control arrangement for the process has been described (Ebner et al., 1996). The canning industry has a high demand for vinegar with very high acetic acid levels (19–20%). Thus the vinegar industry has developed plants that utilize the two-stage submerged-fermentation process to produce this product (Ebner and Follmann, 1983).

A factory for production of glacial acetic acid using the vinegar process was built in Turkey in 1962. This factory had operated for 20 years before production was discontinued in 1981. It produced 2 tons/day of glacial acetic acid from molasses (Ebner and Follmann, 1983). The process included: alcoholic fermentation of a diluted molasses medium; separation of the purified ethanol; submerged-process vinegar production; extraction of acetic acid from vinegar with ethyl acetate; separation of anhydrous acetate from ethyl acetate by distillation; and recovery of the ethyl acetate. Other similar factories erected in other countries have been shut down. The future of such factories depends upon competing oil prices and further developments of the vinegar process.

The total production volume of vinegar output in the European Union and Japan can be fairly well estimated. However, the United States vinegar production figures have not been published since 1987, and production levels in China and Russia are unknown. Excluding China and Russia, a 1996 estimate of worldwide vinegar of 10% acetic acid is given as about 1.9×10^9 liters per yr or 190,000 tons of pure acetic acid (Ebner et al., 1996).

Research and Development

STRAIN IMPROVEMENT: *ACETOBACTER* Odd as it may seem, the vinegar industry has always worked with acetic acid bacteria that are not properly characterized. Industrial submerged fermentations are normally started by inoculation with an undefined sample from a previous running fermentation called "inoculation vinegar." The lack of defined pure starter cultures is due to problems of isolation, cultivation and preservation of the vinegar bacteria. Entani et al. (1985) developed a double-layer agar technique using an acetic acid-ethanol (AE)-medium that permitted cultivation of the *Acetobacter* species capable of producing 10–15% acetic acid in the commercial acetator. Sievers et al. (1992) and Sokollek and Hammes (1997) have perfected techniques for isolation and cultivation of pure colonies of production strains that now can be preserved and identified. In 1992, Sievers et al. described a new species, *A. europaeus*, claimed to be the major component in industrial vinegar "fermenters" in Central Europe (Sievers et al., 1992). However, using the new isolation technology and hybridization with olignuclotide probes constructed on the basis of the 16S rDNA sequences published by Sievers et al. (1994a), only four of ten strains isolated from running industrial acetators in Southern Germany were identified as *A. europaeus* (Sokollek et al., 1998). The other six acetic acid bacteria (presumably *Acetobacter* species) could not be identified by hybridization with the DNA probes available. Starter cultures prepared from both an identified (*A. europaeus*) and an unidentified *Acetobacter* strain when inoculated into a pilot acetator achieved the acetate production characteristic of the industrial process. Thus, it is now possible to carry out useful experiments incorporating genetic technology to improve industrial strains of *Acetobacter*.

The desirable characteristics of acetic acid bacterial strains useful to the vinegar industry are: tolerance to high concentrations of acetic acid and to "total concentration" (acetic acid + ethanol); requirement for low concentrations of nutrients; absence of acetic acid overoxidation; and high productivity. Genetic techniques have been developed over the past 15 years directed toward strain improvement and understanding of the physiology of these characteristics. Also the perceived problems of strain stability as well

Table 4. Comparison of acetic acid productivity between two transformants of *Acetobacter aceti* NBI2099.

Indicators of production and yield	*A. aceti* NBI2099 (pMV24)[a]	*A. aceti* NBI2099 (pAL25)[a]
Acetic acid productivity[b] (g/liter/h)	1.8	4.0
Specific growth rate[c] (/h)	0.072	0.142
Maximum acetic acid concentration (g/liter)	68.4	96.6

[a]PMV 24 is a plasmid vector developed for *Acetobacter* and pAL25 is the recombinant plasmid which was constructed by inserting a gene encoding the 75-kDa subunit of the ALDH complex of *A. polyoxogenes* into pMN24.
[b]Productivity is 20 g of acetic acid /liter.
[c]Growth rate is 30 g of acetic acid /liter.
From Fukaya et al. (1992), with permission.

as development of bacteriophage resistance are being addressed. Development of genetic systems for DNA transfer and expression in acetic acid bacteria has been reviewed (Beppu, 1993; Fukaya et al., 1992). A great deal of work has resulted in the construction of a number of useful chimeric plasmids composed of *Escherichia coli* vectors and cryptic plasmids from *Acetobacter aceti* or *Gluconobacter suboxidans*. Drug-resistant markers on *E. coli* plasmids, such as resistance to ampicillin, kanamycin, chloramphenicol and tetracycline, are expressed in acetic acid bacteria; some of the chimeric plasmids are stable in both hosts without selective pressure. Genetic transformation of acetic acid bacteria has been demonstrated by conjugal transfer from other species of Gram-negative bacteria carrying hybrid conjugal plasmids. In vitro transformation of *A. aceti* by plasmid DNA has been carried out on cells pretreated with calcium ions or polyethylene glycol. Efficiencies of 10^5 transformants per μg plasmid DNA are reported. Electroporative transformation of *A. xylinum* with plasmid DNA is also successful. Insertional inactivation was achieved by transforming a wild-type strain of *A. aceti* using a chimeric plasmid containing a cloned *leuB* gene with an inserted kanamycin resistance gene within the *leuB* sequence. Transformant cells were produced that were both *leuB*-negative and KmR. This work demonstrated both gene disruption and a method for foreign gene insertion (insertional inactivation) as a result of homologous recombination in the *A. aceti* host (Okumura et al., 1988). A third method, spheroplast fusion, has been developed for *Acetobacter* strains. After mixing of spheroplasts of two different strains, regeneration of cells yields a fusion frequency of about 10–5. The purpose here is to obtain fused cells that express desired properties of both the "mother strains" (Fukaya et al., 1989).

Cloning and sequencing of the two *Acetobacter* genes for the two membrane-bound dehydrogenases (alcohol dehydrogenase [ADH] and aldehyde dehydrogenase [ALDH]) have been reported. Characterization of these genes in a number of *Acetobacter* species and in *Gluconobacter suboxydans* is still under study (Beppu, 1993). In a preliminary report, *A. aceti* showed improved acetic acid production and yield after being transformed with a multicopy plasmid carrying the ALDH gene (Table 4). These results suggest that genetic manipulation may lead to improved production strains.

Research on the causes of spontaneous mutation of strains of *A. aceti* and *A. pasteurianus* is underway to determine the nature of the genetic instability of ethanol oxidation during industrial vinegar production. Genetic analysis has implicated insertion of IS element IS1380 into the ADH gene or into the cytochrome *c* gene as being responsible for some cases of mutation. The direction of research on strain instability and on the genetics of tolerance to acetic acid and ethanol has been reviewed (Beppu, 1993).

Strain Improvement: Acetogens The use of the acetogenic bacteria in the anaerobic process of acetic acid production presents three serious problems. The first major concern is low tolerance to acid. The bacterial species listed in Table 3 have been studied in enough detail to indicate that no growth and little acetate production occur at less than pH 4.7. Research over the past 15 years on adaptation or selection of mutants of homoacetogens that tolerate lower pH values has been only partially successful. For example, one strain (mutant C5-3 of *C. thermoaceticum*) grew in continuous culture and (at pH 5.3–5.8) produced up to 3.5–4.5 g of acetic acid/liter. Most recent research on mutation and strain selection has concentrated on *C. thermoaceticum* (Wiegel, 1994). A promising mutant of *C. thermoaceticum* (ATCC49707 or DSM6867) produced up to 55 g/liter, under controlled pH 6.2–6.8, during fed-batch fermentation of glucose. This strain also tolerated dolime (high-magnesium lime) up to 60 g/liter (Parekh and Cheryan, 1991; Parekh and Cheryan, 1994b). This strain is presently under development for

production of calcium-magnesium acetate (CMA; Cheryan et al., 1997).

The second problem for the acetogenic production process is low productivity. In the batch process, rates of 0.5–0.8 g/liter · h are considered unacceptable (Busche, 1991). Only technological advances in the fermentation process, covered below, can improve low productivity. The third problem is that the rate of acetic acid production by acetogens is closely coupled with the rate of growth. *Clostridium thermoaceticum* under fed-batch fermentation conditions and with higher acetate-producing mutants seems to exhibit some uncoupled acetic acid production. However, higher yields of acetic acid and decreased biomass waste would result with an increase in growth-uncoupled acetic acid production (Weigel, 1994).

PROCESS DEVELOPMENT: *ACETOBACTER* Research on development of the acetic acid production process involves many types of bioreactors. In Table 5, the characteristics of some of these experimental bioreactors and processes are compared to three industrial bioreactors in use for vinegar production. The focus of this research is to develop a process that yields a high production rate with a high exit concentration of acetic acid, while maintaining a long period of stable operation. All of these objectives must be realized if the *Acetobacter* process is to be eventually incorporated into a commercial glacial acetic acid production system that will be competitive with the chemical manufacturing processes. Using the commercial submerged vinegar fermentation with a Frings acetator in a semicontinuous mode, the process output is 120–150 g of acetic acid/liter (Table 5). A 64,000-liter unit can produce more than 6,000 lbs of acetic acid in 24 h and is stable for 360 days. The experimental processes listed in Table 5 have achieved much higher productivities than present commercial processes, but in general sacrifice the high acetic acid output. The cell-recycle continuous bioreactors with hollow fiber filtration modules have achieved the highest productivity in the laboratory setting, producing 5–50 times more acetic acid (g/liter · h) than the Frings acetator. However, the output acetic acid is two to three times more dilute (45.7–60 g/liter), thus placing the greater burden on the final separation processes.

Park et al. (1991) investigated a semicontinuous reactor with ethanol fed to maintain 20–30 g/liter for about 50 h when acetic acid accumulated to about 85–90 g/liter (see Table 5). Oxidation of ethanol slowed to zero at this time and the cell culture was rapidly recycled through a hollow fiber filter. The concentrated cells were then diluted with fresh medium and a new cycle started. It was found that the *Acetobacter* cells lost much viability but the nonviable cells continued to oxidize ethanol during the later cycles. A high acetic acid output of about 85–90 g/liter was maintained through six cycles or for about 300 h.

Table 5. Comparison of bioreactors for oxidation of ethanol to acetic acid by *Acetobacter*.

Bioreactor and process	Acetic acid output (g/liter)	Acetic acid productivity (g/liter · h)	Stable operation (days)	References
Commercial processes				
Frings acetator, submerged, and semicontinuous	120–150	1.6–2.0	360	Ebner et al., 1996
Frings Generator, trickling, woodshavings, and recycle	110	0.16	>50[a]	Ebner and Follman, 1981
Continuous, surface, and pellicle	50	0.6	200	Yasui, 1958
Experimental processes				
Cell-recycle-continuous with hollow fiber filtration	52.4	12.7	11	Park et al., 1989
	45.7[b]	10.7	35	Fukaya et al., 1992
	60.0[c]	25		
Cell-recycle-continuous, fed-batch, hollow fiber filtration	90	2.67	14	Park et al., 1991
Surface, shallow flow, and pellicle	57.6	31.7	34	Toda et al., 1989
Immobilized cells on:				
$TiO_2 \cdot H_2O$	69	5.0	61	Kennedy et al., 1980
Fluid-bed, carrageenan	45	5.0	460	Osuga et al., 1984
Fixed-bed, ceramic	34	4.35	270	Gommidh et al., 1986
Fluid-bed, carrageenan	55	3.43	50	Mori, 1993
Fluid-bed, chitosan beads	33	8.28	52	Mori, 1993

[a]Years.
[b]High cell density (3 g-dry/liter) and dilution rate, 3 h^{-1}.
[c]Cell density (0.8 g-dry/liter) and dilution rate, 0.5 h^{-1}.

Upon scale-up, the engineering of these cell-recycle bioreactors promises to be more costly and more complex to operate than the commercial semicontinuous submerged Frings acetators. The experimental shallow-flow bioreactor is designed to give a continuous horizontal flow of medium under a bacterial film with a large surface area of a few hundred cm^2. The liquid depth is less than 10 mm, resulting in high oxygen absorption and an acetic acid output of 57.6 g/liter (Table 5). This apparatus is technically simple and has been shown to yield very high rates of production of acetic acid (31.7 g/liter · h) without the energy required for forced aeration (Toda et al., 1989). Furthermore, the high productivity is dependent on the high surface-to-volume ratio of the shallow liquid flow and the exit acetic acid concentration (50–66 g/liter) does not change very much over a ten-fold variation in liquid residence time.

There have been a great many reports of experimental bioreactors based on the immobilized cell technology. Some of these are listed in Table 5. Many difficulties have been encountered with immobilized cell systems such as poor oxygen exchange and breakdown of gels and beads with consequent bleeding of attached bacteria. The advantage of these bioreactors is that the productivity is three to four times that of commercial processes. None of these immobilized approaches has gone beyond the small pilot-plant bioreactor stage and much more engineering development is necessary for practical commercial application of this technology (Mori, 1993).

Research has demonstrated efficient production of acetic acid from glucose in a coculture of *Zymomonas mobilis* and *Acetobacter* strains (Kondo and Kondo, 1997). Normally, in the vinegar process, the anaerobic ethanol fermentation is carried out separately from the oxidative conversion of ethanol to acetic acid, which requires highly aerobic conditions. Even though *Z. mobilis* grows and produces ethanol from glucose more rapidly in anaerobic culture and *Acetobacter* spp. grow and produce acetic acid under aerobic conditions, the two organisms in fact can coexist in natural settings such as fermentation of plant juices and beers. Also both of these bacteria are tolerant to low pH and high ethanol concentrations (Swings and deLey, 1977). The culture of two bacteria with 100 g of glucose/liter was initially operated at low aeration and low agitation until the glucose reached 5 g/liter, and then the shift to acetic acid production was started by operating at high aeration. The acetic acid yield from glucose was 0.64 g/g (95.5% of theoretical) and thus 62–70 g of acetic acid/liter was produced in about 60 h in these experiments. Further research and scale-up may yield a coculture method for direct manufacture of acetic acid from inexpensive sources of sugar with acetic acid outputs of 120–150 g/liter, similar to production achieved by the Frings acetator (Table 5).

Process Development: Acetogens Development of processes for production of acetic acid by the anaerobic acetogenic bacteria has focused mainly on fermentation of sugars. The advantage of these bacteria (such as *C. thermoaceticum*) is that they are homofermentative, with almost quantitative conversion of substrate to acetic acid. However, as outlined above (see "Strain Improvement: Acetogens"), they are sensitive both to high sugar concentration and to inhibition by acid conditions, resulting in limited acetic acid output (less than 20 g/liter) in batch culture.

The acid tolerant mutant of *C. thermoaceticum* ATCC49707 (see "Strain Improvement: Acetogens") when grown in batch culture with pH controlled at pH 6.3–6.8 showed a much improved acetic acid output of 30–40 g/liter and a productivity of 0.3 g/liter·h (see Table 6). Experimental fermentations have been con-

Table 6. Comparison of experimental bioreactors for acetate production from sugars by acetogenic *Clostridia*.

Bioreactor and process[a]	Acetic acid output (g/liter)	Acetic acid productivity (g/liter · h)	Stable operation (days)	Reference
Batch with YE	35	0.3		Shah and Cheryan, 1995b
Batch with CSL	30–40	0.19–0.33		Shah and Cheryan, 1995a
Fed-batch with YE	55	0.3		Parekh and Cheryan, 1991
Fed-batch with CSL	39.9	0.2–0.3		Shah and Cheryan, 1995b
Cell-recycle-continuous, hollow fiber filtration	25–45	0.4–1.7	54	Parekh and Cheryan, 1994
Cell-recycle-continuous, 2-stage, membrane filtration	37.5	0.75	17.5	Shah and Cheryan, 1995b
Cell-recycle-continuous, with fed-batch, draw and fill	38	0.88	14.6	Shah and Cheryan, 1995b
Immobilized cells, fibrous bed				
Fed-batch	78.2	0.95		Huang et al., 1998b
Continuous	12–23	0.38–0.8	?	Huang et al., 1998b

Symbols and abbreviations: ?, unknown; YE, yeast extract; and CSL, corn steep liquor.

[a]All results were obtained using *C. thermoaceticum* ATCC47909 fermentation of glucose at pH 6.3 to 6.8, except for immobilized cell results, which were *C. formicoaceticum* ATCC 27076 fermentation of fructose at pH 7.6.

ducted to replace expensive additives like yeast extract (YE) with low-cost nutrients. The best source of nutrients found for sugar fermentation using *C. thermoaceticum* is corn steep liquor (CSL), which has been used in industrial ethanol fermentations and for production of pharmaceuticals. This research is summarized by Cheryan et al. (1997). Because *C. thermoaceticum* and other acetogenic anaerobic bacteria are sensitive to high sugar concentrations, experimental fed-batch mode fermentations resulted in higher acetate outputs (see Table 6). Here additional substrate is added as a concentrate at intervals during the fermentation. However, the productivity still remained very low. Continuous fermentation and immobilized whole cell systems have been used to increase productivity in acetogenic fermentations (Table 6).

Several experimental cell recycle bioreactors using a membrane module as a separation device have been developed that yield up to five-fold greater productivity in anaerobic fermentations (Cheryan et al., 1997; see Table 6). Figure 6 is a diagram of a continuous cell-cycle membrane bioreactor. The reactor is connected in a continuous closed loop with the S ultrafiltration membrane module. Product is removed in the permeate and feed is added and volume adjusted in the reactor. Because acetate production is "growth-associated," excess cells must be bled from the retentate. The entire system runs at a high cell density permitting the same high output of fed-batch fermentations with four to five times the productivity. These bioreactors have retained stable operation for greater than 50 days (Table 6). Experiments with the cell-cycle continuous reactors show that (at high dilution rates of 0.03 h^{-1}) the productivity is 1.7 g/liter · h, but the output was only 25 g/liter; at a low dilution rate of 0.007 h^{-1}, productivity dropped to 0.4 g/liter 7 · h, with a high output of 45 g/liter. Thus, a compromise must be set dependent on the other process elements. Variations such as the two-stage continuous cell-recycle and the continuous cell-recycle with fed-batch draw and fill bioreactors are being developed and have both higher output of acetic acid and the higher productivity (see Table 6). Acetic acid production from fructose at pH 7.6 by *C. formicoaceticum* immobilized in a fibrous-bed bioreactor is under development. Both fed-batch and continuous processes were examined (Table 6). Inasmuch as both a higher maximum production rate and higher inhibition rate constant were observed in the fibrous-bed reactor, it was concluded that immobilized cells were less sensitive to acetic acid inhibition than were unattached suspended cells. This improved cell behavior predicts that a fibrous bed reactor may be a tool for adapting these bacteria to acetate-tolerance (Huang et al., 1998b).

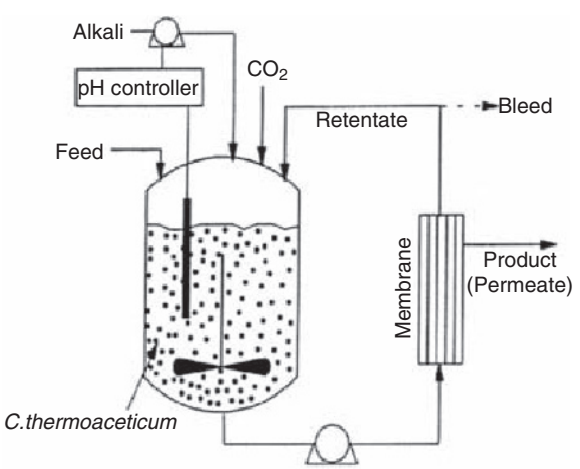

Fig. 6. Production of acetic acid from glucose by *Clostridium thermoaceticum* in a continuous bioreactor. The schematic shows the use of membrane separation for product recovery and cell recycle. From Cheryan et al. (1997), with permission.

Extractant Systems for Acetic Acid Recovery

Fermentation beers are mostly water with a small amount of acetic acid, usually 1.2–3.5%. Recovery costs are significant and they become a major economic consideration for any successful process. The commercial choice for acetic acid recovery has been solvent extraction. Busche (1985) presented an example of an extractive fermentation process along with a cost analysis for a glacial acetic acid plant producing 250×10^6 lbs/y. It is clear from this analysis that recovery costs make up a significant contribution to the overall cost of production. Most important is that product recovery costs are very sensitive to the acetic acid concentration of the fermentation liquor. Busche (1991) compared the economics of manufacture of glacial acetic acid by extractive fermentation using Acetobacter suboxydans with extractive fermentation using *Clostridium thermoaceticum*. A model was designed based on continuous production of acetic acid in fluidized bed bioreactors with recycling beginning with corn syrup-glucose. The ethanol is prepared from corn syrup by fermentation in a continuous bioreactor using *Zymononas mobilis*, and the ethanol beer is directly sent to the *Acetobacter* bioreactor after removal of the *Z. mobilis* cells. Even though the *C. thermoaceticum* fermentation of corn syrup-glucose has a 90% yield, the low output of 3.6% acetic acid results in a higher cost of product purification. The extraction process involves treatment of the broths in a continuous countercurrent multistage extractor using a high-boiling

solvent such as trioctyl phosphine oxide at 50% in 2-heptanone (with a K of 2.5 and a low solubility in water of 1 ppm). The extract is then treated in distillation columns to purify the glacial acetic acid product. The entire technoeconomic analysis showed that the combined *Acetobacter-Zymomonas* scheme for glacial acetic acid production is slightly less costly than the *Clostridium* direct high-yield fermentation ($0.38 versus $0.42/lb). However, neither was competitive with the price at that time of $0.29 to $0.31/lb for synthetic glacial acetic acid. Improvements in bioreactor design were covered in the sections above for oxidation of ethanol by Acetobacter (see "Process Development: *Acetobacter*" in this Chapter) and for sugar fermentation by *Clostridium* (see "Process Development: Acetogens" in this Chapter). These improvements, which produce a higher acetic acid output at an acceptable productivity when combined with downstream organic extractant methods, may eventually result in glacial acetic acid production from biomass competitive with synthetic acetic acid production. Althouse and Tavlarides (1992) made an analysis of 50 organic extractant systems for acetic acid removal. Two candidate extractants (Adogen 381 [40% in cyclohexanone] and Adogen 381 [Xy-Mix]) were selected based on a number of criteria. Either was suggested for the extraction of dilute acetic acid from the outflow of a fermenter. The membrane techniques electrodialysis (ED) and nanofiltration (NF) have been found useful for downstream separation and partially concentrating acetates (Cheryan et al., 1997). Several NF membranes were screened for separation of acetic acids and the technique appeared to be economically sound for consideration in processing fermentation broths (Han and Cheryan, 1996).

Development of Processes for Production of Calcium Magnesium Acetate

The thrust of research and development for production of acetic acid by acetogenic bacteria such as *Clostridium thermoaceticum* and *C. formicoaceticum* is the manufacture of acetate salts. Calcium magnesium acetate (CMA) and potassium acetate (KAc) have been approved by the U.S. Federal Highway Administration as environmentally safe and noncorrosive deicers for winter roadways and for airport runways (Yang et al., 1997). Acetate salts are considered a commodity chemical. Presently, CMA is made from petroleum-derived glacial acetic acid at a cost of about $1,000/ton. Two processes for the production of CMA requiring acetogenic bacteria as biocatalysts have been analyzed to demonstrate technical and economic feasibility.

In a demonstration project report (Basu et al., 1999), a process was outlined whereby municipal solid waste (MSW) coal or sewage sludge was converted to syngas (CO, CO_2 and H_2) by well-known technologies. For example, lignocellulosic biomass may be gasified by the approximate reaction:

$$8CH_2O + 2O_2 + (8N_2) \rightarrow 6CO + 2CO_2 + 6H_2 + 2H_2O + (8N_2) \quad (11)$$

The cooled gas mixture of CO, CO_2, and H_2 was then converted by a new proprietary acetogenic bacterium, ER12, to acetic acid/acetate. As was presented above (see "Scientific Background" in this Chapter), members of acetogenic bacteria such as *A. kivui*, *P. productus*, and *A. woodii* produce acetic acid from either CO or H_2 and CO_2 (see Table 3). Strain ER12, isolated by Bioengineering Resources, Inc. (BRI), is a mesophilic acetogen that rapidly utilizes CO, CO_2 and H_2, has a high tolerance to acetate/acetic acid, and can accumulate 15–20 g of product/liter at pH 4.5–5.0. Production of acetic acid was tested in a continuous stirred tank reactor, with both continuous gas and liquid feed, and with cell recycle. The liquid product stream was fed to an extraction column where a solvent (proprietary) extracted acetic acid to a concentration of 50 g/liter. Dolime was added to produce CMA, the solvent was separated for recycle, and the CMA water stream was fed to a dryer. Figure 7 is a diagram of the process of CMA production from waste biomass.

Economic projections for a facility to produce 100,000 tons/year of CMA from MSW were presented. With no charge or credit for the waste materials used, the operating cost with an included 30% investment return was estimated at $200/ton. This price is less than 28% of the price of the CMA deicer.

Also a detailed process has been developed for production of CMA from cheese whey lactose (Yang et al., 1999). Whey, a byproduct from the manufacture of cheese and casein, contains about 4.5% lactose, 1% protein and some salts and lactic acid. The cheese industry in the United States produces more than 57 billion lbs/year of liquid whey with only one-half used in human food and animal feed. Disposal of the excess cheese whey remains an important problem for the dairy industry. A novel fibrous bed bioreactor has been developed for fermentation of whey lactose to produce acetic acid by a coculture of immobilized cells of *Lactobacillus lactis* and *Clostridium formicoaceticum* (Huang and Yang, 1998a). The coculture consists of a homolactic bacterium and a homoacetic bacterium. These two bacteria sequentially convert lactose to lactate and then lactate to acetate, with an acetate yield from lactose greater than 90%. The perfor-

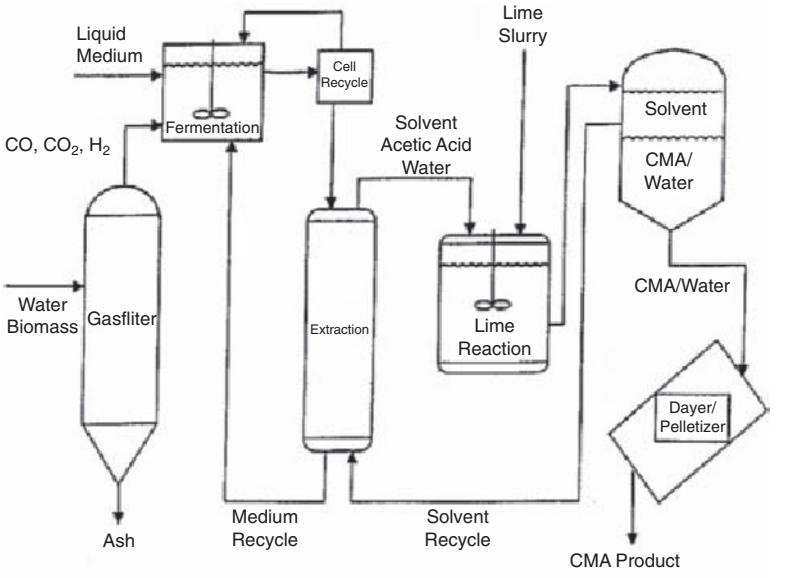

Fig. 7. Process for production of calcium magnesium acetate (CMA) from municipal solid wastes (MSW), sludge or other biomass. Following gasification of the waste, the synthesis gas (CO, CO_2 and H_2) is cooled and fed to a continuously stirred tank reactor containing a liquid stream with nutrient salts and vitamins. The gasses are fermented by an anaerobic acetogenic bacterial culture to form acetic acid. A cell separation system is used to recycle cells back to the reactor, maintaining a high cell concentration and a high production rate and yield of acetic acid. The product stream (15–20 g) acetic acid is fed to an extraction column for solvent extraction of acetic acid yielding 50 g/liter. In a stirred reaction tank, Mg-lime is added to form CMA. The CMA-water stream is fed to a dryer/pelletizer. From Basu et al. (1999), with permission.

mance of *C. formicoaceticum* in the fibrous bed bioreactor was excellent owing to the high cell density (30 g/liter) maintained. In fed-batch operation with corn steep liquor as nutrient at 37°C and pH 7.6, the productivity was about 0.2 g/liter · h and the acetate output was 70 g/liter. Thus, the *C. formicoaceticum* in this bioreactor became tolerant to high acetic acid concentration compared to free cells. The comobilization with the homolactic bacterium, *L. lactis*, in the fibrous bed removed the requirement for purging O_2 from the whey medium to allow growth of the strictly anaerobic *C. formicoaceticum*. *Lactococcus lactis* is not as sensitive to O_2 and removes the residual O_2 from the medium.

Two processes for production of 24 tons/day of CMA from 10^6 lbs of cheese-whey-permeate per day utilizing the fibrous bed bioreactors were evaluated for product quality and manufacturing costs (Yang et al., 1999). Figure 8 diagrams both processes.

In Process 1, the whey is subjected to two tandem fermentations yielding acetic acid, which is then partially extracted from the beer before mixing with dolime to obtain CMA; in Process 2, the whey is fermented by coculture with CaO/MgO added to adjust the pH of the beer. The beer is then evaporated and dried. The CMA produced by both processes had properties very similar to commercial CMA prepared from glacial acetic acid, with a product cost for Process 2 of $291/ton and for Process 1 of $328/ton. The market price for commercial CMA is about $1,000/ton, which is due to the present market price of about $700/ton of synthetic glacial acetic acid (Yang et al., 1999).

Prospects for Bacterial Acetic Acid Production

Specialized Product Areas For the immediate future, chemical synthesis of acetic acid as a commodity chemical from petrochemicals is the major method for production throughout the world. Figure 5 diagrams the major chemical intermediates (precursors of the major industrial products) formed from acetic acid. Production of vinyl acetate, chloroacetic acid, acetic anhydride, and acetate esters requires glacial acetic acid, which is most inexpensively produced by the carbonylation of methanol, the Monsanto process, or by catalytic oxidation of butane (Agreda and Zoeller, 1993). More than 95% of all acetic acid production is directed toward these four precursors.

Is there a niche in which the bacterial processes can be focused on for profitability in the immediate future? First of all, the vinegar industry employing *Acetobacter* species as biocatalysts will certainly continue as a viable presence in the future. Production of vinegar from an expanding variety of fruits and grain sources will always

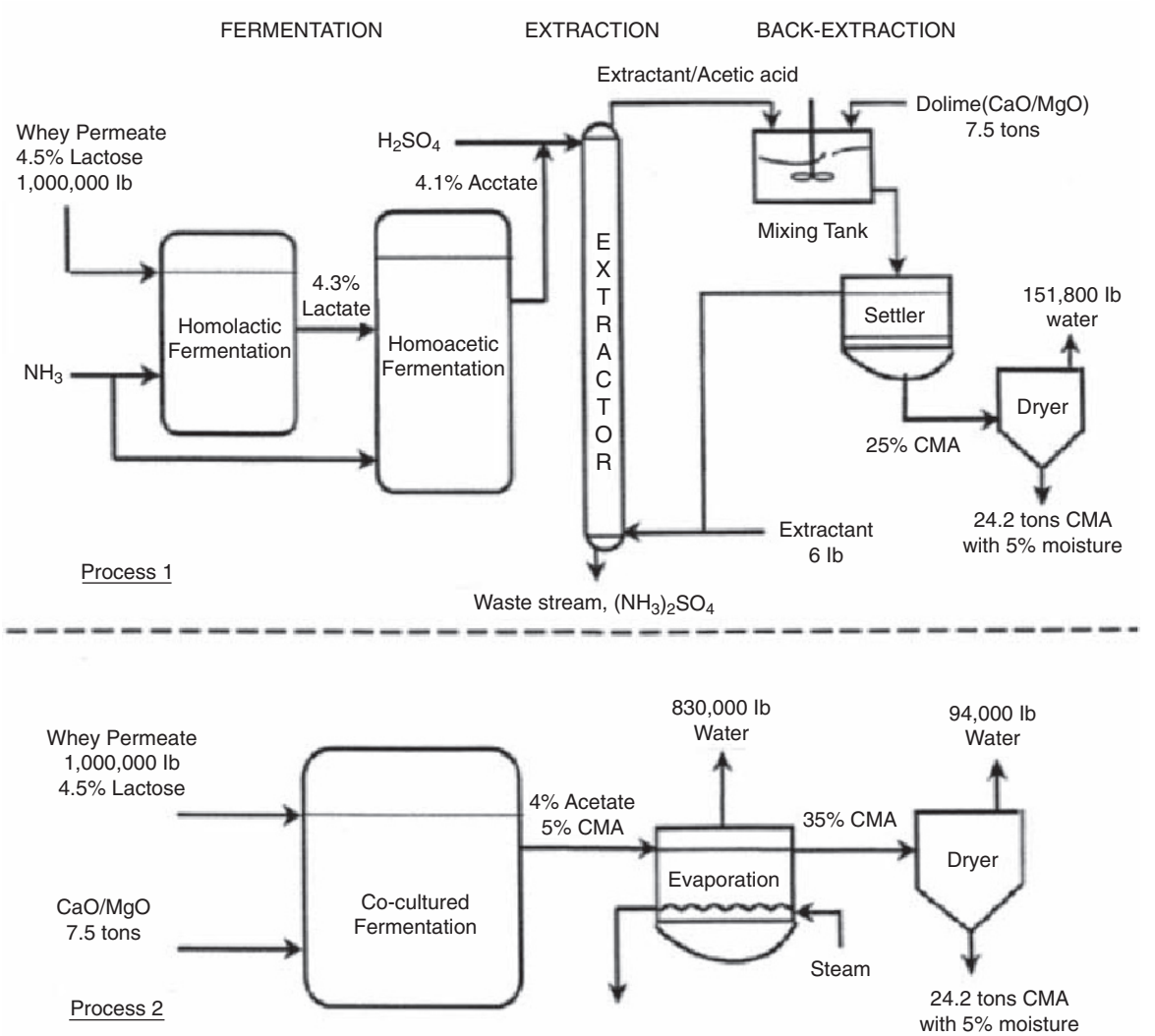

Fig. 8. Two processes for production of calcium magnesium acetate (CMA) from cheese-whey-permeate. In Process 1, the homolactic fermentation of whey-lactose by *Lactococcus lactis* and the homoacetic fermentation of lactic acid by *Clostridium formicoaceticum* are carried out in tandem to produce 4.1% acetate. Acetic acid is concentrated using solvent extraction. Dolime (CaO/MgO) is added to the extractant-acetic acid mixture in a mixing tank. Extractant is recovered. CMA-water is fed to a dryer/pelletizer. In Process 2 the whey-lactose is converted to acetic acid by a simultaneously fermenting coculture of *L. lactis* and *C. formicoaceticum*. Because CaO/MgO is added during the fermentation, the product is 5% CMA (4% acetate). The entire culture beer is steam evaporated to a 35% CMA-water mixture, which is fed to a dryer/pelletizer. From Yang et al. (1999), with permission.

maintain a strong market position in the food industry. The Frings Acetator-based process, utilizing dilute alcohol mashes, remains the most efficient and profitable production method in the food and canning industry (Ebner et al., 1996).

Secondly, the developed bacteria-catalyzed production of inexpensive calcium magnesium acetate (CMA) and potassium acetate (KA) from gasified municipal solid wastes (MSW) or from waste cheese whey is an important product area (see "Development of Processes for Production of Calcium Magnesium Acetate" in this Chapter). The superiority of CMA and KA as deicers for roads and airport runways as well as the economic advantage of the bacteria-based production processes predicts an important area for profitable industrial development for the future. It has been estimated that if only 10% of rock salt is replaced with CMA, the projected annual use of CMA deicer would be $2–2.8 \times 10^9$ lbs/yr. There are also a number of other important industrial uses for acetate salts listed in Fig. 5, such as for heat transfer liquids, meat preservation, and fungicides.

Acetic Acid Production from Bacteria-Based Processes A major decrease in the capacity to synthesize industrial acetic acid from methanol and CO or by other chemical processes may occur at the end of the next century due to depletion of natural gas and petroleum resources together with an increasing demand for these materials worldwide. With price increases accompanying this situation, bacteria-based processes can certainly become major players in the glacial acetic acid market.

Which bacterial process is most attractive for acetic acid production within this future scenario? Ethanol oxidation to acetic acid by the *Acetobacter* process has major advantages over the sugar fermentations to acetic acid by the various acetogens such as *Clostridium thermoaceticum*. Comparing Tables 5 and 6 (see "Current Research and Development"), the former process yields higher output (g/liter) and higher productivity (g/liter · h). In addition, industrial production of glacial acetic acid has been carried out using the *Acetobacter* process (Ebner and Follmann, 1983). Along with the future depletion of petroleum reserves, industrial ethanol production would certainly increase, providing a major source of fuel as well as commodity chemicals. The capacity for fuel ethanol production in the United States as of 1991 was 19×10^9 lbs/yr (Kosaric, 1996). Acetic acid production plants using the Frings Acetator® and new extraction technologies could produce industrial glacial acetic acid directly from the ethanol streams of future ethanol production plants.

A second prospect for acetic acid production is the direct conversion of gasification products, CO, CO_2 and H_2, to acetic acid by anaerobic acetogens. Table 3 (see "Commercial Applications" in this Chapter) is a list of acetogenic bacteria that utilize these gases in addition to methanol, producing almost exclusively acetic acid. The demonstration of acetic acid production from gasified MSW and other carbon sources by the acetogen ER12 showed that these waste sources can be utilized to produce acetate salts efficiently and profitably (see "Development of Processes for Production of Calcium Magnesium Acetate" in this Chapter). Production of glacial acetic acid on a large scale would require development of additional extraction and distillation technologies to separate acetate from the dilute beers. Research efforts focused on increasing acid tolerance of the anaerobic acetogenic production strains by mutation or by immobilization methods will be important to the success of these processes. A future goal will be to develop acetogens that utilize waste sugar sources and/or degrade cellulosic polymers while producing a high proportion of acetic acid (>90%) in the product steam.

Section 2: Lactic Acid

Introduction

With the development of agriculture, at about four to five thousand BCE, the problem of food storage for future use emerged. It was recognized early that fermentation of foods such as milk, vegetables, fruits and meats resulted in both enhanced flavors and better preservation. Food containers dating from 2300 BCE discovered in Egypt with cheese-like residues suggest that lactic acid fermentations have been used for at least four to five thousand years (McGee, 1984). The manufacturing processes applied in these ancient "lactic acid" fermentations were household or small-scale and craft-centered, so improvements were small and slow. But the development of a true technology of the fermentation process had to await the Industrial Revolution in the 18th century, bringing with it the advent of the science of chemistry. Lactic acid (2-hydroxypropionic acid), the major acid in these fermentations, was first identified as a chemical substance in sour milk whey by Carl Wilhelm Scheele in 1780. Scheele was a gifted experimentalist and probably the first organic chemist in history (Benninga, 1990). Besides lactic acid, he also discovered tartaric acid, citric acid, malic acid, and a number of other organic acids.

In 1857, Louis Pasteur was the first to recognize that a lactic fermentation was due to a peculiar gray substance he called a "lactic yeast," which he found was responsible for production of lactic acid. Further, he showed that the lactic yeast was distinctly different from the brewer's yeast that converted sugars to alcohol and CO_2. Later, in 1877, the careful experiments of the famous surgeon, Joseph Lister, demonstrated that only a single bacterium, "*Bacterium lactis*," was sufficient to bring about souring or fermentation of cows' milk. Lister's landmark experiments established not only that one bacterium would cause the lactic acid fermentation, but also that his dilution method made it possible to prepare pure cultures of a single bacterial type. Following Lister's discovery, isolation of pure cultures of lactic acid bacteria became a vital stage in developing a scientific approach to any microbial fermentation process.

The early development of microbial processes for industrial production of ethanol or lactic acid occurred in Berlin at the "Research Institute for Fermentation Industries," directed by Max Delbrück. The major accomplishments of this institution were the preparation of pure cultures of bacteria or yeasts for industrial fermentations and the application of the principle of "natural pure culturing." In "natural pure culturing," the characteristic properties of the desired microbe

are used (in an industrial process) to suppress the growth of undesirable bacteria, thereby allowing its growth to dominate. This principle was applied to lactic acid production by raising the fermenter temperature to 45–50°C, which permitted growth of the lactic acid bacteria while preventing growth of most contaminant microorganisms.

The first industrial fermentation for lactic acid production was at the Avery Lactate Co. in Littleton, Massachusetts. Charles Avery, a chemist who attended Massachusetts Institute of Technology (MIT), discovered that (to produce baking powder) calcium lactate would act as an acidulant in place of the more expensive cream of tartar (potassium bitartrate) when added to sodium bicarbonate. At that time, baking powder had been introduced into the baking industry. However, the new product was a commercial failure owing to both technical problems and economic competition from another effective acidulant, exsiccated alum (Benninga, 1990). Pure cultures of "*Lactobacillus delbrueckii*" and "*Lactobacillus bulgaricus*" were prepared for industrial use by Max Delbrück's Institute for the Fermentation Industries in Berlin from about 1896. Taking advantage of these pure cultures, Albert Boehringer established the first commercially successful large-scale lactic acid production plant in Ingelheim. It came on line in the late 1890s, producing technical grade lactic acid (50 and 80%), which was sold for use in the textile and leather industries, replacing tartaric acid and tartrates. Prior to 1914, five lactic acid production plants emerged in the United States. However, imported German technical grade lactic acid was of the higher quality required for textile, leather and pharmaceutical uses and thus it competed successfully in the United States market. During World War I, the lactic acid industry in the United States expanded rapidly to a total production of 4.6×10^6 lbs/year by 1919, as a result of the disappearance of German competition. However, during the 1920s, the lactic acid fermentation industry in Germany (e.g., Boehringer and E. Merck) recovered and their prewar export markets were rapidly reestablished. Because of the high quality and low prices resulting from new scientific and technical advances and production efficiency, the German exports of lactic acid almost wiped out production in the United States. This lactic acid production (along with worldwide production) again increased, growing to a peak of $7.2–9.0 \times 10^6$ lbs/year during World War II. During this war, sodium and potassium lactate were used as a coolant for armored vehicles in place of glycerol. Between World War II and 1963, lactic acid production in the United States leveled off at about 4.6×10^6 lbs/year.

In 1963, synthetic lactic acid production began on a commercial scale. The chemical synthesis route for synthetic lactic acid yields a racemic mixture of DL-isomers. The commercial process is based on lactonitrile (Holten et al., 1971). Lactonitrile is produced by the base-catalyzed addition of hydrogen cyanide to acetaldehyde. Lactonitrile is then hydrolyzed by strong acid to yield lactic acid, which is purified and recovered. Today, synthetic lactic acid is produced mostly in the United States and Japan, and it accounts for about 50% of total worldwide production. Industrial fermentations also yield about half the world's lactic acid production, and thus are very competitive.

Scientific Background

The physical and chemical properties of lactic acid are extensively covered by Holten et al. (1971). Some of these properties are listed in Table 7.

The two optically active enantiomeric forms are L(+) dextrorotary and D(−) levorotary:

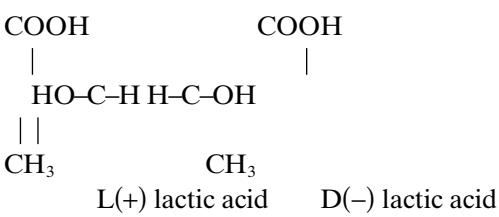

Table 7. Selected properties of lactic acid enantiomers.

Property	Enantiomer and chemical abstracts service registry no.		
	L(+) 79-35-4	D(−) 10326-41-7	DL 598-82-3
Molecular weight	90.08	90.08	90.08
Melting point (°C)	52.8–53.6	52.8–53.6	16.8–33
Boiling point (°C)		103	82–85
Optical rotation $[\alpha]_D^{20}$, in degrees:			
Acid	+2.5	–2.5	
Zinc salt	–8.2	+8.18	
Dissociation constant	1.90×10^{-4}		1.38×10^{-4}
pK (25°C)	3.79	3.83	3.73

From Litchfield (1996), with permission.

Table 7 (from Litchfield, 1996) shows that the zinc salts of the two enantiomers have the reverse optical activity of the free acid. Esters of the two isomers also show the same optical activity shift. Vick Roy (1985) citing Lockwood et al. (1965) states that although the L(+) form appears to be dextrorotatory, it may actually be levorotary as are the salts and esters. The apparent (+) optical rotation may be due to formation of an ethylene oxide bridge between carbons 1 and 2, with a tautomeric shift of the hydroxyl group from carbon 1 to the carbonyl group. Salts and acids cannot form the epoxide ring and are levorotary. Lactic acid is infinitely soluble in water and has low volatility. In solutions of 20% or more, lactic acid polymerizes into linear dimers of lactoyl lactate or higher polymers as well as cyclic dimers of D, D-, L, L-, or D, L-lactides. Lactic acid is both an organic acid and alcohol and therefore can participate in many chemical reactions. The lactic acid bacteria used in industrial fermentations or under development for commercialization are listed in Table 8. The various bacterial strains produce either almost pure lactic acid enantiomers, L(+) or D(−), or a racemic mixture of DL-lactic acid. The present nomenclature (Kandler and Weiss, 1986) lists *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus rhamnosus* (formally *Lactobacillus delbrueckii*) as producers of D(−) and L(+) lactic acid, respectively (see Table 8). In earlier industrial fermentations producing L(+) lactic acid, *L. delbrueckii* was the specified species used. Thus, it is important to be aware of improper identification of strains in the literature, owing to frequent reclassifications. Because of the new markets for specialty polylactic acid polymers, there is a need for L,L-, D,L- and D,D-lactide dimers as precursors of polymers with different physical properties (Datta et al., 1995). Strain selection for commercial scale fermentation focuses on: 1) rapid fermentation of inexpensive carbon sources with low nitrogen content; 2) high yield of stereospecific lactic acid with low amounts of cell mass and other products; and 3) tolerance to low pH (below pH 5) and high temperature (about 45°C). The industrially important organisms are "facultative anaerobes" in that they tolerate oxygen but do not use it as a final electron acceptor in respiration to generate a proton gradient for ATP synthesis. In addition, the fermentation process characterized by high temperature (above 40°C), low oxygen, high lactic acid and low pH tends to restrict contamination by unwanted bacteria.

Lactobacillus rhamosus (formerly *L. delbrueckii*) produces high yields of L(+)-lactic acid from sucrose and glucose at 45°C, making it an excellent commercial production strain (Table 8). But this bacterium will not utilize lactose, so it cannot make use of cheese whey. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. helveticus* both ferment lactose from cheese whey, producing high yields of lactic acid. *Lactobacillus delbrueckii* produces the D(−) enantiomer and *L. helveticus* a racemic mixture of DL-lactic acid. Because most commercial fermentations are developed for L(+)-lactic acid production, strains of *L. casei* and *Lactococcus lactis* are being developed for fermentation of lactose sources such as cheese whey.

There are two pathways for the uptake and metabolism of lactose by the lactose-fermenting lactic acid bacteria summarized in Fig. 9 (Davidson et al., 1995). One pathway transports lactose into the cell using a proton symporter, which is a classical lactose permease. Hydrolysis of lactose is catalyzed by a β-galactosidase to D-glucose and D-galactose. D-Galactose is converted to glucose-1-phosphate by the Leloir pathway (Gottschalk, 1986), and both glucose and glucose-6-phosphate are further metabolized by the Emb-

Table 8. Selected lactic acid bacteria: their fermentation pathways, lactic acid enantiomer produced, and major carbon sources used.

Bacteria	Fermentation pathway	Lactic acid enantiomer	Carbon sources substrates and complex
Lactobacillus delbrueckii subsp. *bulgaricus* (formerly *L. bulgaricus*)	Obligate homolactic	D(−)	Lactose, cheese whey, casein whey, and cheese whey permeate
Lactobacillus rhamnosus (formerly *L. delbrueckii*)	Homolactic-inducible heterolactic	L(+)	Glucose, sucrose and potatoes
Lactobacillus helveticus	Obligate homolactic	D, L	Cheese whey permeate and lactose
Lactobacillus amylophilus	Obligate homolactic	L(+)	Starch
Lactobacillus amylovorus	Obligate homolactic	D, L	Starch
Lactobacillus casei subsp. *casei* and subsp. *rhamnosus*	Homolactic-inducible heterolactic	L(+)	Lactose and cheese whey
Lactococcus lactis subsp. *lactis* and subsp. *cremoris* (formerly *Streptococcus lactis*)	Obligate homolactic	L(+)	Lactose and cheese whey permeate
Streptococcus thermophilus	Obligate homolactic	L(+)	Lactose

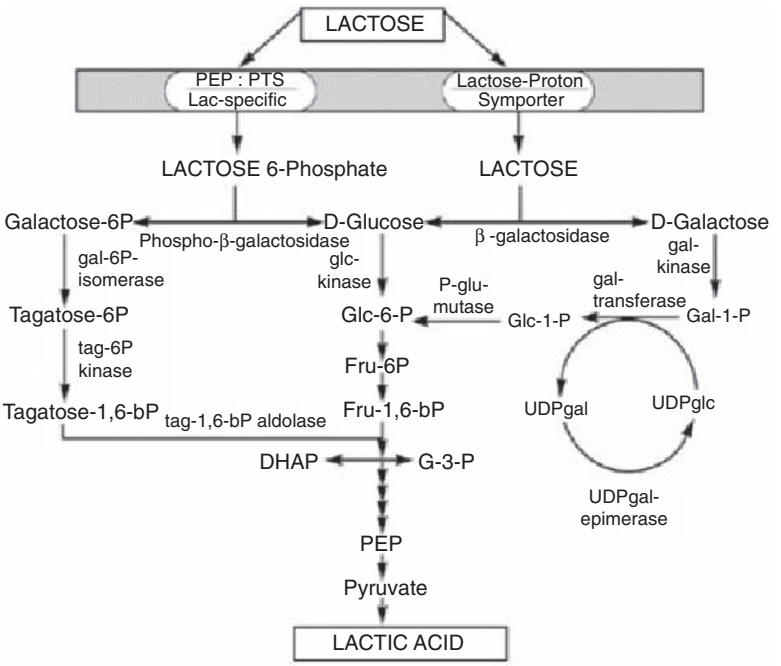

Fig. 9. Pathways for uptake of lactose and metabolism to lactic acid.

den-Meyerhof pathway to lactic acid. The second pathway uses a phosphoenolpyruvate-dependent phospho-transferase system (PEP:PTS, Lac-specific) to form lactose-6-phosphate from lactose as it is transported through the cytoplasmic membrane. The lactose-6 phosphate is hydrolyzed by phosphoryl-β-galactosidase to glucose and galactose-6-phosphate. The galactose-6-phosphate is converted to tagatose-6-phosphate, then tagatose-1, 6-biphosphate, and finally triose-phosphates by a series of enzymes. The triose-phosphates and glucose then enter the Embden-Meyerhof pathway as shown in Fig. 9. *Lactobacillus delbrueckii* and *L. helveticus* contain the lactose-permease-β-galactosidase pathway, whereas *L. casei* and the *Lactococcus lactic* have only the PEP:PTS and phospho-β-galactosidase pathway (Arihara and Luchansky, 1995).

Two species of *Lactobacillus* that utilize starch as a substrate are listed in Table 8. Although starch is not normally utilized by lactic acid bacteria, two species, *L. amylophilus* (Mercier et al., 1992) and *L. amylovorus* (Cheng et al., 1991; Zhang and Cheryan, 1994a), have been isolated and are able to produce a high yield of lactic acid from liquified starch in experimental fermenters.

The industrial strains of lactic acid bacteria can completely ferment a medium of 12–15% sugar in 2–4 days with greater than a 90% yield of lactic acid. All of these bacteria are considered homofermentative in that they utilize a homolactic fermentation pathway, producing two molecules of lactic acid for each molecule of hexose. As

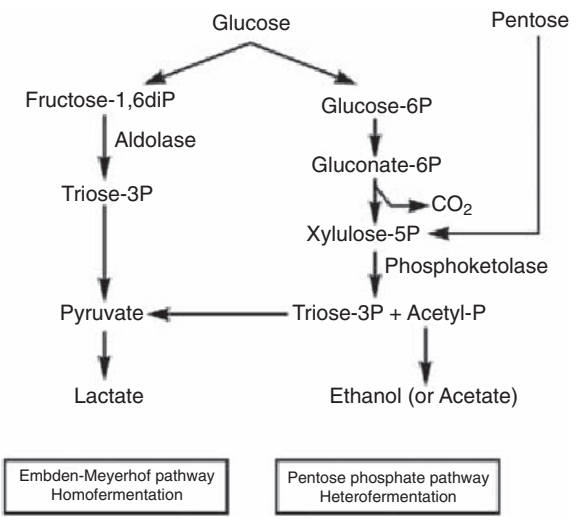

Fig. 10. Metabolic pathways of carbohydrates used by lactobacilli. From Bigelis and Tsai (1995), with permission.

diagrammed in Fig. 10, the Embden-Meyerhof pathway is used in the homolactic fermentation.

The fructose 1,6-diphosphate (FDP) is the key intermediate, and the two enzymes, fructose-6-phosphate kinase and FDP aldolase, are diagnostic for this pathway. In some lactic acid bacteria, the L-lactic dehydrogenase (LDH) is allosterically activated by FDP and/or Mn^{++} ion. The obligate homolactic strains listed in Table 8 ferment sugars (usually hexoses) only by this pathway. Table 8 also lists two homolactic-inducible heterolactic strains, *L. rhamnosus* and *L. casei*. These lactic acid bacteria can use the pentose

phosphate pathway shown in Fig. 10 by inducing the enzyme phosphoketolase. All of the other enzymes needed for this pathway are present in all these bacteria. Induction of this pathway usually requires very low hexose concentrations (and thus lower internal FDP) and/or added pentose. This pathway produces one mole each of lactate, acetate (or ethanol) and CO_2 for each mole of hexose, or one mole each of lactate and acetate for each mole of pentose. A few lactic acid bacteria use exclusively the pentose phosphate pathway because they do not produce the enzyme FDP aldolase. However, these strains are not useful in commercial lactic acid production. The production of a stereospecific product, D(−)- or L(+)-lactic acid, by these bacteria is due to the stereospecific lactic dehydrogenases, L-LDH and D-LDH. It was thought that bacteria such as *L. plantarum* or *L. helveticus* that produce D, L-lactic acid contains a racemase. However, it was shown that *L. plantarum* contains both enzymes, D-LDH and L-LDH, and thus can easily equilibrate added lactic acid enantiomers by catalysis through pyruvate (Dennis and Kaplan, 1960). The two enzymes have quite different properties. Cloning and sequencing of the L-LDH and D-LDH genes revealed no significant homology between them. However, the D-LDH genes from other lactobacilli were found to be homologous (Taguchi and Ohta, 1991).

Lactic acid bacteria are well known for their complex nutrient requirements for growth. An array of vitamins, nucleotides, and amino acids or peptides is essential, and the minimum requirements for growth of each strain can vary (Buchta, 1983). Further, many of the growth factors significantly increase the rate of lactic acid fermentation. Because of the large number of essential and stimulatory factors, it is impractical to cultivate these organisms in chemically defined medium. In large commercial-scale fermentations, complex nutrient sources such as corn steep liquor, malt extracts, malt sprouts or soybean extract are added. Although cheap, the crude materials also may contain specific inhibitors of the fermentation and also increase the cost of lactic acid purification downstream (Bigelis and Tsai, 1995). Thus, a balance must be established between the high rate and yield of a fermentation and the purification costs. Because carbon dioxide (CO_2) fixation is required for many biosynthetic pathways in bacteria, it is not surprising that CO_2 is shown to stimulate growth of many lactic acid bacteria. During a pure homolactic fermentation of sugars, no CO_2 is produced. To prevent a CO_2 limitation, calcium carbonate may be added to neutralize lactic acid for pH control during a fermentation (Bigelis and Tsai, 1995). Some lactic acid bacteria require added fatty acids or fatty acid esters (Tweens). Often fatty acids can be synthesized by these organisms when the vitamin, biotin, is added to the fermentation broth (Bigelis and Tsai, 1995).

The different lactic acid bacterial strains show pronounced differences in proteolytic capacities allowing them to grow on protein sources from meats, vegetables and milks for a supply of amino acids and small peptides. Because these bacteria have multiple amino acid requirements and cannot utilize ammonia as a sole nitrogen source, their growth is dependent upon an efficient system for protein and peptide degradation and transport. The lactic acid bacterial strains selected for a specific industrial fermentation (e.g., sucrose with added corn steep liquor) must have optimal proteolytic characteristics as well as lactic fermentation abilities for the particular set of raw materials used. The efficiency of the fermentation is intimately dependent on the cascade of proteolytic reactions that occur during growth and fermentation. The genetics and physiology of the proteolytic system of lactic acid bacteria have been reviewed (Kok and deVos, 1994). Peptides are sometimes more effective than free amino acids for growth and fermentation. For example, whey protein with an average peptide length of six amino acids (700 kDa) was found most effective for high production rates of lactic acid in *L. delbrueckii* subsp. *bulgricus* (Leh and Charles, 1989).

Microbial production of lactic acid is vulnerable to bacteriophage attack. Industrial fermentations as well as food and dairy fermentation using *Lactobacillus* spp., *Lactococcus* spp. and *Streptococcus* spp. are subject to disruption by bacteriophage infection. Because of the size of this problem and its economic impact, much research has been carried out over the past six decades on the biology of the bacteriophages and the resistance mechanisms mounted by these bacteria to prevent phage infection. Klaenhammer and Fitzgerald (1994) have reviewed and evaluated this research. They point to factors that contribute to the onset of bacteriophage infection in the fermentation process, such as reliance on specialized strains and increasing pressure of manufacturing schedules. The classification of the known lactic acid bacteriophages, how they adsorb to cells, and phage lysogeny and lytic development are fairly well known. The genetics and biochemistry of bacteriophage resistance of lactic acid bacteria suggest some practical applications (Klaenhammer and Fitzgerald, 1994).

The major mechanisms of phage resistance which have been identified in the lactic acid bacteria include blocking of virus adsorption, restriction endonucleases and modification methylases, and abortive infection systems. Over the past ten years, genetic studies of *Lactococcus*

sp. show that these bacteria often carry the genes for these resistance devices on a variety of plasmids. The other lactic acid bacteria have also been shown to carry plasmid-borne resistance mechanisms as well. In their review, Klaenhammer and Fitzgerald (1994) outline "genetic addition strategies" for using these plasmids (carrying restriction systems for transfer and "stacking" into commercial strains) to improve bacterial resistance to multiple phage attack. Advances in the molecular biology of *L. lactis* and its phages have encouraged the emergence of novel recombinant phage defense systems which complement the mechanisms described above. For example, a suicide system has been developed that consists of a cassette with a promoter from a lytic phage (031) linked to a lethal gene (the *LlaI* restriction site; Djordjevic et al., 1997). When the cassette is inserted in the *L. lactis* chromosome, the phage-specific inducible promoter is activated by phage 031 infection, causing expression of the linked lethal gene, which results in death of the host cell and degradation of the phage genome.

Another example of a phage defense system employs antisense mRNA technology as a barrier to phage expression. Production of a specific antisense mRNA may act by competing with normal mRNA at the initiation of translation on the surface of the ribosome. Secondly, the direct binding of antisense RNA to the normal RNA may make it susceptible to degradation by double-stranded RNases in the cell. Walker and Klaenhammer (2000) produced antisense constructs of "late genes" of phage 031 with *ori*31 as a promoter, which were inserted into recombinant plasmids. When *L. lactis* carrying these plasmids are infected with bacteriophage 031, they produce large amounts of late 031 antisense mRNA. This results in significant protection of *L. lactis* against 031 infection. Clearly, in any large-scale commercial fermentation, a carefully planned, long-range phage defense program must be in place.

Commercial Applications

FERMENTATION For commercial scale production of lactic acid by fermentation, there are choices of feedstock, fermentation methods, product recovery, and final uses and applications that must be addressed. The feedstock-carbohydrate to be fermented as well as the enantiomeric form of lactic acid desired (D–, L+, or DL) will determine the organism selected to catalyze the process (see "Scientific Background" and Table 8). The choice of feedstock should be optimized considering 1) low cost, 2) low levels of contaminants, 3) fast fermentation rate, 4) high lactic acid yield, 5) little or no by-product formation, 6) little or no pretreatment required, and 7) year-round availability (Vick Roy, 1985). Long distance transport of feedstocks to the fermentation plant can be costly and should be avoided. Litchfield (1996) stresses that ideally a fermentation of cheese whey to lactic acid should be located close to cheese manufacturing plants because cheese whey is very dilute (4–4.5% lactose) and easily infected with bacteria. Also, refrigeration during storage or transport allows lactose to crystallize out of the whey owing to its low water solubility. Yet cheese whey and cheese whey permeate are low cost available feedstocks. In 1994, in the United States about 57×10^6 lbs (25.9×10^9 Kg) of liquid whey (4–4.5% lactose) was a by-product from dairy product manufacture. Only one half is converted commercially to dried products such as lactose. The rest is waste and has been used in large-scale commercial fermentations for lactic acid by companies such as Sheffield Product Co., Norwich, NY. Complex carbohydrates containing starch or sugar can be used as feedstocks. In early fermentations, beet molasses was used, but a high quality lactic acid was produced only after an added step of solvent extraction during product recovery (Buchta, 1983). Thus, crude feedstocks should be avoided because added chemical contaminants are often difficult to remove during the downstream recovery stage. Purified sucrose from sugar beets and sugar cane, glucose from hydrolyzed starch sources such as corn, and wheys containing lactose are the major raw materials used today. The use of purified feedstocks in the fermentation ensures lower cost of the final product recovery. In the United States, glucose from enzyme-hydrolyzed cornstarch produced in wet-milling is the major substrate for commercial lactic acid formation. Direct fermentations of liquid cornstarch by the amylolytic *Lactobacillus* strains, such as *L. amylophilus* (Mercier et al., 1992) and *L. amylovorus* (Cheng et al., 1991), have been carried out in the laboratory; however, no commercial processes are presently in use. The availability of purified sucrose as a feedstock from beets in Europe and from sugar cane in Brazil permits commercial production of high quality lactic acid in these regions. Nevertheless, sugar prices vary considerably and thus the economics of lactic acid production may be strongly affected.

The major fermentation method used to produce lactic acid on an industrial scale is the batch fermentation process. The factors affecting the batch process have been reviewed (Litchfield, 1996; Vick Roy, 1985). Because of the very corrosive properties of lactic acid, construction materials used for the fermenter and downstream processing equipment are a major cost item. Copper, copper alloys, steel, chrome steel,

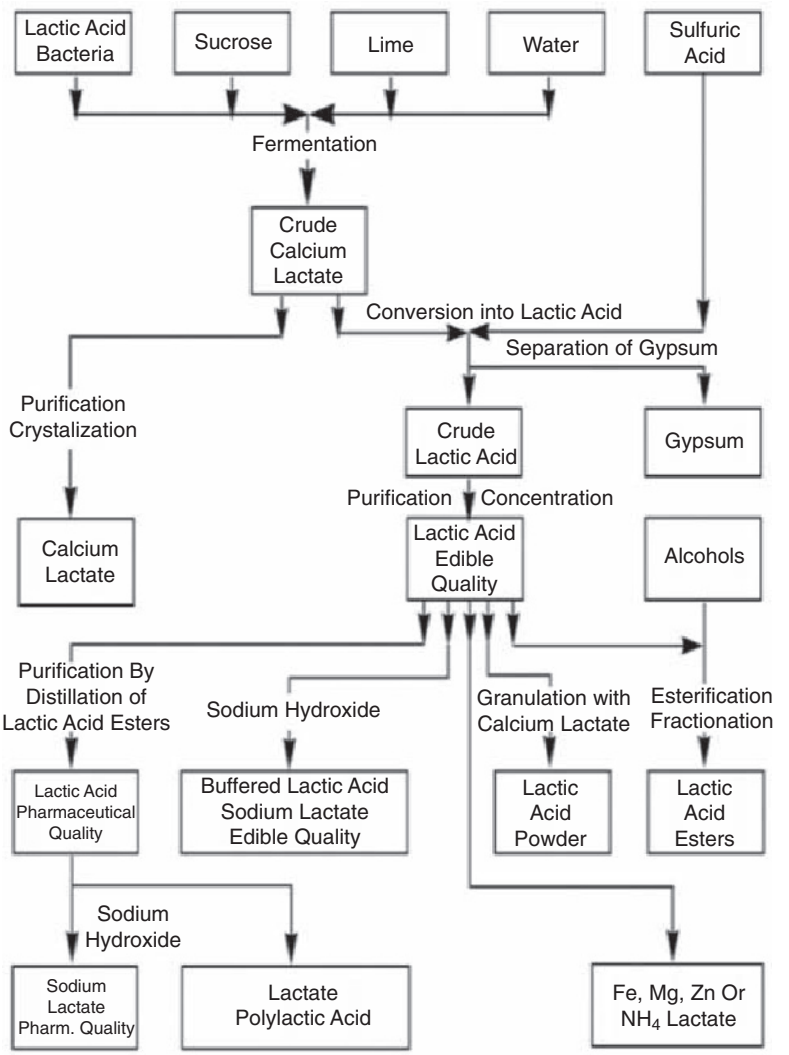

Fig. 11. Flowchart for production of lactic acid and its derivatives by the Purac fermentation process. From Bigelis and Tsai (1995), with permission.

and high nickel steels are all unsatisfactory. High molybdenum stainless steel like SS316 is satisfactory. Plastic linings of fermentation tanks have been used successfully, and new developments in ceramics and plastics may provide future choices (Vick Roy, 1985).

Contamination with other bacteria is not a big problem in commercial fermentation if fermenters are steam-treated or chemically sterilized prior to filling with a heated medium. A pure-culture inoculum is usually used, and the fermentation is run at a high temperature (43–50°C) and with very low oxygen. Eventually a high concentration of lactic acid accumulates. All of these conditions tend to inhibit growth of many potential contaminants. Specific conditions vary with the industrial process. For example, during the fermentation of 15% glucose by *L. rhamnoses* in 30,000-gallon reactors, a temperature of 45–60°C and a pH of 5.0–6.0 were maintained for 4–6 days, producing an 80–90% yield of lactic acid (Inskeep et al., 1952). For fermentation of cheese whey (4–5% lactose) by *L. delbrueckii* subsp. *bulgaricus* in a 5,000-gallon reactor, 43°C and a pH of 5.5 were maintained for 1–2 days with a yield of 85–90% lactic acid (Campbell, 1953). A flow chart of the Purac fermentation process for lactic acid production is diagrammed in Fig. 11. However, companies in the United States starting lactic acid production by fermentation use newly developed proprietary processes (Datta et al., 1995).

Calcium carbonate is usually added during the batch fermentation to control the pH, and the highest concentration of lactic acid (12–15%) is limited by the precipitation of calcium lactate from the broth. Temperature control is maintained with heat transfer coils using circulating water, and the cells are retained in a mixed suspension by mechanical agitators or pump circulation. The productivity of large batch commercial reactors is low, at 1–3 gms/liter · hr.

The final biomass of cells is lower than 30 wt % and usually about 15 wt % of the amount of sugar added initially. The fermentation rate is initially high, but then slows down as nitrogenous sources are depleted and lactic acid accumulates. Litchfield (1996) has reviewed work that shows a significant effect of inoculum size on lactic acid yields obtained with *L. delbrueckii* subsp. bulgaricus, *L. casei* and *L. helveticus* in laboratory-scale fermentation. However, little is reported on the effect of inoculum sizes for commercial-scale lactic acid fermentation.

Product Recovery The recovery of lactic acid from the fermentation medium is a major cost item for the entire process (Vick Roy, 1985; Datta et al., 1995). The product is available in four grades: 1) technical, 2) food (FCC), 3) pharmaceutical (USP) and 4) "heat stable" lactic acid that will not discolor when heated to 200°C for a few hours. The greater the purity, the more complex and expensive the recovery process.

The first step in the conventional recovery processes is heating the fermentation beer to 80–100°C and increasing the pH to 10 to kill the bacteria, coagulate proteins and maintain calcium lactate's solubility in the beer. The crude calcium lactate solution is then filtered, decolorized with activated carbon, and concentrated by evaporation. Lactic acid is recovered by addition of sulfuric acid and then further purified by one of the following routes (Fig. 11):

1) crystallization of calcium lactate (technical);
2) filtration, carbon treatment, and concentration of lactic acid (food quality);
3) formation of lactate esters and fractionation (pharmaceutical quality); and
4) distillation of lactic acid esters (highest purity, heat stable lactic acid).

These purification processes are often combined with liquid-liquid extraction, ion-exchange, and adsorption on solid adsorbants. However, for every ton of fermentation lactic acid produced by the conventional recovery process, about one ton of gypsum ($CaSO_4 \cdot 2H_2O$) by-product is also produced. Ecological and economical disposal thus becomes a problem for a large-scale production facility.

Developments in membrane-based separation and purification of lactic acid have been made. The use of ultrafiltration and electrodialysis provides a new approach for lactic acid recovery that would be low cost and simultaneously avoid the formation of the problem by-product, gypsum (Datta et al., 1995). Advances in membrane technology allowing low-energy desalting-electrodialysis have led to a proprietary method for production of fermentation lactic acid from carbohydrates without calcium salt production (Datta, 1989). This new technology can be operated as a continuous process and scaled up to large-volume recovery of lactic acid from the fermentation beer and manufacture of lactide polymers (Gruber et al., 1992; Gruber et al., 1994). A major commercial effort for lactic acid production and conversion to polylactide by a United States agriprocessor, Cargill Dow, incorporates this technology (Datta et al., 1995; Jarvis, 2001).

Application and Uses The major application of lactic acid and lactic compounds is for food additives. Table 9 presents a list of applications for lactic acid, lactate salts, and various lactylated fatty acid esters in the food industry.

Lactic acid and the lactate salts are generally recognized as safe by the Food and Drug Administration (FDA) in the United States. The stearoyl lactates and other esters used as emulsifiers and bread dough conditioners have also been approved by the FDA. More than 50% of the lactic acid destined for food uses goes into producing these emulsifying agents for bakery goods. The four most important esters of lactate salts are calcium and sodium stearoyl-2-lactylate, glyceryl lactostearate, and glyceryl lactopalmitate (Datta et al., 1995). These products have specific uses in the formulation of cake mixes, dips, and other prepared foods (Table 9). Because production of all of these emulsifiers requires the highest quality "heat stable" lactic acid, only synthetic lactic acid or heat stable fermentation lactic acid grades can be used.

Technical grade lactic acid has been used for a long time in the leather tanning industry as an acidulant. It is used in the finishing of textiles and acid dying of wool. Inorganic acids are used competitively in these processes, but lower cost lactic acid together with environmental regulations against dumping waste salts may favor extensive use of lactic acid in the future.

Lactic acid is used in many small-scale applications such as resin production, solder fluxes, lithographic and textile printing, adhesives, electroplating and electropolishing baths, and detergent formulations. These uses may account for 5–10% of consumption of lactic acid.

There are numerous pharmaceutical and cosmetic applications and formulations containing lactic acid, sodium and calcium lactates, and a range of acyl lactates. Calcium lactate is widely used for treating calcium deficiency. Lactates are added to many cosmetic products as moisturizers, emulsifiers and stabilizers. Lactates have been promoted in skin care products for improving skin texture and appearance (Smith, 1993). Ethyl lactate is an active ingredient in anti-acne preparations (Datta et al., 1995).

There are important medical applications for polylactides prepared from the lactide dimer as

Table 9. Food applications of lactic acids and lactic acid compounds.

Compound (Chemical Abstracts Service Registry no.)	Food application	Code of Federal Regulations[a] reference
L(+)-Lactic acid (79-35-4) > D(−)-Lactic acid (10326-41-7) > DL-Lactic acid (598-82-3)	Antimicrobial agent, curing and pickling, flavoring, enhancer, adjuvant pH control, solvent and vehicle	21 CFR 184.1061
Calcium lactate	Flavoring enhancer, firming agent, leavening agent, nutrient supplement, stabilizer, and thickener	21 CFR 184.1207
Ferrous lactate (5905-52-2)	Nutrient supplements and in infant formula	21 CFR 184.1311
Potassium lactate (996-31-6)	Flavor enhancer, flavoring agent, humectant, and pH control	21 CFR 184.1639
Sodium lactate (72-17-3)	Flavor enhancer, flavoring agent, humectant, pH control, and emulsifier	21 CFR 172.1768
Calcium stearoyl-2-lactylate	Dough conditioner in bakery products, whipping agent in egg products, and conditioning agent in dehydrated potatoes	21 CFR 172.844
Sodium stearoyl lactylate (25-383-997)	Dough conditioner, emulsifier, processing aid in baked products; emulsifier, stabilizer processing aid in milk or cream substitutes, snack dips, imitation cheeses, and dehydrated potatoes	21 CFR 172.846
Lactylated esters of fatty acids	Emulsifiers, plasticizers, and surface active agents in foods	21 CFR 172.848
Lactylated fatty acid esters of glycerol and propylene glycol	Emulsifiers, plasticizers, and surface active agents in foods	21 CFR 172.850
Glycero-lacto esters of fatty acids	Emulsifiers, plasticizers, and surface active agents in foods	21 CFR 172.852

[a]Code of Federal Regulations (1994).
From Littlefield (1996), with permission.

Table 10. Main producers of lactic acid and lactic acid esters.

Synthetic lactic acid: mixture of D and L
Sterling Chemicals, Houston, Texas
Mushashino Chemical Laboratory Ltd., Isohara, Japan
Fermentation lactic acid: L(+), D(−), or DL
Archer Daniels Midland Co., Decatur, Illinois
Cargill, Minneapolis, Minnesota
Ecological Chemical Co.-Conagra/Dupont, Adell, Wisconsin
Chemie Combinatie Amsterdam CCA/PURAC, Corinchem, The Netherlands

well as for lactide-glycolide copolymers synthesized from lactide and glycolide dimers. These polymers are used for procedures requiring surgical sutures, implants, bone plates, and controlled drug release, inasmuch as they are biocompatible, biodegradable, and resorbable materials.

Concern over the negative environmental impact of accumulating nondegradable plastic films and containers has been increasing. Replacement of these petroleum-based plastics with biodegradable plastics may be inevitable within the next 50 years. Lactic acid based bio-plastics will play an important role in future potential products (see "Areas of Research and Development" in this Chapter).

Economics The major commercial producers of lactic acid are listed in Table 10. The worldwide market for lactic acid and lactic acid esters was estimated at 60–65,000 tons/yr (54.5–59 10^6 kg/yr) (Litchfield, 1996). Producers of synthetic and fermentation lactic acid are both included, considering they share the market about equally. The United States consumes almost 40% of annual production (Bigelis and Tsai, 1995). The major producer of fermentation lactic acid in the United States is Archer Daniels Midland, Co., with a capacity of 10–20,000 tons/yr (9.1–18.2 × 10^6 kg/yr; Anonymous, 1993). This proprietary fermentation process is presently nonbacterial. In contrast, Sterling Chemicals, a major producer of synthetic lactic acid in the United States, had an annual capacity of 9.5–10,000 tons (8.6–9.1 × 10^6 kg; Bahner, 1994). The competitive position of fermentation lactic acid over synthetic lactic acid depends upon the ability to selectively produce desired stereoisomers of lactic acid (D– or L+) instead of a racemic mixture produced by the synthetic route. Use of inexpensive carbohydrate feedstocks and advances in separation technologies keep production costs low.

Example prices in 1994 for both fermentation and synthetic food grade 50% and 88% lactic acid were $0.71 and $1.15 per pound (Litchfield, 1996).

The technological and thus economic potential of lactic acid and polylactic acid suggest that the market will expand. A large United States agriprocessing company, Cargill, has constructed a new fermentation facility with a capacity of

125,000 tons/yr of lactic acid, incorporating new separation technologies.

Research and Development

Microbial Genetics and Strain Improvement

Mutants of the lactobacilli can be selected following spontaneous or induced mutagenesis. For example, following mutagenesis with ethyl methane sulfonate, three mutants of *L. delbrueckii* ATCC 9649 were selected, which produced a higher yield of lactic acid than the parent strain in a stirred-tank batch fermentation with 12% glucose. Also, the rate of lactic acid production by the mutant strains was more than twice that of the wild-type (Demirci and Pometta, 1992). *Escherichia coli*, a mix acid fermenter, was converted by mutation to a homolactic strain. A double mutant strain of *E. coli* RR1 was produced which was deficient in both phosphotransacetylase (mutation in *pta*) and in phosphoenolpyruvate carboxylase (mutation in *ppc*). This strain did not produce significant amounts of acetate or succinate, and thus fermented glucose to primarily D(–) lactic acid (Chang et al., 1999). Certainly, mutagenesis followed by selection for properties such as growth at high temperature or high lactic acid concentration remains a standard method for improvement of production strains.

However, since about 1987 there have been dramatic advances in genetic technology and in understanding genetics of the lactic acid bacteria. Reviews of research and applications of genetic technology to lactic acid production have appeared (Gasson and DeVos, 1994; Bigelis and Tsai, 1995; Arihara and Luchaasky, 1995; Davidson et al., 1995; Wang and Lee, 1997a). Research predicts that the tools for genetic engineering of lactic acid bacteria provide for an almost unlimited potential for strain development.

Developing a genetic system for lactic acid bacteria was initially dependent upon optimizing a DNA transfer method. The most significant advancement was the use of electroporative (high voltage) transformation first shown in *Lactococcus lactis* (Harlander, 1987) and then extended to Lactobacillus sp. and other Gram-positive bacteria (Cauvin and Luchansky, 1992; Luchansky et al., 1988). Genetic research with *Lactobacillus* utilizes electrotransformation to transfer plasmid vectors for cloning, expression, integration, and transposon mutagenesis. Often electroporation techniques must be optimized for each bacterial species and new strain under study.

Most plasmid vectors used for cloning, expression, integration, and transposon mutagenesis were initially developed for *Lactococcus lactis* (DeVos and Simons, 1994). Shuttle vectors with lactococcal replicons and staphylococcal resistance markers were initially electroporated into strains of lactobacilli with some success. However, consistent high frequency transformation of lactobacilli has been achieved with several cloning and mutagenesis vectors based on *Lactobacillus* replication regions from cryptic, endogenous plasmids. For example, plasmids pPSC20 and pPSC22 replicated and were stably maintained in *L. lactis*, *Bacillus subtilis*, *E. coli* and a number of Lactobacillus species (Cocconcelli et al., 1991). Table 11 lists some *Escherichia-Lactobacillus* shuttle vectors and their applications.

A list of the plasmids of *Lactobacillus* and construction of useful vectors for commercial applications was reviewed (Wang and Lee, 1997b).

There is a considerable research effort directed toward stabilizing gene expression in both the lactococci and lactobacilli by integrating desired foreign genes into the bacterial genome. Chromosomal insertion of plasmid-carried genes could prevent segregational instability that may be a problem during large-scale or continuous fermentations. The use of integrative gene cloning and nonreplicative integration vectors with

Table 11. *Escherichia-Lactobacillus* shuttle vectors and their applications.

Shuttle vectors	Genes expressed	Lactobacillus hosts	Reference
pGKV210	Catalase gene of *Lactobacillus sake* LTH677	*L. casei*	Knauf et al., 1992
pTRK159	Lactacin F gene of *Lactobacillus acidophilus*	*L. acidophilus*	Moriana and Klaenhammer, 1991
pLP3537	D-Xylose metabolism genes of *Lactobacillus pentosus*	*L. casei*	Posno et al., 1991
pSA3_Endoglucanase gene of *Clostridium thermocellum*_ *L. plantarum*	Batesylase gene of *B. stearothermophilus*	*L. plantarum*	Scheirlinck et al., 1989

From Bigelis and Tsai (1995), with permission.

lactococcal strains is reviewed in detail by Gasson and DeVos (1994). Studies demonstrated that integration occurs via homologous single crossover recombination. Using similar nonreplicative vectors in *L. lactis*, it has been shown that chromosomal genes can be insertionally inactivated and that gene replacement recombination is also feasible. The use of insertional vectors for strain stabilization, gene replacement and gene disruption has been developed for *Lactobacillus* (Arihara and Luchansky, 1995). In *Lactobacillus* stabilization of cloned genes is usually achieved by chromosomal integration based on homologous recombination with a randomly cloned DNA fragment as the integration target inserted in a nonreplicating plasmid. Using this technique, stable chromosomal integration of α-amylase from *Bacillus stearthermophilus* and a cellulase gene from *Clostridium thermocellum* was achieved in *Lactobacillus plantarum* (Scheirlinck et al., 1989). An integrative "food-grade" cloning vector for *L. acidophilus* was constructed using a piece of chromosomal DNA as an integration target (Lin et al., 1999). A second important technique for generation of stable recombinant lactic acid bacterium strains is the use of a bacteriophage integrase-mediated site-specific insertion into the host chromosome (Auvray et al., 1997; Martin et al., 2000). A third innovation is the development of an integrative vector which allows stable gene insertion specifically into the chromosomal lactose operon of *L. casei* (Gosalbes et al., 2000). The integrated foreign genes followed the same expression pattern as for the lactose genes in that they were repressed by glucose and induced by lactose.

Expression studies are encouraging in that more than 65 *Lactobacillus* genes have been cloned and expressed in *E. coli*. Many *Lactobacillus* genes have been expressed in other lactobacilli, and several heterologous genes have been transferred and expressed in *Lactobacillus* strains (Table 11). As lactic acid emerges as a major commodity chemical, research efforts are focusing on direct utilization of inexpensive carbon substrates, such as lactose-containing cheese whey, or starchy and cellulosic biomass sources. Expanding the substrate range of production strains of *Lactobacillus* or building entirely new production strains is now possible by transfer, integration, and expression of the genes for lactose metabolism from *L. casei* combined with other genes essential to catalyze a specific lactic acid production process.

The genes for both of the lactate dehydrogenase enzymes, L-LDH and D-LDH, that control lactic acid stereospecificity in the lactobacilli have been cloned and sequenced. The L-LDH enzyme from a variety of lactic acid bacteria forms a closely related class entirely distinct from the D-LDH enzymes which form a second closely related group of proteins. With broad host-range integration vectors now available, it is possible to alter the LDHs in lactobacilli by gene replacement or gene disruption. Thus, a production strain of *L. helveticus*, which normally produces a mixture of (D, L)-lactic acid, can be converted to produce exclusively either (D–) or (L+)-lactic acid (Bhowmik and Steele, 1994).

Growth of lactic acid bacteria is dependent upon a complex source of organic nitrogen compounds. All of these bacteria are fastidious organisms with multiple amino acid requirements. Thus rapid growth is dependent upon efficient proteolysis of proteins and transport of amino acids and small peptides. Hydrolysis of proteins may be by protease treatment of the medium prior to fermentation or by the action of proteolytic enzymes produced by the lactobacilli or lactococci during fermentation. In all cases, it is essential that the selected fermentation strain and the protein source be compatible for optimal cell growth and fermentation as well as low accumulation of by-products that will interfere with downstream separation of lactic acid. As a consequence, the proteolytic systems of lactic acid bacteria have been the subject of research efforts over the past 15 years. Most of the detailed biochemistry, cell location of enzymes, and genetic engineering of proteolytic enzymes that have been determined for the lactococci is reviewed by Kok and DeVos (1994). The research on proteolytic systems of *Lactobacillus* strains used in fermentation is more limited but developing rapidly (Arihara and Luchansky, 1995).

Overcoming and understanding host-bacteriophage interactions is a major topic of research with the lactic acid bacteria. For example, in *L. helveticus* a plasmid-linked restriction-modification system was identified that blocks fruitful infection by two bacteriophages (Reyes-Gavilan et al., 1990), and in *L. lactis* subsp. *cremoris*, four phage-resistance plasmids have been identified (Forde et al., 1999). Both lytic and lysogenic phages of the lactococci and lactobacilli have been and continue to be under study; genetic strategies for constructing phage-insensitive strains of the lactococci have been formulated (Klaenhammer and Fitzgerald, 1994). Walker and Klaenhammer (2000) reported combining two genetic stratagies to inhibit infection of *L. lactus* by bacteriophage ø31. The bacteria carrying a low-copy-number plasmid, pTRK360, containing the phage ø31 origin of replication, trigger an explosive production of this plasmid upon phage infection. The large number of plasmid-carried *ori31* genes compete with and inhibit phage ø31 DNA repli-

cation, resulting in an efficiency of plaquing of only 0.3. However, these workers combined this strategy with a second strategy employing antisense RNA. To produce a high level of phage ø31 antisense mRNA relative to normal sense mRNA during phage infection, the antisense cassettes containing the late-expressed phage genes (ORF 3 through ORF 6) were subcloned into pTRK360. Infected *L. lactis* carrying this plasmid produced high levels of antisense transcripts later in the lytic cycle. Growth studies at various levels of phage ø31 infection showed that using the dual strategies of competing *ori31* copies and antisense mRNAs of phage ORFs was more effective than using either method alone (Walker and Klaenhammer, 2000). The research on the lactococci is being applied to improving phage resistance of *Lactobacillus*, although presently little is known about phage resistant strategies and phage-coded counter defense mechanisms for this group of lactic acid bacteria.

Advances in Fermentation and Separation Technologies Direct fermentation of starch to lactic acid by lactobacilli is not possible when employing the preferred industrial strains of lactobacilli, considering they use sugars such as glucose or lactose efficiently but do not effectively hydrolyze starch. Although a well established two-enzyme treatment for starch hydrolysis is applied in the corn wet-milling process to produce glucose, such a process may not be usable for other starchy feedstocks used on a smaller scale. Two approaches to this problem are under development. First, lactic acid production using two starch-hydrolyzing strains, *L. amylophilus* (Mercier et al., 1992) and *L. amylovorus* (Cheng et al., 1991), has been demonstrated. Laboratory scale results in batch reactors provided conditions for rather good productivity and a greater than 90% yield of lactic acid. A second approach is an integrated method of simultaneous saccharification and fermentation (SSF) of potato starch by *Lactobacillus delbrueckii* with added glucoamylase (Tsai and Moon, 1998). Prior to SSF the crude starch was mixed with α-amylase and liquefied for 20 min at 103.5°C. The authors predict that the process which eliminates the two-step starch hydrolysis and sterilizer steps will reduce both capital cost and process time.

The productivity of the classical batch-reactor for lactic acid fermentation is lower than desirable for economically viable large-scale production. A great deal of research has been carried out on continuous fermentation processes over the past 10–15 years that promises to raise productivity. The research on continuous processes up to 1995 has been reviewed (Litchfield, 1996).

Added productivity of lactic acid fermentations can be achieved by combining continuous systems with mechanisms that allow higher bacterial cell concentrations. Research is concentrated on two mechanisms: 1) membrane recycle bioreactors (MRBs) and 2) immobilized cell systems (ICSs). The MRB consists of a continuous stirred-tank reactor in a semi-closed loop with a hollow fiber, tubular, flat, or cross-flow membrane unit that allows cell and lactic acid separation and recycle of cells back to the bioreactor. The results of a number of laboratory studies with various MRB systems demonstrate the effect of high cell concentrations on raising lactic acid productivity (Litchfield, 1996). Table 12 lists examples of published results employing various MRB systems.

The volumetric productivity (g of lactic acid/liter · h) is usually greater than 10-fold that of batch or continuous processes. Ceramic tubular membranes have been used which are both steam sterilizable and resistant to mechanical stress (Xavier et al., 1995). Fermentation production of lactic acid directly from starch was optimized in an MRB using *L. amylovorus* (Table 12). No saccharification or pre-liquefication of starch was found necessary (Zhang and Cheryan, 1994a). In summary, the research with MRB systems demonstrates that this is a successful approach to fermentation because both a high productivity is achieved and a high concentration of lactic acid is removed in the final permeate. Long-term performance of these units at high cell densities, however, has been limited to about 10 days owing to filtration-membrane clogging, which requires a cleaning operation or replacement of the membrane unit.

Research has been carried out employing immobilized cell systems (ICSs) with the goal of improving lactic acid production. Bacteria are immobilized either by entrapment in gels such as alginate beads or carrageenan beads or, alternatively, by adsorption as a biofilm on the surface of supports such as porous glass, ceramic beads or polypropylene composites (Table 13).

The results of the major investigations up to 1994 have been reviewed by Litchfield (1996). Normally the immobilized cells are exposed to the substrate-containing medium in a bioreactor where the high concentration of cells catalyzes the production of lactic acid. The spent medium with the lactic acid then passes to a recovery unit to remove the lactic acid. The medium is then renewed by adding back substrate and other nutrients and finally is recycled back to the bioreactor. Both the added sugars and the lactic acid recovered are at relatively high concentrations. In practice the various gels used for entrapment of cells have softened, leaked cells, or prevented rapid outward diffusion of the lactic acid, thus blocking cell metabolism. Also plugging and clogging of column-shaped bioreactors often

Table 12. Examples of lactic acid production in membrane recycle bioreactors.

Bacterium	Type of membrane	Substrate	Product (g/liter)	Cells (g/liter · h)	Productivity (g of lactic acid/liter · h)	Time of operation (h)	Yield (g of lactic acid/g substrate)	Reference
L. delbrueckii (*L. rhamnosus*)	Flat sheet	Glucose	59	118	65	52	0.95	Ohleyer et al., 1985
L. delbrueckii (*L. rhamnosus*)	Hollow fiber	Glucose	40	8	12	220	0.76	Major and Bull, 1988
L. delbrueckii	Tubular ceramic	Glucose	90	136	36	90	0.82	Xavier et al., 1995
L. delbrueckii (*bulgaricus*)	Hollow fiber	Lactose	117	63	84	8	0.99	Mehaia and Cheryan, 1987
L. delbrueckii (*bulgaricus*)	Hollow fiber	Lactose	89	40	22.5	280	0.89	Tejayadi and Cheryan, 1995
L. helveticus	Tubular with electrodialysis	Lactose	27	64	22	144	0.81	Boyaval et al., 1987
L. amylovorus	Hollow fiber	Starch	42	39	8.4	240	0.88–0.9	Zhang and Cheryan, 1994a

Table 13. Lactic acid production in immobilized cell reactors.

Bacterium	Bioreactor and cell support	Substrate	Product (g/liter)	Productivity (g of lactic acid/liter · h)	Time of operation (days)	Yield (g of lactic acid/g of substrate)	Reference
L. casei	Repeated batch, with biofilm on plastic composite supports	Glucose	60	0.8 ± 0.19	72	0.77	Demirci and Pometto et al., 1995
L. casei	Recycle batch, column with biofilm on polyethyleneimine foam coated glass beads	Glucose	95	4.3–4.6	12	0.95	Senthuran et al., 1997
L. casei	Repeated batch, with biofilm on plastic composite supports—s	Glucose	85–95	4.26–3.6	66	0.85–0.95	Ho et al., 1997

occurred. In addition, contamination of difficult-to-sterilize natural gels can be a problem. The use of lactic acid bacterial biofilms adsorbed on inert supports has been more successful (Table 13). Research trials with a composite support of 75% polypropylene and 25% agricultural materials and *L. casei* in a repeated batch fermentation showed excellent biofilm stability for up to 72 days (Demirci and Pometto, 1995). Using a different bioreactor configuration, *L. casei* cells on polyethyleneimine-coated foam glass beads yielded excellent results in a recycle batch fermentation. Both systems can produce an average of 4.3–4.6 g of lactic acid/liter · h (Ho et al., 1997a).

In general, the productivity of the ICSs is far lower than that of the MRB systems. However, the ICSs apparently have the advantage of long-term stability over the MRB systems. The advances in membrane-based separation and purification technologies combined with electrodialysis and ion exchange separation form the basis for scale-up large-volume production. A proprietary process employing these technologies for continuous fermentation of sugar for lactic acid production is under development by a major company in the United States (Datta et al., 1995).

Preliminary research has appeared demonstrating extractive lactic acid fermentation based on aqueous two-phase systems (ATPS). The idea is to provide a technology that simultaneously prevents product inhibition and enhances volumetric productivity. Basically, an ATPS involves an extractive step for removal of the product (lactic acid) at the same time it is being formed during the fermentation stage. For the extractive bioconversion to be effective the product must be partitioned into the phase opposite the one preferred by the microbial cells. Using a two-phase system composed of polycation, polyethyleneimine (PEI) and a neutral polymer, hydroxyethylcellulose (HEC), successful lactic acid partitioning and fermentation by *L. lactis* was demonstrated (Kwon et al., 1996). Following batch fermentation, 75–85% of the lactic acid was in the PEI layer and 90% of the cells were in HEC layer under optimal conditions. Good product yields (0.7–0.85 g/g) and productivity (8–10 g of lactic acid/liter · h) were reported in both batch and continuous fermentations. Other polymer pairs have been tested for a two-phase fermentation-extraction system (Planas et al., 1996). However, no scale-up fermentations or economic analyses of application of these approaches for commercial production have appeared.

Prospects

New Products and Future Demand for Lactic Acid

Up to 1994, the world demand for lactic acid reached about 60–65 $\times 10^3$ tons/yr, with a predicted growth of about 12–15% per year (Bahner, 1994). The major use for lactic acid in the United States is for food applications (about 85%), whereas the remainder is for industrial uses (Datta et al., 1995). Most likely, this situation will change rapidly during the 21st century.

Lactic acid is now recognized as an excellent feedstock in the chemical industry for new products. Based on the potential products from lactic acid (see Table 14), the demand for lactic acid in the future could reach 2.5–3.4 $\times 10^6$ tons/yr. New technologies are under development for manu-

Table 14. Lactic acid: potential products, volumes and value.

Product	Uses	U.S. market[a] volume (10^9 lb/yr)	Selling[a] price ($/lb)	Value ($10^6/yr)
Degradable plastics	Packaging, films	0.3–2.0[b]	0.4–0.6[b]	120–1,200
Oxychemicals:				
Propylene	Polymers, food deicers, humectants, plastic	0.8	0.55	440
Glycol	films, coatings and plastics	1.1	0.65	710
Acrylates		3.2	0.55	1,760
Propylene oxide				
"Green" chemicals and solvents:				
Esters	Plasticizers and food processing packaging	0.1–0.2[c]	0.5	50–100
Ester derivatives	Same as above	0.05–0.1[c]	0.5	25–50
Plant growth regulators:				
Poly-L-lactates	Mulch film for vegetable and fruit crops	0.05–0.1[c]	1.0	50–100
Total		5.6–7.5		3,155–4,360

[a]Market volumes and prices are for 1991 (Chemical Marketing Reporter) unless otherwise stated. Prices and values are in US$.
[b]Estimates from Battelle, SRI, Cargill (1993) announcement.
[c]Argonne's estimates.
From Datta et al. (1995), with permission.

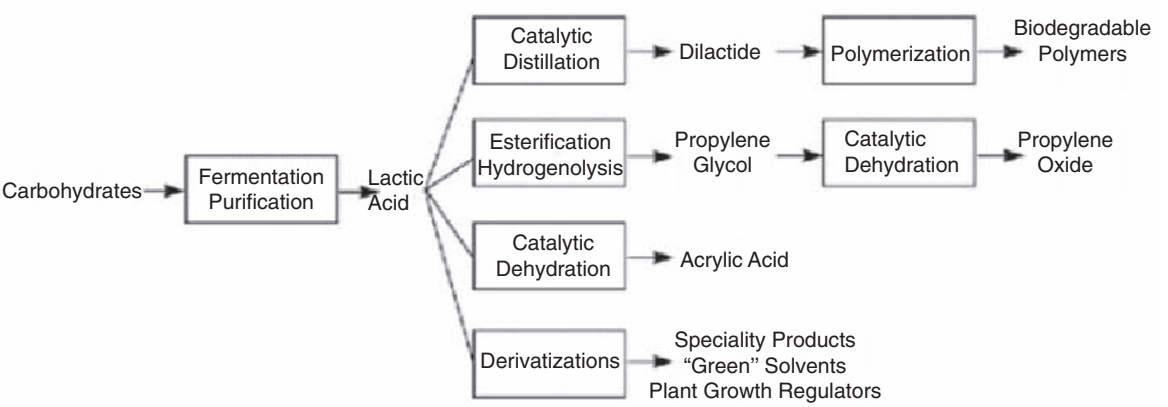

Fig. 12. Lactic acid: potential products and technologies. From Datta et al. (1995), with permission.

facturing lactic acid and for industrial scale processes for product synthesis (Fig. 12).

Research on membrane-based separation methods combined with electrodialysis forms the basis for new processes for lactic acid production. These technological improvements have led to proprietary large-scale production methods for obtaining purified concentrated lactic acid from carbohydrates without excess calcium sulfate (gypsum) or other salts in the waste stream. United States agriprocessing companies are building large-capacity production plants for simultaneous fermentation and separation of lactic acid based on this technology (Datta et al., 1995).

Both DuPont and Cargill are developing large-scale manufacturing processes for conversion of lactic acid to lactides, glycolides and prepolymers. Eventually, the goal is to develop commercial-scale processes for production of biodegradable polymers for a variety of uses. The lactide copolymers have a range of properties and promise to compete well with petroleum-based polymers such as polystyrene, polyvinyl chloride and vinylidine (Tables 15a and 15b).

Lactic acid also may be converted into a variety of other chemicals such as propylene glycol, propylene oxide, and acrylic acid by known technologies (Fig. 12). If potential uses and market volumes appear as predicted (Table 14), then commercial-scale manufacturing processes also must be developed. In the future, lactic acid esters, which are nontoxic and less volatile, may replace more toxic solvents now in use as plasti-

Table 15a. Comparison of a lactide copolymer with vinyl polymers.

Property	Vinylidene chlorine molding compounds	85/15 Copolymer of L-lactide/ε-caprolactone	Flexible PVC
Tensile strength, psi	3,000–5,000	3,200	1,500–3,500
Elongation, %	up to 250	6 to 500	200–450
Initial modulus, psi	50,000–80,000	84,000	50,000–100,000
Impact strength, ft-lb/in.	0.3–1	No break	0.4–7
Hardness, shore D	50	87	20–76
Specific gravity$^{20/4}$	1.65–1.72	1.26	1.16–1.35
Abrasion resistance	Excellent	Excellent	Excellent
DTA m.p., °C		133	None
Compression molding, °C	105–175	125–150	140–175
Ease of molding	Excellent	Excellent	Good
Weather resistance	Good	Fair to poor	Good
Lube oil resistance	Excellent	Excellent	Excellent
Solvents/nonsolvents	Resist most solvents	Benzene, chloroform/heptane, alcohol, and ether	Benzene, chloroform/heptane, alcohol, and ether
Mineral acid	Resistant	Resistant	
Caustic	Resistant	Attacked	

Abbreviations: PVC, polyvinyl chloride; psi, pounds per square inch; and DTA m. p., differential thermal analysis melting point.
From Lipinsky and Sinclair (1986), with permission.

Table 15b. Comparison of a lactide copolymer with polystyrene.

Property	Polystyrene	95/5 Copolymer of L-lactide/ε-caprolactone
Tensile strength, psi	7,000	6,900
Elongation, %	2	1.6
Initial modulus, psi	450,000	112,000
Impact strength, ft-lb/in.	0.25	0.36
Hardness, shore D	85	90
Specific gravity$^{20/4}$	1.08	1.26
DTA m.p., °C	None	145
Compression molding, °C	130–200	160–170
Ease of molding, °C	Excellent	Good
Weather resistance	Good	Fair
Lube oil resistance	Swells	Excellent
Solvents/nonsolvents	BZ, $CHCl_3$, MEK/alcohol, ether, and heptane	BZ, $CHCl_3$, MEK/(swells) alcohol, ether, and heptane
Mineral acid	Resistant	Resistant
Caustic	Resistant	Attacked

Abbreviations: BZ, benzene; MEK, methylethylketone; and see Table 15a for definitions of other abbreviations.
From Lipinsky and Sinclair (1986), with permission.

cizers and films (Table 14). Consumer demand and regulatory agencies already favor safer aerosol-forming solvents.

Competitive Processes Chemical synthesis of lactic acid from lactonitrile is the major alternative process for manufacture in competition with fermentation lactic acid. In 1995, synthetic lactic acid accounted for about 50% of the total world lactic acid production (Litchfield, 1996). The advantage of the chemical synthesis route is the ease of obtaining a highly purified lactic acid which is required for many industrial products. The synthetic lactic acid is a racemic mixture (DL), whereas fermentation lactic acid may be selectively D(−) or L(+) or DL depending on the organism catalyzing the conversion. Controlled optical purity as well as chemical purity of lactic acid is vital for commercial production of lactide polymers with specific properties required for the desired applications and potential uses of the polymers. Also, optically active lactic and lactate esters may be applied to new chiral synthesis routes for specialty chemicals such as drugs and agrichemicals (Datta et al., 1995).

Fungal fermentation of carbohydrates to lactic acid is potentially competitive with the classical *Lactobacillus* fermentation. Presently, Archer Daniels Midland's fermentation facility in Illinois, in the United States, is producing lactic acid

from enzyme-converted corn starch, using a "nonbacterial" propriety fermentation process. The production capacity is reported as 10–20 × 10^3 tons/yr of lactic acid, but details of the process are not available. *Rhizopus* spp. grow aerobically utilizing glucose or sucrose to produce L(+)-lactic acid. Some species, such as *R. oryzae* and *R. arrhizus*, can convert starch or starch-containing complex substrates to L(+)-lactic acid. *Rhizopus* spp. are obligate aerobes and heterolactic. Thus, the highest theoretical yield of lactic acid is about 0.75 g per g of glucose. Practically, experimental yields of 0.5–0.72 g/g are observed, compared to 0.85–0.9 g/g for the *Lactobacillus* fermentations (Litchfield, 1996). However, the yield of lactic acid can be improved and production of side products reduced by the application of genetic engineering to the metabolic pathways. The advantage of the *Rhizopus* spp. is that they grow and produce lactic acid in an inorganic mineral medium containing only ammonia or nitrate as a nitrogen source and no amino acid or vitamin requirements. This property greatly simplifies the downstream separation and purification of the lactic acid product.

Future Lactic Acid Bacteria The future commercial emphasis is on production of lactic acid as a bulk chemical converted from biomass by microbial fermentation. Clearly, future research employing genetic engineering will be aimed at converting bacteria into more efficient chemical reactors for this process. The desired properties of a future engineered lactic acid bacteria are listed in Table 16. Only the first four of these properties are characteristic of the present industrial strains of *Lactobacillus* and related lactic acid producers. The problems of productivity and product tolerance are limiting factors in batch fermentations. The desired high productivity and tolerance to lactic acid are now being dealt with using new continuous membrane-based fermentation-separation technology (FST), applied to commercial production. The last three properties listed (Table 16) limit the effective use of the *Lactobacillus* strains.

Transfer of metabolic pathways and phage-resistance properties between bacteria using genetic engineering technologies (GET) is available for developing future super bacteria. This new set of industrial lactic acid bacteria will probably not be based on the present *Lactobacillus*, inasmuch as overcoming the complex growth requirement is an enormous task. Conversion of a chosen bacterium into a true homolactic L(+)- or D(−)-lactic acid producer strain is now achievable through genetic engineering technology (GET).

Section 3: Gluconic Acid

Introduction

Two reviews cover the microbial production processes for conversion of glucose to gluconic acid and its calcium and sodium salts (Milson and Meers, 1985; Roehr et al., 1996). Hlasiwetz and Habermann (1870) first identified gluconic acid as a major product of the chemical oxidation of glucose. Boutroux (1880), while studying the microbial fermentation of glucose in the presence of calcium carbonate, isolated calcium gluconate. The gluconic acid-producing organism was later identified as a strain of *Mycoderma aceti*, most probably a *Gluconobacter* sp. Forty-two years later, Molliard (1922) reported that gluconic acid was produced by *Sterigmatocystis nigra* (probably *Aspergillus niger*) during growth with sucrose. Within a few years, gluconic acid production was associated with many fungi, such as *Penicillium* species, strains of *A. niger*, and strains of aerobic bacteria such as *Pseudomonas*, *Gluconobacter*, and *Zymomonas*.

The list of uses of gluconic acid and its derivatives is astoundingly diverse and has continued to grow in the food, pharmaceutical, and metal treatments industries throughout the 20th century (see "Commercial Applications" in this Chapter). As a result, much attention has been focused on processes for production of gluconic acid and its salts by oxidation of glucose. There

Table 16. Future lactic acid bacteria.

Desired properties	Present bacteria (Lactobacillus)	Developing technologies
High yield, >0.9g of lactic acid/g of substrate	Homolactic	GET
Optical purity D(−) or L(+) options	Yes	GET
High temp. (42–55°C)	Grow and ferment	FST
Anaerobic metabolism	Yes	FST
Product tolerance	About 10%	FST
High productivity >10g of lactic acid/liter · h	Low	FST
Broad substrate use (cellulose, starch, etc.)	Limited	GET
Grow in mineral salts	Complex requirements	GET
Virus infection control	Liable to infection	GET

Abbreviations: GET, Genetic Engineering Technologies; and FST, Fermentation and Separation Technologies.

are four categories of commercial processes: chemical processes; the *A. niger* process and other fungal processes; the *Gluconobacter* process and other bacterial processes; and the immobilized enzyme processes.

The chemical conversion of D-glucose to D-glucono-δ-lactone and thence to D-gluconic acid has been used in manufacturing processes. Electrochemical oxidation in the presence of bromide (Isbell et al., 1932) and oxidation with air or oxygen (employing a catalyst; deWilt, 1972) are examples of methods in use. However, because of the appearance (during chemical production) of unwanted side-products that require difficult downstream purification, the more specific microbial "fermentations" and other biochemical processes are definitely competitive.

Aspergillus niger was the major organism in the development of microbial processes for conversion of glucose to gluconic acid. Bernhauer (Bernhauer, 1924; Bernhauer, 1928) demonstrated that selected strains of *A. niger* carried out this conversion in high yields when the acid produced was neutralized, usually by calcium carbonate. Research on the technology of gluconic acid production started in the United States Department of Agriculture (USDA) in 1926. About this time at the USDA, a pilot plant process was developed for gluconic acid production using selected strains of *Penicillium luteum purpurogenum* in a surface-type "fermentation" (Herrick and May, 1928). But the critically important development was the production of gluconic from glucose by the submerged culture technology using stirring and forced aeration in the reactor (May et al., 1934). With either *A. niger* or *P. luteum* used in the oxidation process, a 90% yield in only 48–60 h was reported. Employing a strain of *A. niger* (strain 67) with the modern submerged reactor technology, pilot plant studies yielded 95% conversion of glucose (in a 15–20% solution) in 24 h (Moyer et al., 1937). The commercial-scale technique utilizes a 25% glucose solution, achieves a 95% conversion in the submerged reactor, and involves neutralization by calcium carbonate as well as reuse of mycelium in 24-h cycles. Sodium gluconate is the product desired for most commercial uses. Blom et al. (1952) developed the basic process for sodium gluconate production in which gluconic acid is neutralized (~pH 6.5) with sodium hydroxide during the microbial conversion. This process is essentially that used by most production plants today. A continuous gluconic acid production process was developed in Japan by Fujisawa Pharmaceutical Group (Yamada, 1977). This process employed *A. niger* and was carried out using conventional fermentation techniques. In 1977, production of gluconic acid in Japan had reached 8,500 tons/yr.

Production methods employing the bacterial strains *Acetobacter* (*Gluconobacter*) were developed and patented early in the last century (Currie and Carter, 1930; Verhave, 1930). The genus *Gluconobacter* and the genus *Acetobacter* are closely related and are accepted as two separate genera in the family Acetobacteraceae, primarily based on the proposal by Asai (1968). The main distinction is that the *Acetobacter* sp. rapidly oxidize ethanol to acetic acid and only slowly oxidize glucose to gluconic acid, whereas *Gluconobacter* sp. rapidly oxidize glucose to gluconic acid. The *Gluconobacter oxydans* ATCC621H (formerly *A. oxydans*) was the most frequently used strain in early gluconic acid production processes. The family grouping Acetobacteraceae is supported by 16S ribosomal DNA sequence similarities of eight species (Sievers et al., 1994b). However, this molecular analysis does not present a convincing case for separation of *G. oxydans* from the species *Acetobacter*. The first production techniques employed a modified "quick vinegar" process used early in production of acetic acid (see the "Acetic Acid" in this Chapter). However, a more modern submerged fermentation process utilizing *Gluconobacter oxydans* was also developed (Currie and Finlay, 1933). This process is very much like the present method used for vinegar acetic acid production by *Acetobacter* (see the "Acetic Acid" in this Chapter). Increases in gluconic acid concentration and in productivity have been achieved in continuously performed processes combined with immobilization of *Gluconobacter* cells on carriers (Shiraishi et al., 1989a). Research on process development has proceeded and is covered in "Areas of Research and Development."

Interest in a gluconic acid production process using a novel acetic acid bacterium, *Acetobacter methanolicus*, has emerged (Uhlig et al., 1986). *Acetobacter methanolicus* is a facultative methylotrophic bacterium that can grow on methanol as a sole carbon and energy source. Several patents document the possible future use of this organism (Babel et al., 1986; Babel et al., 1987; Babel et al., 1988; Babel et al., 1991).

Acetobacter diazotrophicus is an isolated aerobic nitrogen-fixing acetic acid bacterium (Gilles et al., 1989). It grows well in simple mineral salts medium and oxidizes glucose at high rates in acid conditions (pH 3.5) and (unlike *G. oxydans*) in the presence of high concentrations of gluconic acid (Attwood et al., 1991). Because of these characteristics, the use of *A. diazotrophicus* is of interest for bulk production of gluconic acid.

A fourth bacterial candidate, *Zymomonas mobilis*, can rapidly convert mixtures of glucose and fructose to gluconic acid and sorbitol with almost a 100% yield. This coupled oxidation-reduction process depends upon the *Z. mobilis*

enzyme glucose-fructose oxidoreductase present in cells grown with these sugars (Zachariou and Scopes, 1986). Several protocols for cell treatment and cell immobilization are under study for development of commercial processes leading to large-scale production of the two products sorbitol and gluconic acid (see "Areas of Research and Development" in this Chapter).

A fourth process for gluconic acid production is the direct use of the enzyme glucose oxidase (GO) as a catalyst to carry out the rather simple reaction:

$$\text{D-glucose} + O_2 \xrightarrow{GO} \delta\text{-gluconolactone} + H_2O_2$$

Glucose oxidase isolated from *Aspergillus* or other fungi has been immobilized on a number of supports, and studies show that it is quite sensitive to the hydrogen peroxide formed during glucose oxidation. Therefore, a number of methods for co-immobilizing GO together with catalase have been developed (e.g., Bucholz and Goedelmann, 1978). This approach is, in theory, similar to the immobilization of *A. niger* mycelia, either flocculated (as pellets) or covalently bound to various supports (Sakura et al., 1989). Systems employing other enzymes that oxidize glucose also have been studied. A coupled system that simultaneously oxidizes glucose to gluconic acid and transfers electrons via NAD(H) to reduce fructose to mannitol has been proposed. Bacterial glucose dehydrogenase (GH) and mannitol dehydrogenase (MH) from different organisms have been immobilized to carry out the following reactions:

$$\text{D-glucose} + NAD^{+GH} \rightarrow \text{D-gluconolactose} + NADH + H^+$$

$$\text{D-fructose} + NADH + H^{+MH} \rightarrow \text{D-mannitol} + NAD^+$$

Membrane reactors have been employed that allow retention and recycling of NAD (Howaldt et al., 1988; Howaldt et al., 1990; Oben et al., 1996). This approach is probably technologically too complex to permit scale-up for bulk commercial production. A more likely candidate for commercial production by an immobilized enzyme process is the use of the single enzyme glucose-fructose oxidoreductase (GFOR) of *Zymomonas mobilis* (Nidetzky et al., 1997; Silva-Martinez et al., 1998).

In summary, considering the production of gluconic acid, the *A. niger* process appears to dominate present-day manufacturing methods. Likewise, the continuing research and development efforts utilizing the *Gluconobacter* process are clearly evident. In addition, the emergence of two new bacterial processes employing *A. methanolicus* and particularly *Z. mobilis* has opened promising new routes for production that may be competitive in the future. These bacterial processes are emphasized in the sections below.

Scientific Background

D-Gluconic acid is one of the oxidation products of D-glucose. There are three major reactions catalyzed by microorganisms for oxidation of D-glucopyranose to yield D-glucono-δ-lactone by removing two hydrogens (Fig. 13).

D-Glucono-δ-lactone is in equilibrium with gluconic acid in water solution. Also gluconic acid is in equlibrium with D-glucono-γ-lactone; in addition, gluconic acid ionizes in water to yield a gluconate anion and a hydrogen ion. Thus, a water solution of gluconic acid is a rather complex system depicted at the bottom of Fig. 13. The equilibria shown influence the apparent acid dissociation constant with an approximate pKa of 3.7. Thus, gluconic acid acts as a weak acid in solution and is highly soluble in water. The equilibria between the gluconolactones and the free gluconic acid are attained very slowly. However, a lactonase, produced by most glucose-oxidizing bacteria and fungi, catalyzes a rapid hydration of the lactones (Fig. 13). The sodium salt of gluconic acid has the property of

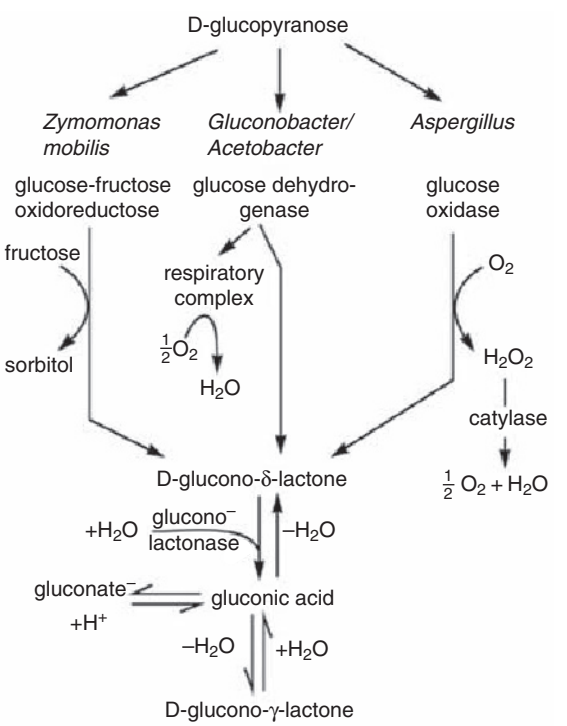

Fig. 13. Major routes of enzymatic oxidation of glucose to gluconolactone by bacteria and fungi. Gluconolactonase, present in all of these organisms, catalyzes formation of gluconic acid. Gluconic acid is in equilibrium with the lactones and gluconate in water solution.

forming complexes with various metal ions. The high metal sequestering action of sodium gluconate has led to important commercial uses in many varied processes and products (see "Commercial Applications" in this Chapter).

The biochemical pathway of glucose oxidation to gluconic acid by *Aspergillus niger* and most other fungi is catalyzed by glucose oxidase followed by a catalase which in turn removes H_2O_2 (Fig. 13). The details of the physiology and biochemistry of the fungal process for gluconic acid production were reveiwed by Roehr et al. (1996) and will not be covered here.

The bacteria employed or in processes under development for gluconic acid production are listed in Table 17. *Gluconobacter oxydans*, *A. methanolicus* and *A. diazotrophicus* are closely related strains (Sievers et al., 1994b). All three species utilize the membrane bound D-glucose dehydrogenase enzyme pathway for oxidation of D-glucose to D-gluconic acid (Fig. 13).

The biochemistry and suggested bioenergetics of the glucose-oxidizing system and respiratory chain for *Gluconobacter* and *Acetobacter* have been extensively studied and reviewed (Matsushita et al., 1994). Figure 14 is a cartoon of the

Table 17. Bacteria used in developing processes for gluconic acid production.

Organism	Properties	References
Gluconobacter suboxydans		
or *G. oxydans* subsp. *suboxydans*		
or *G. melanogenus*		
ATCC 621H, IFO 3290	Grows with vitamins	Sievers et al., 1994b
IF03293, ATCC9937	Produces ketogluconates and gluconic acid from glucose	
Acetobacter methanolicus		
MB58, IMET10945	Produces only gluconic acid from glucose	Uhlig et al., 1986
LMG 1668	Grows on methanol, requires pantothenic acid	Sievers et al., 1994c
Acetobacter diazotropicus		
ATCC 49037	Can grow aerobically with N_2 as sole N source Simple mineral medium	Gillis et al., 1989
Zymomonas mobilis	Produces ketogluconates and gluconic acid from glucose	
DSMZ473	Produces only gluconic acid and sorbitol from glucose	Zachariou and
Strains ZM6, ATCC 29191	and fructose	Scopes, 1986

Abbreviations: ATCC, American Type Culture Collection, Rockville, MD; DSMZ, Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany; IFO, Institute for Fermentation, Osaka, Japan; IMET, Institute für Mikrobiologie und Experimentelle Therapie, Jena, Germany; LMG, Laboratory of Microbiology, University of Ghent, Belgium; and ZM, Zentralinstitute für Mikrobiologie und Experimentelle Therapie, Jena, Germany.

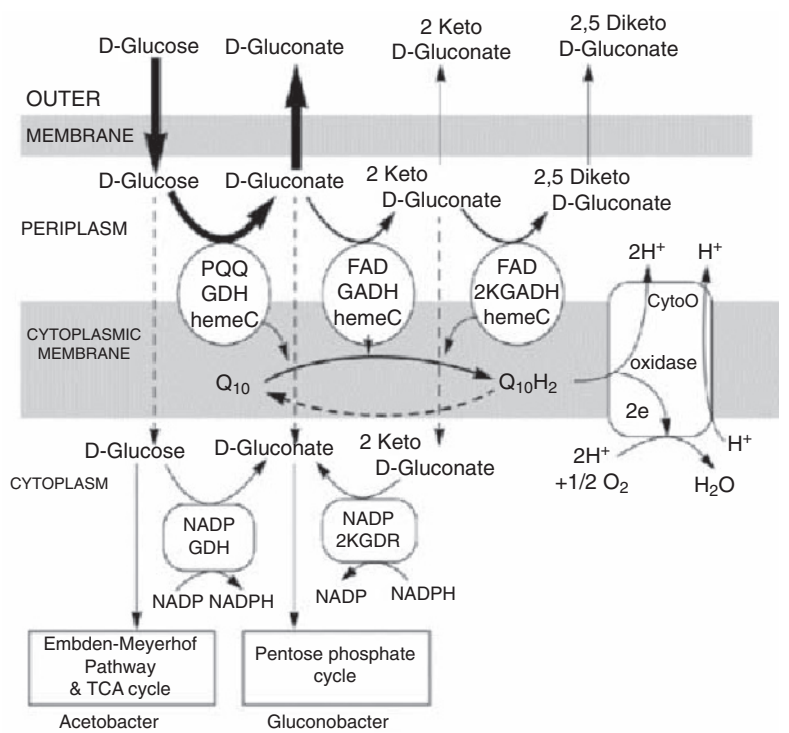

Fig. 14. Oxidation of glucose to gluconic acid by *Gluconobacter* and *Acetobacter*. Quinoprotein D-glucose dehydrogenase (GDH) and flavoproteins D-gluconate dehydrogenase (GADH) and 2-keto-D-gluconate dehydrogenase (2kGADH) are located on the outer surface of the cytoplasmic membrane. Cytochrome *o* (Cyto *O*) accepts electrons from the dehydrogenases via ubiquinone (Q_{10}) and cytochrome *c* (hemeC). Ubiquinol ($Q_{10}H_2$) is oxidized by cytochrome *o* oxidase (or Q oxidase) to generate a proton gradient. NADP-dependent D-glucose dehydrogenase (GDH) and NADP-dependent 2-keto-D-gluconate reductase (2KGR) work in the cytoplasm. Redrawn from Matsushita et al. (1994), with permission.

proposed arrangement of the elements of the glucose-oxidizing system.

These bacteria produce gluconic acid from glucose mainly via a membrane-bound enzyme, D-glucose dehydrogenase (GDH), with pyrroloquinoline quinone (PQQ) attached as a prosthetic group. *Gluconobacter oxydans* also produces varying amounts of 2-keto-D-gluconate and 2,5-diketo-D-gluconate by further oxidation of gluconic acid catalyzed by the two enzymes gluconate dehydrogenase (GADH) and 2-keto-D-gluconate dehydrogenase (2KGADH). These two enzymes are also membrane bound, each containing three subunits: a cytochrome *c*; a flavoprotein with a covalently bound FAD; and a third subunit. These oxidations take place on the outer surface of the cytoplasmic membrane. Mostly gluconic acid together with smaller amounts of the other two oxidation products accumulates in the beers. The dehydrogenases are linked to the respiratory chain through ubiquinone (Q_{10}) in the cytoplasmic membrane (Fig. 14). A set of $NADP^+$-dependent GDH and GADH enzymes is located in the cytoplasm, where it functions to interchange glucose and the gluconates. D-Glucose is metabolized primarily through the Embden-Meyerhof pathway and tricarboxylic acid (TCA) cycle by most *Acetobacter* sp. Alternatively, *Gluconobacter* sp. convert glucose to gluconate, which is metabolized through the pentose phosphate pathway (Fig. 14). Gluconate production by *G. oxydans* is largely catalyzed by the membrane-bound GDH which has 30 times the activity of the cytoplasmic $NADP^+$-dependent GDH (Pronk et al., 1989). *Gluconobacter* and some species of *Acetobacter* can accumulate high concentrations of gluconic acid and smaller amounts of ketogluconates outside the cells. The oxidative reactions carried out by the sugar-oxidizing systems are coupled to the respiratory chain in which the electron transfer can develop a proton-electrochemical gradient across the cytoplasmic membrane, as diagrammed in Fig. 14. The proton gradient is used for ATP production and energetic events for cell growth and maintenance. But it has been shown that the coupling of glucose oxidation to the growth yields for *G. oxydans* is very inefficient at pH values less than 5.0 and at high glucose concentrations (Olijve and Kok, 1979b). Thus, a property of the respiratory chains of both *Gluconobacter* and *Acetobacter* is the rapid oxidation of high concentrations of alcohols and sugars while not permitting a high rate of energy generation. How is the potential for producing or utilizing the proton gradient (shown in Fig. 14) somehow uncoupled? In the case of *Gluconobacter* a bypass, cytochrome δ oxidase system, is apparently induced at low pH, which allows a high rate of sugar (or alcohol) oxidation without producing a large proton gradient (Matsushita et al., 1994). But many *Acetobacter* spp. do not produce the bypass cytochrome oxidase system, and it remains unclear how uncoupling of energy production occurs. These acetic acid bacteria have evolved to live in specific environments, such as surfaces of fruit and flower parts, where high concentrations of sugars and alcohols exist under aerobic conditions.

The further oxidation of gluconic acid to the ketogluconic acids by *Gluconobacter* or *Acetobacter* (illustrated in Fig. 14) is a potentially undesirable reaction when using these organisms for gluconic acid production. Suppression of most ketogluconate formation has been achieved by maintaining the pH at low levels (Roehr et al., 1996). The best results for gluconate production by *G. oxydans* depend upon the presence of high glucose concentrations, low pH and high aeration.

Uhlig et al. (1986) described a novel acetic acid bacterium (*A. methanolicus*) that is a facultative methylotroph (Table 17). This pink-pigmented bacterium grows well on methanol or glycerol or glucose in a mineral medium containing only pantothenic acid as a growth factor. More important, glucose is strongly oxidized to gluconate, but 2-, 5-, or 2,5-ketogluconates are not formed. Based on 16S-rRNA sequences, *A. methanolicus* was found to be closely related to other species in the genus *Acetobacter* and also to *G. oxydans* (Sievers et al., 1994c). *Acetobacter methanolicus* is different from other acetic acid bacteria in that it contains a methanol oxidase respiratory chain, as do other methylotrophs, in addition to sugar and alcohol oxidase respiratory chains. The methanol oxidase system consists of methanol dehydrogenase, which is a quinoprotein dehydrogenase (PQQ), and cytochromes c_L and c_H located free in the periplasm together with a membrane-bound cytochrome *c* oxidase which is either cytochrome *co* or aa_3 (Matsushita et al., 1994). In contrast, as depicted in Fig. 14, all of the primary dehydrogenases that are part of the sugar- and ethanol-oxidizing systems are membrane-bound and donate electrons to ubiquinone and a terminal ubiquinol oxidase such as cytochrome *o*, *a* or *d*. Methanol is assimilated as formaldehyde using the ribulose monophosphate pathway through fructose-1, 6-biphosphate.

An interesting aerobic nitrogen-fixing bacterium, *A. diazotrophicus*, was isolated from sugar cane in Brazil. DNA-rRNA hybridization placed this organism in the genus *Acetobacter* (Gilles et al., 1989). Additional characteristics of *A. diazotrophicus* include growth on a simple mineral media, high rates of gluconic acid formation, growth and gluconic acid production at high glu-

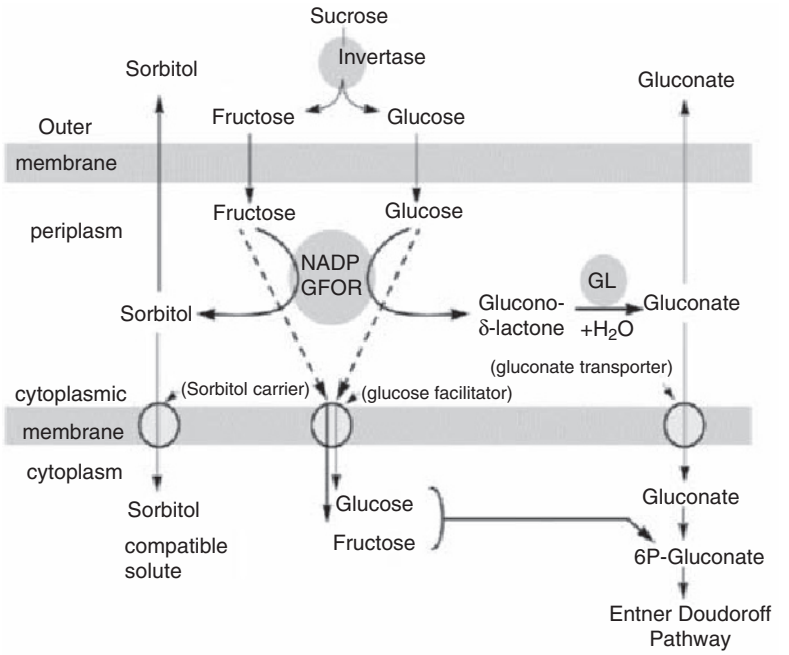

Fig. 15. Zymomonas mobilis production and utilization of gluconate and sorbitol from glucose and fructose. Sucrose may be hydrolyzed to glucose and fructose by an extracellular invertase. The periplasmic enzyme glucose fructose oxidoreductase (GFOR) with a tightly bound NADP converts the two sugars to D-gluconolactone and D-sorbitol. Gluconolactone is hydrolyzed to gluconate (gluconic acid) by a periplasmic gluconolactonase (GL). During normal metabolism, gluconate is utilized for carbon and energy via the Entner-Doudoroff pathway. Some glucose and fructose also are utilized. The sorbitol taken into the cell acts as an osmoprotectant. When normal metabolism is blocked, gluconate and sorbitol are manufactured and exit the cell's periplasm in equimolar amounts. Redrawn from Loos et al. (1994), with permission.

cose concentrations of 15% and at low pH, down to pH 3.5. These properties indicate that *A. diazotrophicus* may be of interest in commercial production (Attwood et al., 1991). In nature, *A. diazotrophicus* forms a symbiotic association with the sugar cane plant. During bacterial nitrogen-fixation, about one half of the fixed nitrogen is exported and made available as an organic nitrogen source for the host. The plant provides mainly sugars that are oxidized by the bacterium, some of which provide carbon and energy for growth. Glucose metabolism in *A. diazotrophicus* (like in *G. oxydans*) proceeds exclusively by the pentose-phosphate pathway. No enzymes peculiar to the Embden-Meyerhof or Entner-Douderoff pathways were detected (Attwood et al., 1991). However, the major portion of the glucose is oxidized via the membrane-bound PPQ-GDH and cytochrome quinoloxidase system. This oxidation pathway must provide a means to maintain an anaerobic environment within the cell so that the O_2-sensitive nitrogenase remains active. When bacteria are grown at low NH_3 (1 mM) the glucose dehydrogenase activities are 6.8- to 10-fold higher than when cells are grown in high NH_3 (40 mM). Isolated membranes from bacteria grown with low NH_3 showed an equivalent higher glucose oxidase activity (Flores-Encarnacion and Contreras-Zentella, 1999). Thus the respiratory system appears to be adjusted for scavenging oxygen to protect the nitrogenase, considering nitrogenase activity is much higher in cells with low NH_3 and growth is N_2-dependent.

The well-known ethanologenic bacterium, *Zymomonas mobilis*, has attracted attention as a candidate for development of processes for gluconic acid production (Table 17). Using glucose-fructose oxidoreductase (GFOR), an enzyme with a tightly coupled NADP as a hydrogen carrier (Fig. 15), this organism can convert a mixture of glucose and fructose, or sucrose, into gluconono-δ-lactone and sorbitol.

In addition, the enzyme glucono-δ-lactonase (GL) ensures rapid hydrolysis of the lactone to gluconic acid (Zachariou and Scopes, 1986). Under normal conditions the gluconate is taken up by these cells, phosphorylated, and utilized via the Entner-Doudoroff metabolic pathway for energy and carbon compounds required for growth. However, procedures are under development to permeabilize pregrown cells for production of gluconic acid and sorbitol, beginning with high concentrations of glucose and fructose (Roehr et al., 1996). Permeabilized cells leak their cofactors such as ATP and NADH, and thus the gluconic acid produced is not further metabolized. Silveira et al. (1999) reported that they obtained high yields of gluconic acid and sorbitol with untreated cells of *Z. mobilis* when high concentrations of glucose plus fructose (650 g/liter) were present. The high yields of gluconic acid were found to be due to inhibition of normal metabolism, permitting diversion of most substrates through the GFOR/GL pathway (Silveira et al., 1999).

Zymomonas mobilis can grow anaerobically in media containing very high concentrations of

sugars, such as glucose and sucrose. In natural habitats, such as fruit juices, plant saps and honey, the bacteria may encounter dry periods producing environments with high concentrations of sugars. It was demonstrated that sorbitol promotes growth of *Z. mobilis* in high-sugar solutions. It was proposed that probably sorbitol functions to counteract osmotic stress by acting as a compatible solute (Loos et al., 1994). The enzyme, GFOR, is located in the periplasm of *Z. mobilis* and makes up about one percent of the total cell protein (Fig. 15). The GFOR/GL system has a dual role. It converts fructose into sorbitol, which acts as a compatible solute permitting growth and survival of *Z. mobilis* in solutions of 30% sugar or higher. Simultaneously, glucose is converted into gluconic acid, some of which may be transported into the cell, phosphorylated and become available to the Entner-Doudoroff metabolic pathway for cell growth (Fig. 15). Clearly the bulk of both sorbitol and gluconic acid exits the cell under conditions where metabolism is blocked and fructose plus glucose concentrations are high.

Commercial Applications

Uses of Gluconic Acid and Gluconates Brief summaries are found in Milson and Meers (1985) and Roehr et al. (1996). D-Gluconic acid is a nontoxic and noncorrosive weak acid that is very soluble in water. It also forms water-soluble complexes with heavy metal and alkaline metal cations. Because gluconic acid is considered either a safe food additive or even a "food" in most countries, it has been of use in many processes related to the food industry. In the dairy industry, gluconic acid added to cleaners prevents calcium salt deposition in milking machinery and milk storage vessels. It is used in gentle metal-cleaning operations for aluminum and steel containers and equipment in food processing plants. Gluconic acid addition to various foods and beverages prevents cloudiness, complexes trace amounts of heavy metals and improves the food by adding a mild sourness to the taste. Gluconic acid is usually marketed as a 50% water solution, whereas the D-glucono-δ-lactone is commercially available as a crystalline solid. The glucono-δ-lactone is added as a slow-acting acidulant in baking powders for prepared instant cake mixes or instant bread mixes. Glucono-δ-lactone also is added during production of cured meat products such as in sausage preparations.

Sodium gluconate is the major commercial product used today, available either as a solid or in solution. The multiple uses of sodium gluconate depend upon its sequestering action with calcium, magnesium and iron in the presence of alkaline solutions of sodium carbonate or sodium hydroxide. For example, cleaning solutions are marketed to remove oxide scales or paint and lacquer residues from metal or glass surfaces. Also, sodium gluconate solutions are used in the textile industry to prevent iron deposition. Sodium gluconate acts as a plasticizer and a retardant of the setting process when mixed into concrete. Calcium gluconate is widely used for antacid and calcium supplement preparations. Ferrous gluconate is used in dietary iron supplement preparations. Pharmaceutical preparations containing nitrogen bases are often marketed as gluconates, as are disinfectants such as chlorohexidine gluconate.

Industrial Production The total world production of gluconic acid and gluconate in 1996 was estimated as $100–120 \times 10^6$ lbs/yr, of which 80% was sodium gluconate (Roehr et al., 1996). The standard for commercial production of gluconic acid or gluconates is the *Aspergillus niger* process. Although this process is basically a biochemical conversion, it is presently competitive with chemical, electrochemical, or catalytic oxidation techniques because of their lower yields and side reactions.

The *A. niger* process is presently superior to the older *Gluconobacter* and *Acetobacter* processes for a number of reasons summarized here and previously mentioned (Milson and Meers, 1985; Roehr et al., 1996). First, the requirements for *A. niger* growth are only mineral salts, a carbon source (such as glucose) and aerobic conditions. In contrast, the *Gluconobacter* species usually require a number of vitamins for growth. Secondly, for *A. niger*, the theoretical yield of gluconic acid from glucose is 100%, although a practical yield is usually between 90 and 95%. The yield for *Gluconobacter* is usually lower (about 75–80%) because of further enzymatic oxidation of gluconic acid to 2-keto-gluconate and 2,5-diketogluconate (Fig. 14). By careful attention to maintaining a moderately high glucose concentration and controlling the pH, the appearance of ketoacids can be minimized to less than 10% of the product (Noury and Van der Lande, 1962). But these ketoacids must then be removed during downstream processing. Thirdly, pregrown *A. niger* mycelia suspended in production media with glucose (250–380 g/liter) at pH 6.5 will produce almost 95% gluconic acid at a productivity of 13 g/liter · h, whereas strains of *Gluconobacter* are usually sensitive to glucose concentrations greater than 100 g/liter. Ziffer (1971) described a fed-batch method by which *A. niger* converted glucose (600 g/liter) to gluconic acid in 60–70 h. Crystallization of the sodium gluconate was prevented by allowing the pH to slip down to 3.2–3.5 during the process. In sum-

mary, the *A. niger* process is used for commercial production because it can produce (at a high productivity) a high yield of gluconic acid or gluconates in a relatively pure state requiring a minimum of downstream processing or purification. However, the competitive position of the *A. niger* process has been challenged by two new bacterial processes (see "Areas of Research and Development" in this Chapter).

The selling price of gluconic acid (50% solution, technical grade) was \$0.50–0.56 per lb and of sodium gluconate (powder, technical grade) \$0.66–0.70 per lb (Anonymous, 2000).

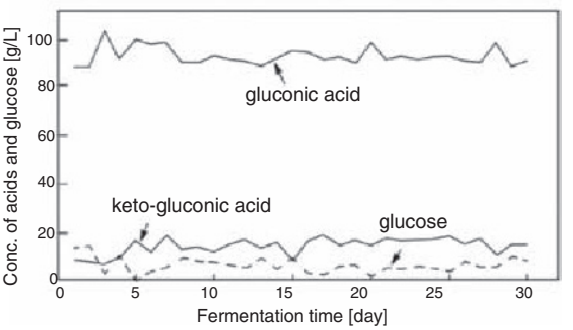

Fig. 16. Continuous production of gluconic acid from glucose by *Gluconobacter suboxydans* immobilized by adsorption on a ceramic honeycomb monolith reactor. Production was for one month at glucose, 100 g/liter; reactor residence time, 3.5 h; and aeration rate, 900 cm^3/min. The high productivity was 26.3 g/liter · h, with a yield of 84.6% gluconic acid. From Shiraishi et al. (1989b), with permission.

Areas of Research and Development

GLUCONOBACTER AND ACETOBACTER STRAINS AND PROCESS DEVELOPMENT The bacterial strains presently being considered for incorporation into future processes for gluconic acid production are listed in Table 17. Initially, *G. oxydans* was used for production; it became evident that this organism presents important barriers to commercial process development.

First, the rapid growth requirement for vitamins and (for some strains) other organic nitrogen sources complicates the growth medium and later the downstream purification of the final product. Second, this bacterium is sensitive to low pH and to high concentrations of glucose and gluconic acid, resulting in low productivity and low final concentrations of gluconic acid in the beer. Third, the significant further oxidation of gluconic acid to 2-keto and 2,5-diketogluconate lowers the final yield and adds by-products that must be removed in later purification.

Process development and employment of alternative bacterial strains have bypassed some of these problems. Shiraishi et al. (1989b) investigated continuous production of gluconic acid with *G. suboxydans* IFO 3290 immobilized by adsorption on ceramic honeycomb monoliths. This type of ceramic with uniform 1- or 2-mm channels was found to be ideal for allowing rapid gassing (up to 900 cm^3/min) as well as effective immobilization and metabolism by aerobic bacteria. Using a laboratory-scale ceramic reactor (immobilized *G. suboxydans*), a continuous conversion of glucose (100 gm/liter) was performed for 30 days, during which time the adsorbed cells remained stable (Fig. 16).

A gluconic acid yield of 84.6% and glucose conversion of 94% were achieved. The calculated productivity of 26.3 g/liter · h was the highest ever reported for *Gluconobacter*. Unfortunately, the production of keto-gluconic acid was about 9%. Attempts to reduce the ketogluconate concentration using this continuous immobilized-cell method (changing the system to a three-stage reactor) were only partially successful (Shiraishi et al., 1989a).

Transposon, Tn*5*-induced mutagenesis of *G. oxydans* was demonstrated (Gupta et al., 1997). Using this method, Gupta et al. (1999) have produced a mutant of *G. oxydans* ATCC 9937 (mutant C11) that is defective in gluconic acid dehydrogenase (GADH) and thus produces only gluconic acid from glucose without further oxidation of gluconic acid to 2-ketogluconate or 2,5-diketogluconate (see Fig. 14). Mutant C11 will certainly be attractive for development of a gluconic acid production process free of ketogluconate synthesis.

During the 1980s and 1990s, the Institute of Biochemistry in Leipzig began developing a process for gluconic acid production carried out with a methanol-utilizing strain Acetobacter methanolicus MG58 (IMET 10945T; Babel et al., 1986; Babel et al., 1991). The organism was grown on methanol and the growth process ended owing to a deficiency of nitrogen and phosphorous. Cells (5–20 g of dry biomass per liter; BM) were suspended in the reactor vessel (F) and 150–250 g of glucose/liter was added. During the batch phase (10–20 h), glucose was converted into gluconic acid and the pH dropped to about 1.5–2.0. A continuous process was started, adding glucose (G) to the recycled permeated stream to maintain a level of gluconic acid at 200 g/liter (CGA) in the reactor (Fig. 17).

Even at this high concentration of gluconic acid, the *A. methanolicus* cells, when continuously fed glucose, maintained a synthesis rate of gluconic acid at 20–30 g/liter · h for seven days (Poehland et al., 1993). The medium retention time in the reactor (F) and the permeate stream

rate were controlled by pumps in the recycle circuit, which runs through a microfiltration module (MF). This arrangement continuously removes gluconic acid and other ingredients from the medium. The ultrafiltration output was passed continuously into a electrodialysis apparatus (ED) to concentrate and purify the gluconic acid (GA). Optimization experiments were carred out in a laboratory-scale system with a 30-liter reactor vessel. Figure 18 illustrates a continuous gluconic acid process employing *A. methanolicus* and lasting more than five days.

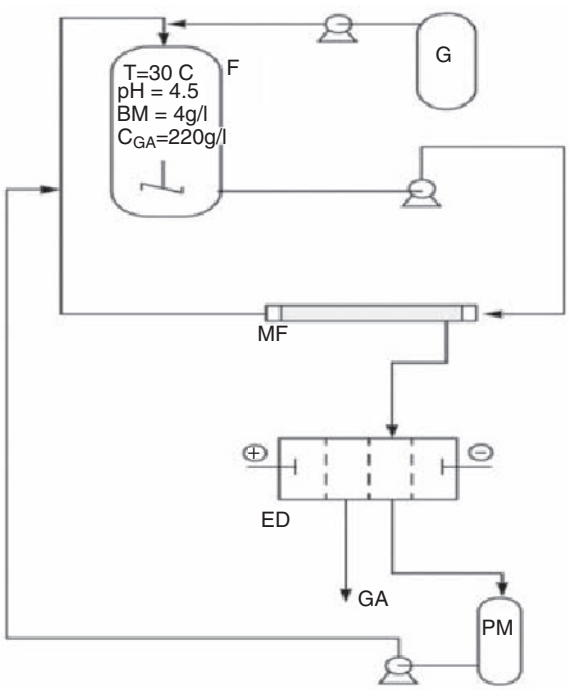

Fig. 17. Flow diagram of the gluconic acid synthesis process utilizing *Acetobacter methanolicus*. See the text for details. Redrawn from Poehland et al. (1993), with permission.

The published results are encouraging for further process development because this organism can withstand high concentrations of gluconic acid and glucose while continuously producing gluconic acid at high rates and at low pH values. Most important, the formation of 2-ketogluconate or 2, 5-diketogluconate does not occur using this organism, so that downstream removal of these by-products is unnecessary (Uhlig et al., 1986).

Acetobacter diazotrophicus, an isolated nitrogen-fixing acetic acid bacterium, shows high rates of gluconic acid production in preliminary studies (Attwood et al., 1991). In contrast to *G. oxydans*, *A. diazotrophicus* grows rapidly on simple mineral medium, tolerates acid to pH 3.5, and also tolerates high glucose and gluconic acid concentrations greater than 150 g/liter. Apparently, aerobic utilization of dinitrogen at low ammonia concentrations depends upon an extremely high rate of respiratory electron transport utilizing glucose oxidation coupled to oxygen uptake (Flores-Encarnacion, 1999). The physiologic conditions for a maximum rate of gluconic acid production using this bacterium may be developed easily, considering a simple batch culture with 150 g of glucose/liter at high ammonia produced an 80% yield of gluconic acid at a productivity of about 15 gm/liter · h (Attwood et al., 1991). Unfortunately, *A. diazotrophicus* produced some 2-ketogluconate during gluconic acid production. However, genetic methods are available to knock out the gluconate dehydrogenase gene or develop mutant strains as reported for *G. suboxydans* (Gupta et al., 1999).

Zymomonas mobilis Process for Gluconic Acid and Sorbitol Production *Zymomonas mobilis* contains the periplasmic enzyme, glucose-fructose oxidoreductase (GFOR),

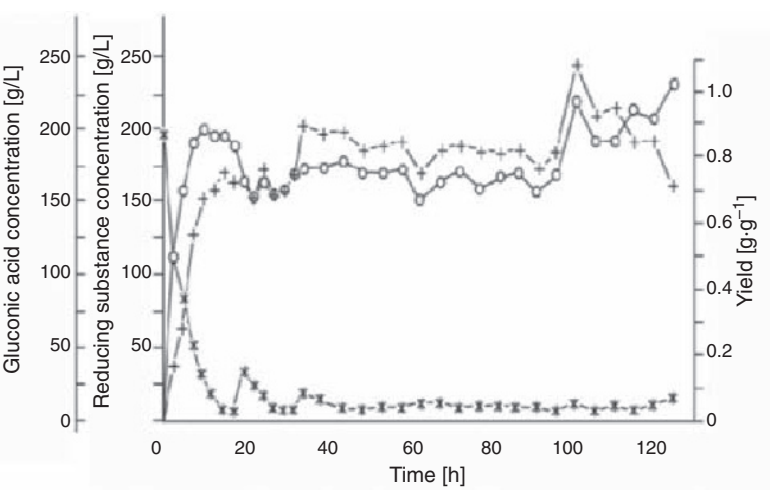

Fig. 18. An example of a continuous process for conversion of glucose to gluconic acid by Acetobacter methanolicus: –o–, yield; –+–, gluconic acid concentration; –∗–, reducing substance concentration (glucose). From Poehland et al. (1993), with permission.

together with gluconolactonase (GL; Zachariou and Scopes, 1986). (See "Scientific Background" of gluconic acid production for a description of the biochemistry and physiology of the *Z. mobilis* system.) Subsequently, it has been shown that this organism can totally convert equimolar fructose and glucose solutions of over 50% into sorbitol and sodium gluconate. The advantages of *Z. mobilis* for process development are:

1) no requirement for high aeration rates in contrast to aerobic processes using *A. niger* or *G. suboxydans*;
2) productivity is enhanced by high concentrations of sugars; and
3) both sorbitol and sodium gluconate have industrial value and can be separated downstream (Chun and Rogers, 1988).

The chemical technology for manufacture of sorbitol requires H_2 gas reduction at high pressure (40–50 atm) and high temperature (140–150°C) of a 70% glucose solution with a Ni^{+2} ion catalyst. The sorbitol syrup produced must be passed through several ion exchangers to reduce the residual nickel below 5 ppm prior to use of the sorbitol in the food industry (Phillips, 1963). In contrast, under the mild conditions, *Z. mobilis* produces equimolar quantities of gluconic acid (or sodium gluconate) and sorbitol at high yields and high productivity. Also, considering selling prices, production of sorbitol (powder, $0.73–0.96/lb, or crystals, $0.73–1.03/lb) from fructose (powder, $0.39/lb) rather than glucose (powder, $0.51–0.54/lb) as a starting material may be more economical (Anonymous, 2000).

Zymomonas mobilis normally utilizes much of the gluconate produced for metabolism via the Entner-Doudoroff pathway (see "Scientific Background" in this Chapter). To develop a process that produces high quantities of both gluconate and sorbitol, the metabolic utilization of gluconate must be suppressed or bypassed. Taking advantage of the perplasmic position of the GFOR and GL enzymes together with methods for preventing metabolism of gluconic acid, three approaches have been used. First, it was found that *Z. mobilis* cells treated with toluene were permeabilized, and thus leaked cofactors such as NAD and NADP. These cells were unable to metabolize gluconate, but retained the full ability to convert glucose to gluconate. This was because the NADP cofactor remains tightly coupled to the GFOR enzyme. Table 18 illustrates data from a number of workers showing that toluene permeabilized cells carry out production of sorbitol and gluconate at high rates and at greater than 90% conversion in a batch reactor. These cells immobilized in calcium-alginate or in carrageenan had similar production characteristics. Using these immobilized cells in continuous or packed-bed continuous recycle reactors resulted in longer running times, up to 75 days (Rehr et al., 1991). However, the productivity and/or the percent conversion was somewhat lower than for batch operation (Table 18). Continuous production of gluconic acid and sorbitol from alternative substrates has been carried out using toluene permeabilized *Z. mobilis* cells co-immobilized with chitin-enzyme complexes in aginate beads (Kim and Kim, 1992a; Ro and

Table 18. Experimental production of gluconic acid and sorbitol by *Zymomonas mobilis*.

Cell treatment	Reactor	Run time	Substrates glucose and fructose g/liter	Production of gluconate-sorbitol Conversion rate		References
				Rate[a] g/liter · h	Percent %	
Toluene permeable	Batch	16 h	600	17.7	95–94	Chun and Rogers, 1988
Toluene permeable in calcium alginate beads	Batch	16 h	600	17.7	94–93	Chun and Rogers, 1988
	Continuous	125 h	200	7.2–7.6	80–85	
CTAB permeable in carrageenan beads	Continuous (two stages)	75 d	200	4.8–6.2	>98	Rehr et al., 1991
Toluene permeable + inulinase-chitin in calcium alginate beads	Continuous	2 d	200[b]	19.2–21.3	50	Kim and Kim, 1992a
	Recycle-packed bed	10 d	200[b]	23–26	44	
Toluene permeable + invertase-chitin in calcium alginate beads	Batch	22 h	200[c]	18	93	Ro and Kim, 1991
	Continuous	2 d	200[c]	4.5–4.6	70	
	Recycle-packed bed	10 d	200[c]	5.1–5.2	57	
Untreated cells (30 g/liter)	Batch	8 h	300	39–50	40–83	Silveria et al., 1999
	Batch	8 h	650	48–45	91–91	

Abbreviation: CTAB, cetyltrimethylammonium bromide.
[a]The rate applies to each product separately.
[b]Jerusalem artichoke inulin (100 g/liter) added instead of free fructose.
[c]Sucrose added instead of fructose and glucose.

Kim, 1991). With immobilized inulase, Jerusalem artichoke inulin provided fructose, and with immobilized invertase, sucrose substituted for both glucose and fructose. The preliminary results are interesting and may provide a framework for further process development using co-immobilization techniques (Table 18).

A second approach to efficient synthesis of gluconic acid and sorbitol has been the use of cell-free GFOR from *Z. mobilis*. Using a crude extract of *Z. mobilis* in a continuous ultrafiltration membrane reactor, excellent substrate conversion and enzyme stability were maintained for about 10 days (Silva-Martinez et al., 1998). However, it has been concluded that the strict requirements for enzyme stability would be costly for commercial production and downstream processing, which is a strong argument favoring use of permeable cells (Nidetzky et al., 1997).

Finally, a third approach to achieving high production rates and conversion yields was demonstrated using untreated cells of *Z. mobilis* (Silveira et al., 1999). In batch runs for 8 h with 30 g of pregrown cells/liter and glucose plus fructose at 650–750 g/liter, yields reached 91% for both products with productivities of 48 and 45 g/liter · h for gluconic acid and sorbitol, respectively (Table 18). Experiments showed that the main reason for the high yields for batch runs with substrate at 650 g/liter or greater but not at lower concentrations, such as 300 g/liter, was the inhibition of normal metabolism of glucose and gluconates (see Table 18). High concentrations of either substrate or products (gluconate and sorbitol) can cause this inhibition effect, which results in preferential conversion of substrates by the glucose-fructose oxido-reductase/glucono-δ-lactonase system (Silveira et al., 1999). The best conversion yield reported for untreated cells is 91% for gluconic acid and sorbitol, which is lower than 94–98% for permeabilized *Z. mobilis* with or without immobilization (Table 18). However, large-scale permeabilization and immobilization of cells would add to production costs. On the other hand, long runs (up to 75 days) in a two-stage continuous system were achieved with cetyltrimethylammonium bromide (CTAB)-permeable cells immobilized in carrageenan (Rehr et al., 1991). Yet, all of the published continuous processes shown in Table 18 operate with low concentrations of products (about 100 g/liter), increasing costs for water removal.

Although numerous patents have been issued on various methods for the production of sorbitol and gluconic acid using various *Z. mobilis* cell preparations, clearly further process development must occur before a viable commercial process will emerge.

PATENTS The use of *Gluconobacter* (*Acetobacter*) *suboxydans* for production of gluconic acid (by modification of the "quick" vinegar process or by the submerged fermentation process [the Frings process]) is documented in early patents (Currie and Carter, 1930; Currie and Finlay, 1933). However, because of the difficulty in controlling the simultaneous appearance of about 10% ketogluconic acids along with gluconic acids, as well as other serious problems (mentioned in "Scientific Background" and "Areas of Research and Development"), at present, *Gluconobacter* is not considered for development of commercial processes. Thus, *Gluconobacter* spp. do not appear in the patent literature concerning gluconic acid synthesis.

However, numerous patents utilizing *Acetobacter methanolicus* for gluconic acid production have appeared recently. Babel et al. in Germany have been most active in developing the technology for this process described in more than eight patents. Three patents from this group are listed in Table 19. The 1991 patent describes a two-phase process (possibly utilizable for commerical production) culminating in production of a very high yield of gluconic acid (see "Areas of Research and Development" in this Chapter).

There has been a great deal of interest in the possible commercialization of the *Zymonmonas mobilis* catalyzed process for oxidation of glucose to gluconic acid combined with the reduction of fructose to sorbitol. Sorbitol as well as sodium gluconate is used in a varety of industrial applications. The six patents listed in Table 19, from 1988 to 1995, reflect examples of processes employing permeabilized cells of *Z. mobilis*.

Because of the characteristics of this system, permeable cells produce high yields at high productivities of both gluconic acid and sorbitol (see "Scientific Background" and "Areas of Research and Development" in this Chapter). In two of the patents (Rehr et al., 1991; Roehr and Sahm, 1995), immobilization techniques are described that increase the total time duration for a stable process. The last patent listed in Table 19 (Silveira et al., 1994) describes the conditions for use of untreated cells of *Z. mobilis* for production of gluconic acid at high yield.

In addition to the patents emphasizing manufacturing methods for bacterial production of gluconic acid, there are many patents on applications of gluconic acid in industry. A brief survey revealed that over the past ten years, more than 70 U.S. patents appeared for use of gluconic acid and sodium gluconate in pharmaceutical products, food preparations, cleaning of metallic circuits and delicate metal parts, leather treatments, and other products and processes.

Table 19. Selected patents for gluconic acid production.

Patent number	Year	Inventors and (applicants)	Organism and technology
DD 236,754	1986	Babel et al. (Akad.Wissenshaften)	*A. methanolicus* Batch process, high-yield gluconate
DD 253,836	1988	Babel et al. (Akad.Wissenshaften)	*A. methanolicus*, *Pseudomonas* sp. Continuous culture
DD 293,135	1991	Babel et al. (Chemie A.-G., Bitterfeld-Wolfen)	*A. methanolicus* Two-phase process: gluconic acid yield 220g/liter
US 47555467	1988	Scopes et al. (Unisearch Ltd., Australia)	*Z. mobilis* Original description, permeabilized cells and cell free
US 5017485	1989	Bringer-Meyer and Sahm (Forschungscentrum, Juelich)	*Z. mobilis* Process: freeze-thaw permeable cells
US 5102795	1992	Rehr and Sahm (Forshungs centrum, Juelich)	*Z. mobilis* Process: surfactant (CTAB) permeable cells
US 5190869	1993	Rehr and Sahm (Forschungs centrum, Juelich)	*Z. mobilis* Process: immobilized, permeable cells
EP 427150B1	1995	Rehr and Sahm (Forshungs centrum, Juelich)	*Z. mobilis* Manufacture: immobilized, permeable cells
BR 9403981	1994	Silveira et al.	*Z. mobilis* Two-phase production: untreated cells

Prospects At present, there are no bacterial conversion processes applied to large-scale commercial production of gluconic acid. Industrial manufacture mainly employs the fungal process with *Aspergillus niger* and *Aureobasidium pullulaus* (Roehr et al., 1996; Anastassiadis et al., 1999).

Two bacterial-based processes are under development that may turn out to be competitive in the future. Acetobacter methanolicus supercedes *Gluconobacter suboxydans* and related strains. The attributes of this organism that eliminate the disadvantages of *G. suboxydans* are dealt with in "Scientific Background" and "Areas of Research and Development." A two-phase process has been developed in which *A. methanolicus* cells can be grown on methanol in phase one, and then high glucose concentrations can be converted rapidly to pure gluconic acid during the production phase (Poehland et al., 1993; Babel et al., 1991). In experimental reactors with continuous-feed recycle, high yield and high productivity were maintained for 7 days (see "Areas of Research and Development" in this Chapter).

The *Zymomonas mobilis* production system described above (in "Areas of Research and Development") has the advantage of producing both sodium gluconate and sorbitol under anerobic conditions. The lack of an aeration requirement during the production process certainly lowers costs when compared to other bioprocesses. Secondly, both sodium gluconate and sorbitol are marketable chemicals with a number of applications. The development of processses using permeabilized cells, untreated cells, or immobilized cells in continuous or fed-batch reactors is still in the experimental stage. The results promise high yields of gluconic acid at high productivities under conditions that may be cost-effective and competititve with other processes for gluconic acid manufacture.

Section 4: Succinic Acid

Introduction

Succinic acid (1,4-butanedioic acid) is a four-carbon acid which occurs as a constituent of almost all plant and animal tissues and microorganisms. It has been of considerable interest in recent years as a renewable feedstock for various petrochemical-based large-volume chemicals, owing to the reactivity of its functional groups. Succinic acid received its name from the Latin name "Succinum" meaning "amber," from which it was first isolated. National Aniline began production in the United States in 1929 using an electrolytic reduction of maleic anhydride. The fermentative production of succinic acid was studied in Japan in the 1970s utilizing a variety of feedstocks such as paraffins, sucrose and isopropyl alcohol (Kirk-Othmer, 1983).

Succinic acid also is produced as a by-product of adipic acid manufacture and can be sold as a mixture with other organic acids from this process. Annual world production of adipic acid exceeds a billion pounds and a large amount of by-product succinic acid is obtained. Succinic acid is also sold as a purified product and sells in the range of $2.50 to $2.80 per lb, as listed in a

Chemical Prices section of the Chemical Marketing Reporter of January 10, 2000.

The fermentation processes of *Escherichia coli* and other enterobacteria have been studied since the time of Pasteur, and much is known about fermentative metabolism and the generation of fermentation end products. In the oxidation of hexose, the fermentation products of *E. coli* are made up of a mixture of ethanol, acetic, formic, lactic and succinic acids. *Escherichia coli* adjusts the proportion of products to attain redox balance and generally succinic acid makes up only 5–10 mol% of the fermentation products (Bock and Sawers, 1996). Progress in metabolic engineering has helped raise the interest of various groups in a biological-based industrial process for the overproduction of succinic acid that might compete with petrochemical processes generating comparable four-carbon feedstocks.

Owing to the reactivity of succinic acid, it can be converted to a variety of products, including large volume chemicals such as 1,4 butanediol and tetrahydrofuran. The use of renewable resources and carbohydrates derived from agricultural materials such as corn has increased the interest in making intermediates such as succinic acid in an industrial fermentation process. The potential for industrial succinic acid fermentation was discussed as long as 20 years ago by Zeikus (1980). The various biotechnological methods for production and the markets for succinic acid and its various derivatives were reviewed (Zeikus et al., 1999).

Succinic acid was identified as a target chemical by the United States Department of Energy Alternative Feedstocks Program. Their initial activity focused on the development of decision-making tools, which led to the commencement of process development research in 1993 for succinic acid (Schilling, 1995). A consortium of industry, academic and government scientists in conjunction with the National Corn Growers put together "Vision 2020," based on the use of renewable material for chemical production. One of the projects listed in Vision 2020 is a biocatalyst for the production of succinic acid from biomass (Reisch, 1999). The future need to replace petrochemically derived chemicals for more green technologies will be a driving force in advancing technology for the production of fermentation-derived chemicals such as succinic acid, as well as push the process development for catalytic conversion of succinic acid to other products.

Scientific Background

Succinate, along with a mixture of other products, is produced during fermentative metabolism by a variety of microorganisms. In *Escherichia coli*, glucose is transported into the cell via the phosphotransferase system and metabolized to pyruvate. The NADH produced during glycolysis must be recycled by conversion of pyruvate to fermentation products so that glycolysis may continue. Pyruvate dehydrogenase, which oxidatively decarboxylates pyruvate, is negatively regulated by NADH and is repressed during fermentative growth. Pyruvate formate lyase, which is functional only under anaerobic conditions, converts pyruvate to acetyl CoA and formate. The cell only turns on one of these enzymes at a time, dependent on the redox status of the cell, allowing the cell to balance NADH during aerobic or anaerobic growth (Bock and Sawers, 1996).

During anaerobiosis, the enzyme pyruvate formate-lyase is used by *E. coli* to form one molecule of formate and one molecule of acetyl CoA from one molecule of pyruvate. Formate can either be excreted from the cell or decomposed to carbon dioxide and dihydrogen. Two NADH molecules are consumed through the reduction of acetyl CoA to ethanol, which is the most highly reduced major fermentation product of *E. coli*. This conversion occurs through two dehydrogenation reactions catalyzed by alcohol dehydrogenase (Clark, 1989).

The production of lactate also uses reducing equivalents, and lactic acid is formed by the enzyme lactate dehydrogenase. Lactate is not

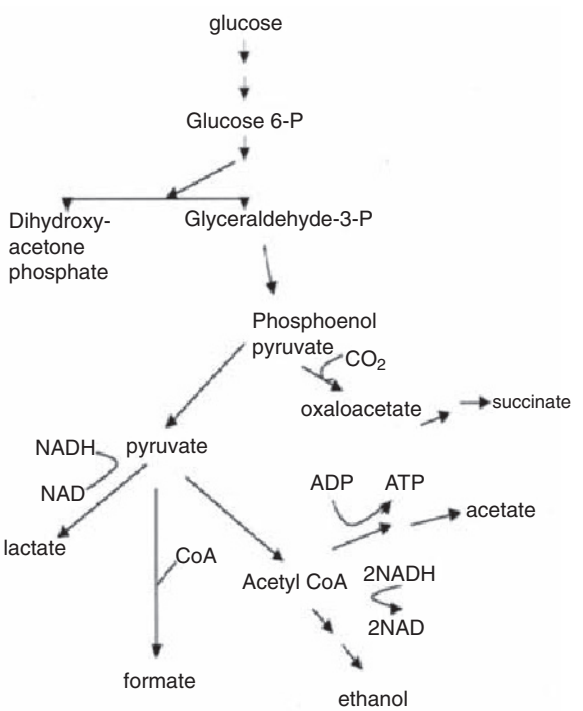

Fig. 19. Mixed acid pathways of *Escherichia coli*.

essential to balance fermentative growth, and the fermentative NADH-dependent lactate dehydrogenase does not appear to be necessary for anaerobic growth (Mat-Jan et al., 1989).

The production of succinate and the enzymatic reactions of malate dehydrogenase and fumarate reductase use two reducing equivalents per succinate formed. The carboxylation of phosphoenolpyruvate (PEP) is the first step in this reaction, with metabolically generated CO_2 limiting the amount of succinate formed (Bock and Sawers, 1996). The reactions below show the mixed acid pathways of *E. coli*.

Typically wild type *E. coli* generates fairly small amounts of succinic acid. Under "Research and Development," the development of *E. coli* strains that produce large quantities of succinic acid will be discussed in greater detail.

Many succinate-producing bacteria so far described have been isolated from the rumen. Propionic acid, which is used by the animal for biosynthetic precursors and for energy, is produced by the decarboxylation of succinic acid. A review written by Gokarn et al. (1997b) covers in more detail the diversity of microorganisms that can be isolated. The most predominant cellulolytic organism in the rumen is *Fibrobacter succinogenes*. It can use a variety of complex substrates and fixes CO_2 during succinate production. Other succinate producers in the rumen include but are not limited to *Ruminobacter amylophilus* and *Wolinella succinogenes* (Gokarn et al., 1997b).

The productivity of selected rumen bacteria for organic acid production was investigated using a variety of cellulosic substrates. The rumen bacteria *Fibrobacter succinogenes* S85 was shown to produce succinate with a yield of 0.38 g/g of glucose and with an acetate yield of 0.11 g/g of glucose consumed. This organism could use cellobiose to produce succinic acid but at slightly lower yields. Because succinate has been found to be a major end product of cellulosic substrates (Walseth cellulose, microcrystalline cellulose, pulped paper and steam-exploded yellow poplar), the use of cellulosic materials for succinic acid production is promising. In contrast, the major end product of *Ruminococcus flavefaciens* on cellobiose, Walseth cellulose, and microcrystalline cellulose was acetate, whereas on pulped paper and steam-exploded yellow poplar, succinate was the major end product. In general, the *Fibrobacter* strain produced more succinate at a higher rate than did the *Ruminococcus* (Gokarn et al., 1997a).

Selenomonas ruminantium, a Gram-negative anaerobe and one of the major species present in the rumen, accumulates succinate as a major end product in the presence of lactate, if malate is added to stimulate lactate uptake. Without malate present, the major end products are acetate and propionate. When succinate reached the concentration of the added malate, malate utilization ended. From a practical standpoint, malate could be added to ruminant diets high in rapidly fermentable carbohydrates. Because high rumen lactate is undesirable, the presence of malate could stimulate lactate utilization (Evans and Martin, 1997).

Actinobacillus sp. 130Z, a facultative anaerobic microorganism that was isolated from the rumen, produces high levels of succinate, and also accumulates some formate and acetate. This strain was classified as *Actinobacillus succinogenes* sp. nov. (Guettler et al., 1999), and owing to its ability to make high levels of succinate under a variety of conditions, it has been the subject of intensive work as well as patent activity. Studies involving this strain will be discussed in greater detail under "Research and Development."

Propionibacterium can ferment glucose or glycerol to make fairly high levels of succinic acid (Gottschalk, 1985), which is a characteristic component of Swiss-type cheese. High concentrations of CO_2 promote this activity (Wood and Werkman, 1938; Wood and Werkman, 1940). *Propionibacterium freudenreichii* subsp. *Shermanii* metabolizes aspartate primarily to succinate and ammonia during fermentation of lactate. When fed lactate with sparged CO_2, however, only a small concentration of succinate is formed and propionate is the main product. This indicates that propionibacteria may use combinations of pathways such as the citrate and transcarboxylase cycles to generate different mixes of end products (Crow, 1987).

The production of succinate by *Lactobacillus* has not been widely studied. Kaneuchi et al. (1988) screened 86 isolates of *Lactobacillus* that were isolated from fermented cane molasses using de Man-Rogosa-Sharpe (MRS) broth. Approximately one-third of the strains screened produced succinate, which is of interest owing to the importance of succinic acid as a flavor ingredient in dairy products. They determined that the diammonium citrate in the MRS broth was the precursor to succinate.

The metabolism of carbon dioxide is a critical aspect for the successful production of succinic acid. Three enzymes that have been examined in the overproduction of succinate can fix CO_2. They catalyze the reactions listed in Table 20.

Actinomyces viscosus, which is associated with periodontal disease and is a succinate producer, incorporates labeled $NaHCO_3$ into succinate but not lactate. High levels of the enzyme phosphoenolpyruvate (PEP)-carboxykinase were found in cell extracts in this study. This enzyme condenses carbonate and PEP to oxalacetate, which

Table 20. Carbon dioxide utilization reactions involved in succinate production.

Substrates	Enzyme	Products	Cell use
Oxalacetate and NTPs	PCK E.C. 4.1.1.49	PEP, NDPs and CO_2	Functions during gluconeogenesis to form PEP
PEP, CO_2 and water	PPC E.C. 4.1.1.31	OAA and orthophosphate	Functions aerobically to replenish OAA Under fermentative conditions, directs PEP to succinate
Pyruvate, ATP and HCO_3^-	PYC E.C. 6.4.1.1	OAA, ADP and orthophosphate	Can be used to enhance succinate production

Abbreviations: NTP, nucleotide triphosphate; PCK, phosphoenolpyruvate carboxykinase; NDP, nucleotide diphosphate; PEP, phosphoenolpyruvate; PPC, phosphoenolpyruvate carboxylase; OAA, oxalacetic acid or oxalacetate; and PYC, pyruvate carboxylase.

can then be converted to either succinate through malate dehydrogenase and additional metabolic steps or aspartate catalyzed by the enzyme glutamate aspartate aminotransferase (Brown and Breeding, 1980).

The microorganism *Anaerobiospirillum succiniciproducens* forms succinate in high amounts and can make greater than 30 g/liter (Datta, 1992a). Several studies have focused on the effect of the supply of CO_2-HCO_3^- on succinate production (Samuelov et al., 1991; Nghiem et al., 1997; Lee et al., 1999a). A pH of 6.2 and high CO_2-HCO_3^- levels promote succinate as the major product, and the levels of PEP-carboxykinase are high while lactate dehydrogenase and alcohol dehydrogenase levels decrease. Under high CO_2-HCO_3^-, the growth rate of *A. succiniciproducens* is high and cell yield doubles. Lactate and ethanol are formed as electron sink products if CO_2-HCO_3^- are low, and a lower growth rate is observed (Samuelov et al., 1991).

The PEP-carboxykinase (PCK, EC 4.1.1.49) was purified from *A. succiniciproducens*. High levels of this enzyme are made during succinate production, and roughly 10% of the crude cell protein is PCK. The enzyme has an absolute need for divalent cations and synergistic effects of various cations were demonstrated. Despite the fact that the fixation of HCO_3^- is important in succinate production, the K_m value for HCO_3^- is similar to those in other reported enzymes in vertebrates which function in a reverse direction. The authors speculate that high external concentrations of HCO_3^- in the environment push the reaction towards oxalacetate (Podkovyrov et al., 1993).

The PCK-encoding gene was cloned from *A. succiniciproducens* and expressed in *E. coli*. Sequence analysis showed that it is closely related to other PCKs of Gram-negative bacteria and that it is 67.3% identical to the PCK of *E. coli*. This is surprising in that the PCK gene of *E. coli* is not crucial for succinate production, and one would not expect such high homology. The authors compare kinetic constants with other ATP/ADP-dependent PCKs, although the comparison is not exact because of the differing conditions of analysis. The *A. succiniciproducens* enzyme appears to have a higher V_{max} for CO_2 fixation than those of *E. coli* and Trypanosoma cruzi and a higher K_ms for PEP and ADP. The authors conclude that sequence and structural features are probably not responsible for the differing activities of the various PCKs (Laivenieks et al., 1997).

The PCK of *A. succiniciproducens* requires the nucleoside triphosphate ADP, and a pyruvate kinase-like activity of this enzyme was identified. In gluconeogenesis, PCK catalyzes the irreversible decarboxylation of oxalacetate, and in *A. succiniciproducens*, AMP was shown to activate this oxalacetate decarboxylase reaction (Jabalquinto et al., 1999).

The enzyme PEP-carboxykinase also appears to play an important role in other high-succinate producers besides *A. succiniciproducens*. The availability of CO_2 in the medium affects succinate production, and a direct relationship between CO_2 added and succinate production is observed. In a comprehensive comparison between enzyme activities of Actinobacillus sp. 130Z and *E. coli* K-12 grown anaerobically, the specific activity of the *Actinobacillus* PCK was shown to be thousands-fold higher than that of *E. coli*. This is in contrast to the many other enzymes that showed similar activity and indicates that PEP-carboxykinase is produced constitutively in this microorganism (Van der Werf et al., 1997).

In contrast to the previous two examples on the importance of PCK in succinate production by *A. succiniciproducens* and Actinobacillus, overexpression of PCK in *E. coli* had no effect. This result is surprising in that the *E. coli* PCK enzyme has similar if not better kinetic characteristics than the *A. succiniciproducens* enzyme, and why this enzyme does not play an important role in *E. coli* succinate overproduction is uncertain. In *E. coli*, a different CO_2-fixing enzyme appears to play a role, as overexpression of PEP-

carboxylase, produced during growth on glycolytic substrates, increased succinate production from 3.27 to 4.44 g/liter and did not alter the distribution of fermentation products (Millard et al., 1996).

A similar pathway for the formation of succinate in both *Actinobacillus* sp. 130Z and *A. succiniciproducens* has been proposed. The formation of oxalacetate from PEP via CO_2 fixation is the first key step. The enzymes malate dehydrogenase, fumarase and fumarate reductase, all enzymes of the tricarboxylic acid (TCA) cycle, work in a reductive fashion towards succinate (Van der Werf et al., 1997; Samuelov et al., 1991). The reactions catalyzed are as follows:

$$\text{Phosphoenolpyruvate} + CO_2 + \text{NDP} \rightarrow \text{Oxaloacetate} + \text{NTP} \quad (1)$$

$$\text{Oxaloacetate} + \text{NADH} \rightarrow \text{L-Malate} + \text{NAD} \quad (2)$$

$$\text{L-Malate} \rightarrow \text{Fumarate} + H_2O \quad (3)$$

$$\text{Fumarate} \rightarrow \text{Succinate} \quad (4)$$

Enzymes utilized are 1) phosphoenolpyruvate carboxykinase, 2) malate dehydrogenase, 3) fumarase and 4) fumarate reductase.

The drive towards succinate production is influenced by environmental factors that can be controlled in a fermentation process. This aspect will be discussed under "Areas of Research and Development."

Commercial Applications

Succinic acid is sold into four major existing markets. The largest is as a surfactant and foaming agent. The other uses are as a chelator in electroplating, an acidulant and flavoring agent in food, and in the production of health-related agents. The estimated market size for these four areas is more than $400 million per year (Zeikus et al., 1999).

The promise of large-scale low-cost fermentations from renewable resources, especially corn, has spurred interest in the United States to develop chemical production for large-volume chemicals using bio-based processes. Succinic acid can be converted by hydrogenation to 1,4-butanediol, which has a world market in excess of 500,000 metric tons. Butanediol is used to produce polybutylene terephthalate (PBT) resins that have desirable mechanical and thermal properties and are a high-performance version of polyethylene terephthalate resins (PET). Also, 1,4-butanediol is a precursor of tetrahydrofuran, which can be polymerized to polytetrahydrofuran (PTHF). Gamma butyrolactone (GBL) can also be derived from 1,4-butanediol, and much of GBL is used to manufacture the solvent *N*-methyl-2-pyrrolidone (Szmant, 1989).

Butanediol is manufactured primarily through a process called "the Reppe Process," which reacts acetylene and formaldehyde at high pressure. Alternative chemical technologies such as a maleic anhydride process and a process using ester hydrogenation technology have come on line (Tullo, 1999). The raw materials used in the chemical process are large volume and low cost. For example, maleic anhydride sells in the range of about $0.50/lb. This means that a fermentation process must be very efficient with low raw material costs to be able to compete with a chemical-based process.

A methodology was described to assess the feasibility of success in making commodity chemicals from renewable resources. The methodology uses a five-step process in the assessment. The first step is portfolio selection, and some of the key selection criteria are high theoretical yields from substrate, high market interest and volume. The second phase involves initial economic screening and uses an economic criterion called "the Fraction of Revenue for Feedstock" (FRF). In this calculation, the cost of the feedstock is divided by the value of all the products, and the products that show the most promise are those where the fraction is smallest. This value takes into account the yields of the products derived from the various feedstock components. The third phase is a comparative analysis of bioprocessing routes that uses a raw material cost ratio, which in essence compares the raw material costs for a bioprocess versus those for the petrochemical route. Phase four is a qualitative analysis, and phase five is a detailed economic analysis requiring a significant effort to classify and do economic assessment of the process technologies. A number of products in an initial portfolio were assessed, then narrowed down to a shorter list which includes acetic acid, 2,3-butanediol, malic acid, and propylene glycol to name a few. Succinic acid was identified as a potential near-term opportunity because it had one of the best FRFs and raw material cost ratios (Landucci et al., 1994). Despite the attractiveness of succinic acid, at this writing an industrial-scale bioprocess has not been attained. However, improvements in the process technology and strains (as evidenced in the patent literature) indicate the potential for a succinic acid bioprocess in the near future.

The other important aspect of the development of the succinic acid market is the chemical conversion of succinic acid to other products. Selective and low cost catalyst development is needed to enable lower cost economics. The chemistry of succinic acid catalysis has been reviewed by Varadarajan et al. (1999) and the

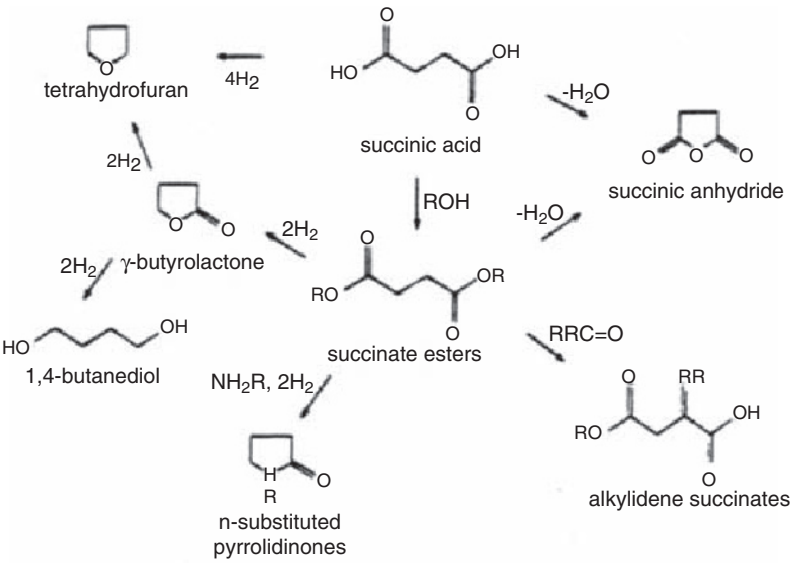

Fig. 20. Summary of catalytic conversion pathways and potential products derived from succinic acid. From Varadarajan et al. (1999), with permission.

summary of the various derived products that follows is from that work. Succinic acid can be readily converted into alkyl esters that have uses as industrial solvents and paint removers. Succinic acid, its anhydride or its esters, can be hydrogenated to the product family of 1,4-butanediol, however, this conversion has not been as well-studied as the hydrogenation of maleic acid or anhydride. A third important product family that can be derived from ammonium succinate, succinimide, or succinic acid is based on 2-pyrrolidinones. They are used for polyvinyl pyrrolidinone (PVP) production, which has an estimated minimum market value of $150 million per year. Other uses for 2-pyrrolidinones include solvents and plasticizers. The commercial production depends upon petrochemical-based materials. A summary of the potential products that can be derived from succinic acid is presented in Fig. 20 (Varadarajan et al., 1999).

The major economic issue in whether succinic acid can compete is the low starting cost of petrochemical raw materials. The target cost for succinic acid from a bioprocess is estimated to be around $0.25/lb for a large-scale plant (Zeikus et al., 1999; Varadarajan et al., 1999). This target cost is necessary to compete with low-cost petrochemical raw materials.

Despite the major interest in the large-volume uses of succinic acid, other new specialty markets of interest should not be ignored. A succinate-based animal feed additive from whey fermentation has been described (Samuelov et al., 1999). The entire fermentation is dried down to a product, which is about one-third succinate, and also contains protein, other organic acids and salts. Succinate is desirable as a feed additive as it is rapidly converted to propionate, which in the rumen improves efficiency and contributes to the overall health of the animal.

Ethylenediamine-*N*,*N*′-disuccinic (EDDS) acid is a chelator that can be used in detergents and is a potential replacement for ethylenediaminetetraacetic acid (EDTA) (Lin et al., 1996). It is also biodegradable and various optical isomers have different susceptibilities to biodegradation (Takahashi et al., 1997). Succinic acid also can be used in the manufacture of biodegradable plastic. A novel biodegradable plastic, called "Bionolle," invented in the early 1990s is based on poly(butylene succinate), poly(butylene adipate succinate), or poly(ethylene succinate). These plastics have been produced on a pilot plant scale and show good processability. They can be processed with conventional equipment and can be made into products such as bottles and foam (Fujimaki, 1998). A copolymer can be made with succinic acid and 1,4-butanediol (Takiyama and Fujimaki, 1994). Bionolle has been shown to be biodegradable in a variety of environments, including activated sludges, soils and compost. Many strains were isolated that can degrade Bionolle (Nishioka et al., 1994).

A novel food use of succinic acid was also identified which is attractive for consumers who would like to reduce their salt consumption. A salt substitute comprised of succinic acid, chloride ion and lysine has been patented (Turk, 1993). The use of organic acids as environmentally friendly promotants of plant growth is an attractive use that bears further exploration. As an example, succinate addition can improve the growth of plants such as potato (Andrianova et al., 1998). Clearly, the growth of succinic acid use as an intermediate in a wide variety of both

specialty and commodity markets shows considerable promise for the future viability of a succinic acid bioprocess.

Research and Development

Although at this time, there is no commercial-scale succinic acid bioprocess, interest remains high, as shown by the activity in the patent and scientific literature. Three different organisms which all show promise have been the focus of the strain improvement research and process development.

Strain Improvement *Actinobacillus* 130Z was isolated from bovine rumen at the Michigan Biotechnology Institute. It was later identified as *Actinobacillus succinogenes* sp. nov., and it is a facultatively anaerobic Gram-negative rod. It is one of the most promising succinic acid producers studied to date, as it can use a variety of sugars and produce greater than 70 g of succinic acid/liter (Guettler et al., 1999). It was isolated in enrichment vials containing an ionophore and fumarate. An atmosphere enriched in carbon dioxide is necessary for rapid fermentative growth (Guettler et al., 1998). The enzyme activities of *Actinobacillus* 130Z and *E. coli* K-12 were compared under the same growth conditions. As described earlier, *Actinobacillus* had increased levels of the CO_2-fixing enzyme PEP-carboxykinase (which appears to be made constitutively) as well as high levels of L-malate dehydrogenase, fumarase, and fumarate reductase, which are all involved in the conversion of PEP to succinate. *Actinobacillus* also had high malic enzyme and oxalacetate decarboxylase activities in contrast to *E. coli* (Van der Werf et al., 1997).

Improvements to *Actinobacillus* were obtained by growing the organism on a source of fermentable carbon as well as sodium monofluoroacetate and by selecting for fluoroacetate-resistant mutants. These mutants give higher succinate production, reduced amounts of acetate and formate, and can produce more than 100 g of succinic acid/liter in 48 h with a yield as high as 97 wt. % on glucose. Presumably carbon flow has been diverted from acetate and formate into the production of succinate (Guettler et al., 1996b).

Anaerobiospirillum succiniciproducens is another high succinate producer that has been the focus of several patents and process development. One patent describes a method for making mutants of *A. succiniciproducens* similar to the method for deriving mutants of succinate-producing *Actinobacillus*. The technique involves the selection of fluoroacetate mutants of *A. succiniciproducens*. The mutant isolated produces less acetate, and in the fermentation, the succinate/acetate ratio is as high as 85 : 1. The lowering of acetate in the fermentation is important in that it is a problem contaminant in the electrodialysis separation process for purifying succinic acid from the fermentation. Reduced toxicity and inhibition of the fermentation by acetate are other advantages of this mutation. It appears that in the mutant, termed "FA-10," pyruvate production has increased while acetate production decreases and succinate levels stay about the same (Guettler and Jain, 1996a).

A metabolic engineering approach towards improvement of *Anaerobiospirillum succiniciproducens* was attempted by cloning and overproducing the PEP-carboxykinase gene. This gene, as described previously, catalyzes the addition of carbon dioxide to PEP to form oxalacetate and can work physiologically in both directions (Laivenieks et al., 1997). This is the first *A. succiniciproducens* gene to be cloned and sequenced. Codon usage is similar to *E. coli* except for a few amino acids, and the putative promoter region also shows many similarities. The gene was overexpressed successfully in *E. coli* so that further kinetic studies could be done. The authors did not look at the effects of overexpression in *A. succiniciproducens*, but now that work has commenced on identifying key genes in this organism, it is likely that future work will focus on improvements of the organism for succinic acid production.

As mentioned earlier, *E. coli* produces succinate but usually at very low levels. One strategy employed by various groups has been the overexpression in *E. coli* of various enzymes that direct carbon flow to succinate. As mentioned previously, uptake and utilization of carbon dioxide is necessary for high yields of substrate and attention has been focused on increasing the level of CO_2-fixing enzymes. Phosphoenolpyruvate carboxylase, which functions aerobically to produce oxalacetate and fermentatively to direct PEP to succinic acid, was overexpressed in *E. coli* and a significant increase in succinate production was seen. The authors cited an average increase from 3.27–4.44 g of succinic acid/liter formed (Millard et al., 1996). When PEP-carboxykinase, which catalyzes the phosphorylation and decarboxylation of oxalacetate to form PEP, was overexpressed in *E. coli*, the distribution of fermentation products remained the same and the amount of succinic acid did not increase. That the overexpression of PEP-carboxykinase did not improve succinate production in *E. coli* is rather surprising, as *A. succiniciproducens* uses a PEP-carboxykinase in its succinate production. The authors speculate that some as yet unknown regulatory control may prevent the *E. coli* PEP-carboxykinase from operating in the reverse direction (Millard et al., 1996).

As *E. coli* does not contain pyruvate carboxylase, a heterologous gene from Rhizobium etli was introduced into *E. coli*. A 50% increase in succinate levels and about a 20% decrease in lactate concentration were observed. Glucose uptake was not affected, and thus this approach may have advantages over increased PEP-carboxylase, which may have undesirable effects in reduction of glucose membrane transport and the phosphotransferase system (Gokarn et al., 1998). The *R. etli* pyruvate carboxylase requires acetyl CoA for activation and is inhibited by aspartate and may be expressed at low levels under typical conditions for succinate production. Use of an alternate pyruvate carboxylase that is not affected by either acetyl CoA or aspartate such as the *pyc* gene from *Pseudomonas fluorescens* was proposed (Gokarn et al., 1999).

Much of the focus on succinate production has been with the use of glucose as a substrate. However, fumaric acid has also been explored as a possible substrate. Fumarate reductase was overexpressed in *E. coli* strains that were fed fumaric acid. High cell density and the presence of glucose were required for increased succinate yield and rate of production. The recombinant strains with enhanced fumarate reductase levels had a succinate molar yield of 120% after four days. Without glucose present, however, mostly malate was produced (Goldberg et al., 1983). Multicopy fumarate reductase genes were also expressed in *E. coli* fed varying concentrations of glucose and fumaric acid. Fumaric acid at 90 g/liter inhibited succinate production, whereas succinic acid production was rapid in the range of 50–64 g/liter. Like the work mentioned in the above paragraph, the authors found that glucose addition in the presence of fumaric acid was necessary and as little as 5 g of glucose/liter could substantially increase the production. The authors speculated that glucose was used as the hydrogen donor for the conversion of fumaric to succinic acid (Wang et al., 1998b).

Another overexpression strategy was tried with the NAD^+-dependent malic enzyme of *E. coli*. Thermodynamically the reduction of pyruvate to malate is favored but in nature this reaction does not occur. A double mutant of *E. coli*, NZN111, which is blocked in both pyruvate:formate lyase (*pfl*) and lactate dehydrogenase (*ldh*), was used as the host. It is unable to grow anaerobically because its pyruvate metabolism is blocked by the fermentation end products acetate, formate, ethanol, and lactic acid. The mutant NZN111 with multiple copies of malic enzyme accumulated succinic acid as a major end product only if the cells were switched to anaerobic metabolism gradually by metabolic depletion of oxygen in a sealed tube (Clark et al., 1988). Mutant strains blocked in either *pfl* or *ldh* did not alter their distribution of fermentation products when overexpressing malic enzyme.

The importance of reducing power was shown for NZN111, as addition of hydrogen gas to the media increased the apparent yield of succinate from 0.65 g per g of glucose to 1.2 g per g of glucose. Although succinate production increased with expression of malic enzyme, NZN111 fermented glucose very slowly, making it less attractive for commercial production (Stols and Donnelly, 1997a). Overexpression of a heterologous malic enzyme from *Ascaris suum* yielded similar results (Stols et al., 1997b).

A novel succinate pathway in *E. coli* was discovered in the NZN111 mutant. Using site-directed mutagenesis, mutations were made in malate dehydrogenase, which catalyzes the interconversion of malate and oxalacetate. These mutant malate dehydrogenases had activity towards pyruvate instead of oxalacetate and provided a low lactate dehydrogenase-like activity. Fermentative growth appeared to be limited by the low lactate dehydrogenase-like activity, so directed evolution was used to select a variant enzyme with improved lactate dehydrogenase activity. After several rounds of subculturing, a mutant termed "AFP111" (named after the Alternate Feedstocks Program of the United States Department of Energy) was isolated based on accelerated glucose catabolism. From one mole of glucose, this mutant yields 1 mole of succinic acid, 0.5 moles of acetic acid and about 0.5 moles of ethanol. This is the first example of an *E. coli* strain that produces succinic acid as its major fermentation product. Donnelly et al. (1998a) proposed that glucose forms two PEPs, one of which is converted to succinate and the other to ethanol and acetate (Fig. 21).

The new strain AFP111 appears to be attractive for commercial use, owing to the lack of strict anaerobic culturing conditions needed, the use of low cost growth medium, and its ability to grow on xylose and other biomass-derived sugars. This strain was shown to produce up to 45 g of succinic acid/liter (Donnelly et al., 1998b).

A similar approach in a different laboratory towards constructing strains of *E. coli* was done by creating a double mutant with mutations in the phosphotransacetylase gene (*pta*) and the *ldh* gene. The *pta* gene catalyzes the conversion of acetyl CoA to acetyl phosphate and a mutation in this gene blocks the fermentative pathway to acetate. When grown under anaerobic conditions, this mutant produced about four times more succinate than the wild type produced as well as significant pyruvate levels. Lactate production was not detected and acetate production was considerably reduced, which may be of interest in light of downstream separation processes (Pan et al., 1999).

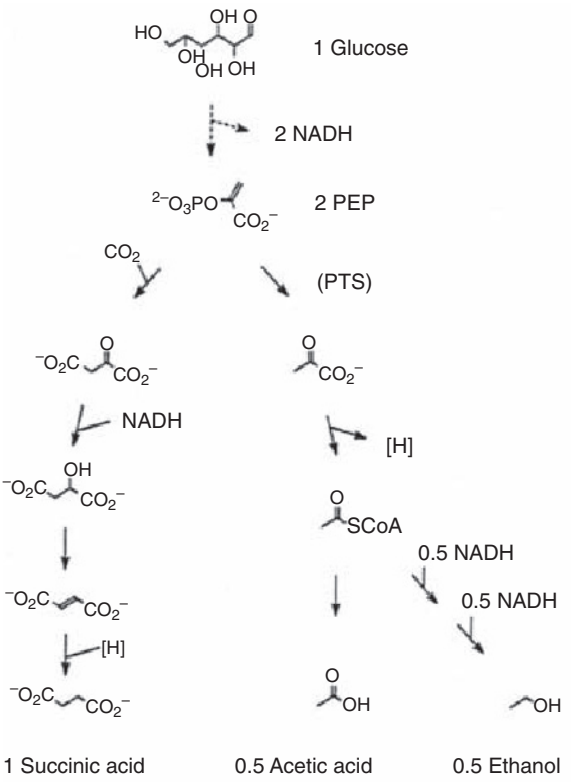

Fig. 21. Pathway for succinic acid production from glucose by *E. coli* strain AFP111. From Donnelly et al. (1998a), with permission.

Process Development There is at this writing no commercial-scale process for the production of succinic acid. There has been activity over the last several years, which is primarily found in the patent literature, to improve the fermentation as well as the downstream process. A great deal of effort was initially spent on *Anaerobiospirillum succiniciproducens*, as this was the first organism observed which could produce succinic acid in levels high enough to envision a commercial process.

Anaerobiospirillum succiniciproducens is a strict anaerobe and grows at an optimal temperature of 39°C. A typical fermentation medium contains dextrose, peptone, yeast extract and salts. The optimal pH range for this organism was determined to be between 5.8 and 6.4. This organism can produce approximately 30 g of succinate/liter from a starting glucose concentration of 50 g/liter. Calcium hydroxide is added to produce a calcium succinate product, which can be precipitated from the broth (Datta, 1992a).

Low cost commercial ingredients can be used as part of an *A. succiniciproducens* fermentation, using dextrose and corn steep solids from a corn wet milling plant. The pH adjustment can be done using high purity industrial grade lime. High initial concentrations of dextrose were inhibitory unless tryptophan was added at 25 ppm. A continuous multistage fermentation with simultaneous precipitation of calcium succinate was shown to be feasible, and a productivity of 2.0 g/liter·hr was observed. Another cost saving measure, the use of spent filtrate from a previous fermentation, was shown to increase fermentation productivity when added to a fermentation up to a level of 50% (Datta et al., 1992b).

The effect of medium components on the production of succinic acid by *A. succiniciproducens* was systematically studied. Sodium ion is essential for glucose transport and an optimal level of 4 g of NaCl/liter was determined. In contrast, magnesium ion did not appear to affect growth or succinic acid production. A variety of complex nitrogen sources were also assessed, and a mixture of polypeptone, yeast extract, soytone and peptone gave better results than any of these ingredients used alone (Lee et al., 1999b).

A large batch was run in an 80-liter fermenter. A high succinate yield of 87 wt. % of added substrate carbon was obtained in a fermentation time of 22.5 h (Datta et al., 1992b). Biotin was shown to be an important microingredient. At 50 mg of added biotin/liter, an increase in glucose consumption was seen and both succinic and acetic acid were increased by 17% and 30%, respectively. Biotin can be found in corn steep liquor, however, the heat treatment needed to sterilize corn steep liquor may inactivate the biotin (Nghiem et al., 1996).

One of the most important factors in the success of succinate fermentation by *A. succiniciproducens* is the addition of carbon dioxide in some form to the media. Using CO_2 supplied as solid magnesium carbonate at pH 6.2 (a molar ratio of CO_2-HCO_3^- to glucose of 1.0 to 0.5), 90% of the glucose was consumed and 65% of the carbon was converted to succinate. When the molar ratio of CO_2-HCO_3^- to glucose was lowered to 0.065, only 45–60% of the glucose was consumed and 8% of the carbon was converted into cell mass. The molar yield of ATP also drops significantly in the lower CO_2-HCO_3^- concentrations. At high CO_2-HCO_3^- levels, PEP-carboxykinase is present in high amounts (Samuelov et al., 1991).

Some uncertainty exists in how the CO_2 should be optimally supplied to the fermenter. Nghiem et al. (1997) found that supply of CO_2 via 1.5 M Na_2CO_3 was adequate and that sparging CO_2 gas into the fermenter at 0.025 liter/min lowered the rate and yield of succinate production, whereas acetate production was not affected. This is in contrast to the work of Datta (1992a), who found that the fermentation did not start as rapidly without a CO_2 sparge. In Datta's patent, increas-

ing the partial pressure of CO_2 produced better results, and at 100% CO_2, which is a partial pressure of 1 atmosphere, the fermentation was finished in 21 h, in contrast to a fermentation time of 42 h at 30% CO_2 (Datta, 1992a). Possibly CO_2 solubility is a factor in the different results, as at pH 6.5, CO_2 is three times more soluble than at pH 6.2 (Jones and Greenfield, 1982).

In comparison to glucose, succinic acid is a highly reduced fermentation product. The effect of supplying hydrogen gas with CO_2 was evaluated. The greatest effect appeared to be on the rate of succinate production. At an optimal H_2/CO_2 ratio of 5:95, the rate of succinate production is 1.8 g/liter · h. Varying glucose concentrations also were studied, as higher substrate concentration would help reduce fermentation costs. The optimal glucose concentration of 20–40 g/liter is in the range of previously observed values, and at the higher glucose concentration of 80 g/liter, rate and yield of succinate were lowered (Lee et al., 1999a).

Not as much work on the process development of an *Actinobacillus* 130Z fermentation has been described. This situation will likely change, as this organism shows very promising attributes for an industrial process. *Actinobacillus* 130Z is not a strict anaerobe and does not require the careful culture conditions as does *A. succiniciproducens*. *Actinobacillus* 130Z is tolerant to very high succinate concentrations and will initiate growth in up to 130 g of magnesium succinate/liter. In contrast, *A. succiniciproducens* will not initiate growth in media with 20 g of disodium succinate/liter. *Actinobacillus* 130Z prefers to obtain its nitrogen from organic sources, and succinate production is increased by eliminating ammonium sulfate. It is more tolerant than *A. succiniciproducens* to pH and can produce succinic acid at high amounts in the pH range of 6.2–7.2. Control of pH can be done with either sodium carbonate or sodium hydroxide. *Actinobacillus* 130Z also can use a wide variety of substrates, including arabinose, fructose, xylose, sucrose and glucose (Guettler et al., 1996c).

Actinobacillus 130Z requires the addition of carbon dioxide to the medium and about 0.3 atmosphere partial pressure is used. Because it is not sensitive to oxygen, pretreatment of the media to remove oxygen need not be done. It is able to use low-cost media ingredients such as corn steep liquor from a corn wet milling plant. Compared to *A. succiniciproducens*, it demonstrates unusually high tolerance to substrate concentrations and can produce succinic acid at glucose levels as high as 150 g/liter (Guettler et al., 1998). This ability to tolerate high glucose concentration is desirable in that less fermentation capital equipment needs to be employed, reducing the overall cost of the process.

Actinobacillus 130Z produces the highest reported amounts of succinic acid. In serum vials, it can produce nearly 80 g/liter and in a fermenter produced more than 60 g/liter in less than 36 h. With continued incubation, it was able to produce 75–79 g of succinate/liter (Guettler et al., 1998).

A novel approach towards supplying reducing power to *Actinobacillus succinogenes* was attempted using electrically reduced neutral red. An electrochemical bioreactor with anode and cathode compartments separated by a cation-selective membrane was constructed. The redox dye mediates electron transfer from the electrode into cellular metabolism. Electrically reduced neutral red enhanced the growth of *Actinobacillus* along with glucose consumption and succinate and ethanol production, whereas formate and acetate production was decreased. Neutral red is not toxic to the cells and can bind to the membrane, providing electrons to fumarate reductase. Neutral red appears to replace quinones in the fumarate reductase complex of *Actinobacillus* (Park and Zeikus, 1999). The approach of using electrical reducing power may have applications in other industrial fermentations besides succinic acid production.

The other succinate process that has been the focus of process development in the patent literature uses *E. coli* AFP111. A two-stage process in which the organism is grown aerobically to generate biomass and switched to anaerobic growth to turn on succinate production has been described. Hydrogen gas can be used as a reductant by the microorganism. The authors report that succinic acid concentration can reach 45 g/liter in a 100% carbon dioxide atmosphere and the weight yield of succinate to glucose can reach 99% (Nghiem et al., 1999).

The typical medium described for *E. coli* consists of tryptone, yeast extract, glucose and salts. A fed-batch fermentation can be run using a glucose and corn steep feed mixture and during the fermentation the glucose concentration is maintained at less than 1 g/liter. *Escherichia coli* could produce succinic acid in the pH range of 6.2–7.4, but the optimal pH appears to be close to 6.6. The microorganism produced high levels of succinic acid in a corn steep liquor medium, with a fermentation time of approximately 99 h (Nghiem et al., 1999).

There appears to be at present three candidates for the industrial production of succinic acid. Each has advantages and disadvantages for commercial production. A simple comparison of the three strains can be made based on yield and fermentation time and is shown in Table 21.

As yet, all examples in the literature have been laboratory or pilot scale, and it is unknown what

Table 21. Summary of high succinic acid producers.

Organism	Succinic acid titer (g/liter)	Fermentation time (h)
Actinobacillus succinogenes 130Z	~100	36–40
Anaerobiospirillum succiniciproducens	~35	36–40
Escherichia coli AFP111	~45	99

challenges will occur when these fermentations are scaled up to greater volumes.

The cost of recovery of succinic acid will be of prime importance in determining whether a cost-effective process will allow succinate-derived chemicals to compete with petrochemicals. Most succinate fermentations need to be run at or near a neutral pH and require the addition of a salt to maintain pH. Removal of the salt to achieve the isolation of the desired acid is one of the challenges of developing a low-cost separation process. Various approaches towards the recovery of organic acids have been tried, but it is beyond the scope of this chapter to review organic acid separation technology. Some work has focused specifically on succinic acid and that is what will be covered here.

As mentioned previously, succinic acid can be precipitated from fermentation broth with calcium hydroxide. A calcium succinate cake can be recovered from the fermentation broth. Acidification of this cake with sulfuric acid yields succinic acid and calcium sulfate solids (gypsum). After this stage, the succinic acid contains many impurities and can be further purified by ion exchange on a strongly acidic cation exchange resin that removes calcium and other cations. Use of a weakly basic anion exchange resin in the next step will remove sulfate and other anionic impurities (Datta et al., 1992b). One drawback of the calcium precipitation process is that it produces gypsum as a by-product.

Another approach to purify succinic acid is the use of electrodialysis technology. One patent describes an integrated process in which the fermentation broth goes through an electrodialysis membrane to concentrate the succinate salt, and then that feed subsequently goes through a water-splitting electrodialysis membrane to form a base and the succinic acid product. The base is recycled to the fermenters for pH control, and the succinic acid is further purified with ion-exchange chromatography to remove residual sodium ion, amino acids and sulfate ions. The other major fermentation product, acetate, is retained through this process with the succinic acid (Glassner and Datta, 1992).

Water-splitting electrodialysis can be improved by using a supersaturated solution of the free acid. Sodium acetate inhibits crystallization of succinic acid, but free acetic acid, which is produced by water-splitting electrodialysis, promotes crystallization of succinic acid (Berglund et al., 1991).

Another separation strategy uses the formation of diammonium succinate, where the ammonium ion is used to control the pH in the fermentation. The diammonium succinate can be concentrated and reacted with a sulfate ion at low pH to yield ammonium sulfate and succinic acid. Succinic acid has very low solubility in aqueous solutions with a pH below 2 and can be crystallized. The succinic acid can be purified with methanol and the ammonium sulfate is thermally cracked into ammonia and ammonium bisulfate. The ammonia can be fed back into the fermenter and the ammonium bisulfate can be recycled for use in succinic acid crystallization (Berglund et al., 1999).

It is likely that process development for succinic acid will continue to be explored. Desirable features of an economic process would include a high yielding fermentation that can tolerate process conditions, low-cost substrate, and an integrated separation process that can recover and purify succinic acid at low cost.

Patents and Regulatory Issues

There are a number of patents on the microorganisms, fermentation process, and downstream separation of succinic acid. In addition, there are patents that cover the further conversion of succinate to other products and many patents on the competitive chemical processes that make large volume chemicals such as 1,4-butanediol. A useful summary of the downstream chemical conversion patents appears in Varadarajan and Miller (1999). While patents have been referenced throughout this section on succinic acid, it is of interest to summarize succinic acid fermentation, strain development, and downstream separation patents here.

Prospects for Succinic Acid Production

At this writing, there is no commercial large-scale process for succinic acid production by fermentation. To make large-scale commodity chemicals derived from succinic acid, the price of the acid needs to be in the range of less than 50

Table 22. Patents for succinic acid production.

Assignee	Patent number	Year	Organism	Technology
Applied Carbochemicals	U.S. 5958744	1999	Not applicable	Downstream purification
University of Georgia Research	PCT WO99/53035	1999	*E. coli*	Strain improvement
Foundation Lockheed Martin Energy	U.S. 5869301	1999	*E. coli*	Fermentation
Research Corp. Korea Institute of Science and Technology	PCT WO99/06532	1999	*E. coli*	Strain improvement
University of Chicago	U.S. 5770435	1998	*E. coli*	Strain improvement
Michigan Biotechnology Institute	U.S. 5723322	1998	*Bacterium* 130Z (*Actinobacillus*)	Fermentation
Michigan Biotechnology Institute	U.S. 5521075	1996	*Anaerobiospirillum succiniciproducens*	Fermentation
Michigan Biotechnology Institute	U.S. 5504004	1996	*Bacterium* 130Z (*Actinobacillus*)	Strain
Michigan Biotechnology Institute	U.S. 5573931	1996	*Bacterium* 130Z (*Actinobacillus*)	Strain improvement Fermentation
None cited	U.S. 5143833	1992	*Anaerobiospirillum succiniciproducens*	Downstream purification
None cited	U.S. 5168055	1992	*Anaerobiospirillum succiniciproducens*	Downstream purification
None cited	U.S. 5143834	1992	*Anaerobiospirillum succiniciproducens*	Downstream purification

cents per pound. This is due to the competition with petrochemically derived raw materials that are also sold in this price range or at an even lower price.

The specialty markets for succinic acid such as surfactants, food uses, and health-related ingredients may benefit from having a "natural" succinic acid produced by fermentation. As the prices of these materials are much higher than commodity chemicals, a fermentation-based process may be able to make product at a cost competitive for these markets (Tsao et al., 1999).

What are some of the technology developments needed to make the production of succinic acid by fermentation an attractive economic option? The theoretical yield of succinic acid is greater than one kilogram of succinic acid per kilogram of glucose. To achieve this theoretical yield, efficient delivery of CO_2 for fixation by the organism is necessary. Also, strains that can use H_2 to supply reducing equivalents may yield economic improvements in the fermentation process.

Strain development is well underway with organisms that can produce as much as 100 g of succinic acid/liter. Certain strains appear to be robust enough to flourish under conditions of high substrate, high final product concentrations, and they do not require special handling, such as total removal of oxygen. Some organisms also can grow on a wide variety of carbon sources and sugars. This opens up the opportunity to use low-cost cellulosic-based materials such as agricultural waste products as a very cheap source of starting raw materials.

The costs of separation of succinic acid will be a critical component in economical production of this acid. As acids are neutralized in the fermentation to maintain an appropriate pH for the microorganism, the salts formed must eventually be removed and can be a waste generation problem. Promising separation technology is the use of liquid-liquid extraction and water-splitting electrodialysis. Key also to lowering separation costs is the development of microorganisms that produce as few by-products as possible which need to be removed from the final product stream.

The other key issue for production of large volume chemicals is the technology needed to further convert succinic acid to other products such as 1,4-butanediol. This process technology must be very competitive with other processes that start with low-cost petrochemical feedstocks. One potential avenue to reducing costs may be the use of crude fermentation broth that is chemically converted and then purified in some further downstream process.

The use of fermentation-derived succinic acid is one of the most promising bio-based processes for large-scale commodity chemical production and may be competitive to petrochemical processes at some future date. The development of this process at a large scale can open up new markets for agriculturally derived raw materials and be a "green" chemical based on renewable resources.

Section 5: Polyhydroxyalkanoic Acids

Introduction

Polyhydroxyalkanoic acids (PHAs) are polyester storage material synthesized by a wide range of microorganisms when their growth in medium containing excess carbon and energy sources is restricted by the lack of an essential nutrient. The PHAs also are formed as reduced end products in some organisms when there is a deficiency in O_2 as the terminal electron acceptor for the cell to achieve a redox balance in metabolism. Some PHAs have physical properties comparable to thermoplastics produced from petrochemicals. The best known PHAs are the homopolymer polyhydroxybutyrate and the copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate) or BIOPOL, which was produced by the bacterium *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) and saw applications in a range of consumer products (Steinbüchel, 1996). The process for BIOPOL production was developed by Zeneca BioProducts (formerly ICI of the United Kingdom) and later sold to Monsanto of the United States (Lee, 1996b; Steinbüchel, 1996). The commercial production of BIOPOL ended in 1998 because of high production cost. However, active research is under way worldwide to improve the physicochemical properties of PHAs as well as the economics of producing better PHAs. Also, novel applications for PHAs have been proposed for the future when PHAs with new properties are synthesized and the commercial importance of their chiral monomers is recognized.

One salient property of PHAs is their biodegradability. Until the introduction of man-made plastics, manufactured goods using materials derived from plants and animals were biodegradable and completely recyclable in the environment. Although plastics have dramatically improved human life, the durability of discarded plastic wares in the environment has created a serious waste-management problem. Use of the biodegradable PHAs as a substitute for the petroleum-based plastics can help preserve the quality of life as well as the natural environment, if PHAs can be produced economically.

Despite their undesirable durability after disposal, plastics are hard to replace because of their low cost and versatility. Plastic wares are inexpensive mainly because they can be mass-produced from historically inexpensive raw materials. The thermoplastics have allowed the use of machines and procedures to mass-produce a very wide range of consumer products. It is thus obvious that if a low-cost, environmentally degradable thermoplastic is available, the new material will allow the continued production of inexpensive but nonpolluting plastic wares for the future generations. This need is the driving force for active research on PHAs.

During the past decade, books (Doi, 1990a; Doi and Fukuda, 1994; Mobley, 1994), a special issue of FEMS Microbiology Reviews (volume 103, pp. 91–364, 1992), and many reviews (Anderson and Dawes, 1990; Aminabhavi et al., 1990; Steinbüchel, 1991; Steinbüchel, 1996; Poirier et al., 1995; Lee, 1996a; Lee, 1996b; Lee, 1997; Ishizaki et al., 1997; Steinbüchel and Füchtenbusch, 1998; Madison and Huisman, 1999) were devoted to PHAs. The following publications may be consulted for a more in-depth coverage of the microbiology of PHA-producing organisms (Steinbüchel, 1991), the biosynthesis and degradation of PHAs (Anderson and Dawes, 1990; Steinbüchel, 1991; Steinbüchel, 1996; Hocking and Marchessault, 1994; Madison and Huisman, 1999), genes for PHA formation (Madison and Huisman, 1999), the physicochemical properties of PHAs (Hocking and Marchessault, 1994; Steinbüchel, 1996), analytical methods for PHAs (Anderson and Dawes, 1990; Hocking and Marchessault, 1994; Steinbüchel, 1996), commercial production and economic considerations (Doi, 1990a; Byrom, 1992; Hrabak, 1992; Hocking and Marchessault, 1994; Lee and Chang, 1995a; Poirier et al., 1995; Lee, 1996a; Lee, 1996b; Steinbüchel, 1996), metabolic engineering of bacteria and plants for PHA production (Madison and Huisman, 1999; Poirier et al., 1995), and patents for PHA production and application (Steinbüchel, 1996).

Scientific Background

History Among the naturally occurring PHAs, the best known is the polyhydroxybutyric acid, which is commonly abbreviated as "poly(3HB)" or "PHB." The discovery of poly(3HB) in bacteria is attributed to Maurice Lemoigne of the Pasteur Institute, who in 1925 reported the presence of 3-hydroxybutyric acid in the autolysate of *Bacillus megaterium* (Lemoigne, 1925) and in 1926 reported the isolation from *B. megaterium* of a polyester with the empirical formula of $(C_4H_6O_2)n$ and identified the material as poly-3-hydroxybutyrate (Lemoigne, 1926). In a note published in 1943, Lemoigne indicated that the occurrence of a PHB-like material in *Azotobacter* was reported by M. W. Beijerinck as early as in 1901, but it had been overlooked until it was reported by Stapp in 1918 (Lemoigne, 1943).

Schlegel et al. (1961) presented a landmark study on the formation and utilization of poly (3HB) by three strains of hydrogen-oxidizing or knallgas bacteria belonging to the genus "*Hydrogenomonas*," which has since been

renamed as "*Alcaligenes eutrophus*" and as "*Ralstonia eutropha*" (Yabuuchi et al., 1995). Electron micrographs of thin sections of *Hydrogenomonas* H16 showed that the inclusions of poly(3HB) could accumulate to occupy the bulk of the intracellular space, and the amount of poly(3HB) reached 65% of the dry weight of these cells. Poly(3HB) purified from *Azotobacter chroococcum*, *Bacillus megaterium* and *Hydrogenomonas* (strains H1, H16 and H20) by these workers had the expected chemical composition and identical infrared spectra. The melting points ranged from 150–155°C (*A. chroococcum*) to 168–173°C (*B. megaterium*), and the variation was attributed to different degrees of polymerization, an explanation that was validated by later studies. The earlier workers found that poly(3HB) is soluble in chloroform but not in ethyl ether, which provides an effective method for the purification of poly(3HB) from lyophilized cells.

Chemical Structure PHAs are hydrophobic linear polyesters with the general structure of

$$-[-O-CHR-(CH_2)-CO-]_{100-30,000}$$

For biosynthetic PHAs, n can vary from 1 (poly-3-hydroxyalkanoate) to 4 (poly-6-hydroxyalkanoate). The hydroxyl-substituted carbon atom (the chiral center) of the acid monomer and the corresponding carbon atom in the PHAs are in the *R* configuration. Over 120 different acid monomers have been found as constituents of PHAs (Anonymous, 1999). Furthermore, the PHAs may be homo- or heteropolymers. The *R* groups or side chains of the acid monomers may be aliphatic or aromatic, saturated or unsaturated, linear or branched, and halogenated or epoxidized. These side chains also can be modified chemically (Madison and Huisman, 1999). A simpler side chain can range from a hydrogen atom to an aliphatic chain of up to 13 carbon atoms, i.e., these PHAs are the condensation product of monomers ranging from 3-hydroxypropionic acid to 3-hydroxyhexadecanoic acid (Steinbüchel, 1996; Madison and Huisman, 1999). Because of the diversity of the side-chains in the PHAs, the physicochemical properties of PHAs vary significantly to allow a broad range of possible applications.

Physicochemical Properties For practical purposes, the most important properties of PHAs are the thermoplasticity and elasticity. The PHAs are thermoplastic materials, meaning that they are resins that become highly viscous and moldable at temperatures close to or above the melting point. The properties of poly(3HB) are often compared to those of polypropylene (Hocking and Marchessault, 1994), as both polymers have similar melting points, degrees of crystallinity, and glass-transition temperatures. However, poly(3HB) is both stiffer and more brittle than polypropylene, resulting from the presence of large crystals in poly(3HB). Poly(3HB) has a lower solvent resistance but a much higher resistance to ultraviolet weathering than polypropylene. The solubility of poly(3HB) in a wide range of solvents has been determined (Steinbüchel, 1996). The crystal structure of PHAs has been discussed in some detail by Hocking and Marchessault (1994).

The material properties of poly(3HB) are greatly improved by the incorporation of 3-hydroxyvaleric acid to form the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) or poly(3HB-co-3HV) copolymer (Hocking and Marchessault, 1994). By manipulating the ratio of the two monomers in the copolymer, a range of physical properties may be obtained. Because organisms other than *R. eutropha* have PHA polymerases that can incorporate a variety of 3-hydroxyalkanoic acids into the copolymer, application of genetic engineering should allow the production of PHAs with superior physical properties as well as biodegradability.

Poly(3HB) and poly(3HB-co-3HV) are piezoelectric materials, whereas the piezoelectric properties of other PHAs have not been investigated (Steinbüchel, 1996). The piezoelectric materials produce electric charges on parts of their surface when mechanical pressure is applied to the crystalline material, and an electric current will result from the charges if the crystal is short-circuited. Conversely, application of a voltage between certain faces of the material produces a mechanical distortion (a deformation) of the material. Piezoelectric materials have important applications in electromechanical transducers, such as microphones. In medicine, chemically synthesized piezoelectric polymers such as polyvinylidone fluoride stimulated bone growth. The piezoelectric property of poly(3HB) may be important for some medical applications (Steinbüchel, 1996).

PHA-Producing Organisms Most genera of bacteria and members of the halophilic Halobacteriaceae of the Archaea synthesize PHAs. A comprehensive listing of PHA-producing organisms can be found in Steinbüchel (1991), which illustrates the wide distribution of the capacity to synthesize PHAs among microbes and also suggests the physiological importance of PHAs to the producing-organisms. Table 23, adapted from Lee (1996b) and Steinbüchel and Füchtenbusch (1998) and also with newer findings, lists the salient features of representative natural and recombinant strains of PHA-producing bacteria. It should be noted that the name of the PHA-

Table 23. Production of polyhydroxyalkanoic acid by natural and recombinant strains of bacteria.

Bacterium	PHA	Carbon source	Cell density (g/liter)	PHA content (%)	References
Alcaligenes latus	Poly(3HB)	Sucrose	112	88	Wang and Lee, 1997
Azotobacter vinelandii	Poly(3HB)	Glucose	40.1	79.8	Page and Cornish, 1993
Chromobacterium violaceum	Poly(3HV)	Valeric acid	41	65–70	Steinbüchel and Schmack, 1995
Escherichia coli, recombinant	Poly(3HB)	Glucose	117	76	Kim et al., 1992b
Escherichia coli, recombinant	Poly(3HB)	Whey	194	87	Ahn et al., 2001
Escherichia coli, recombinant	Poly(3HB-co-3HV)	Glucose + propionic acid	203.1	78.2	Choi and Lee, 1999b
Klebsiella aerogenes, recombinant	Poly(3HB)	Molasses	37	65	Zhang et al., 1994
Methylobacterium organophilum	Poly(3HB)	Methanol	250	52	Kim et al., 1996
Protomonas extorquens	Poly(3HB)	Methanol	233	64	Suzuki et al., 1986
Pseudomonas oleovorans	Poly(3HHx-co-3HO)	Octane	40	35–40	De Koning et al., 1997
Pseudomonas oleovorans	Poly(3HHx-co-3HO-co-3HD)	Glucanoic acid + octanoic acid	54	66	Kim et al., 1997
Pseudomonas putida	Poly(3HHx-co-3HO-co-3HD-co-3HDD-co-3HTD)	Oleic acid	93	45	Weusthuis et al., 1996
Pseudomonas putida, recombinant	Poly(3HB-co-3HV-co-4HV-co-3HHx-co-3HO)	Octanoic acid + levulinic acid	20	43	Steinbüchel and Gorenflo, 1997
Ralstonia eutropha	Poly(3HB)	Glucose	164	74	Kim et al., 1994
Ralstonia eutropha	Poly(3HB)	Carbon dioxide	91.3	67.8	Tanaka et al., 1995
Ralstonia eutropha	Poly(3HB-co-3HV)	Glucose + propionic acid	>100	70–80	Byrom, 1992

Abbreviations: PHA, polyhydroxyalkanoic acid; 3HB, 3-hydroxybutyric acid; 3HD, 3-hydroxydecanoic acid; 3HDD, 3-hydroxydodecanoic acid; 3HTD, 3-hydroxytetradecanoic acid; 3HHx, 3-hydroxyhexanoic acid; 3HV, 3-hydroxyvaleric acid; 3HO, 3-hydroxyoctanoic acid; and 4HV, 4-hydroxyvaleric acid.

producing organisms can be a potential source of confusion because bacterial taxonomy is a work in progress. Both the name and the taxonomic position of an organism are subject to change. For example, the name of the best-known PHA-producing organism has been changed from *Hydrogenomonas* H16 to *Alcaligenes eutrophus* to *Ralstonia eutropha*. A literature search based on any one of these names will miss a large amount of pertinent information.

Besides heterotrophic bacteria and archaea, the phototrophic bacteria, including phototrophic purple bacteria and cyanobacteria, are also PHA producers. A study of 15 strains of nonsulfur purple bacteria and 15 strains of sulfur purple bacteria showed that all of them produced poly(3HB) when the growth medium was supplemented with acetate (Liebergesell et al., 1991). When supplemented with propionate, valerate, heptanoate or octanoate, most of the strains produced poly(3HB), and the nonsulfur purple bacteria produced poly(3HB-co-3HV) copolymers even with acetate as the carbon source. The production of PHA by cyanobacteria was first reported by Carr (1966), and the list of PHA-producing cyanobacteria includes species of *Anabaena*, *Aphanothece*, *Chloroglea*, *Gloeocapsa*, *Oscillatoria*, *Spirulina*, *Synechococcus* and *Synechocystis* (Hein et al., 1998; Taroncher-Oldenburg et al., 2000). Production of PHAs by cyanobacteria for commercial purposes has attracted attention lately because the phototrophic organisms use CO_2 instead of the more expensive organic compounds as the carbon source.

Besides the PHA-producing organisms available in pure cultures, various environmental samples, such as the sludge from sewage digesters, contain characteristic PHAs that have not been observed in pure cultures (Steinbüchel, 1996). The biosynthetic capabilities of these not yet characterized PHA-producing organisms may greatly expand the possibility of producing novel chiral monomers for the chemical and

pharmaceutical industries (see "Production of Chiral Monomers" in this Chapter).

Physiological Functions of PHAs The bulk of PHAs is synthesized as a storage material for carbon and energy or as the result of the cell's need to use an alternative route to dispose of excess reducing power under O_2 deficiency. These PHAs occur as granules or inclusion bodies in the cell and contain non-PHA substances for the assembly of such structures (Steinbüchel, 1996). In addition, PHAs also are produced by other organisms, including *Escherichia coli*, but to a much lower level and in complexes with calcium and polyphosphates. It has been proposed that complexes of poly(3HB), calcium ions, and polyphosphates form a helical tubular structure which is located in the cytoplasmic membrane of *E. coli* (Reusch and Sadoff, 1988). Because transformation efficiency correlated with the concentration of poly(3HB) in the membrane, it was speculated that the tubular structure in the membrane might play a role in conferring a cell's competency in taking up extracellular DNA (Reusch, 1992a). However, this model has been challenged (Müller and Seebach, 1993).

Unbound or complexes of PHAs also occur in eukaryotic organisms, including baker's yeast and tissues of various plants and animals, but the concentrations were one or two orders of magnitude lower than those in competent cells of *E. coli* (Reusch, 1992a; Reusch and Sparrow, 1992b). The function of PHA in eukaryotes is not known (Steinbüchel, 1996).

Biosynthesis The biosynthesis of PHAs involves three key reactions:

1) the condensation of two acyl-CoA molecules to form a 3-ketoacyl-CoA, with the release of a CoA,

2) the reduction of 3-ketoacyl-CoA to the chiral (*R*)-(−)-hydroxyacyl-CoA monomer, and

3) the polymerization of the monomers to PHA. Two non-chiral compounds, acetyl-CoA and its condensation product acetoacetyl-CoA, are the most common metabolic intermediates serving as the precursors for the synthesis of poly(3HB) or other PHAs. Stereospecific reductases and hydratases convert acetoacetyl-CoA into chiral (*R*)-(−)-3-hydroxybutyric acid, which is polymerized into poly(3HB). From different starting substrates, different pathways are used for the synthesis of acetyl-CoA, and different organisms may use distinct pathways to form (*R*)-(−)-3-hydroxybutyric acid (Steinbüchel, 1996). Besides acetyl-CoA, longer-chain acyl-CoAs are synthesized via specialized reactions when appropriate substrates are available. The actual composition of the PHA formed thus depends on the types of monomers that are present in the cell, which in turn depends on the carbon substrates that are provided in the growth medium. This flexibility allows one to direct an organism to synthesize PHA copolymers with tailored monomer compositions that confer desirable properties.

Among the different pathways for PHA biosynthesis, the pathways for the formation of poly(3HB) are the best characterized. In *R. eutropha*, the synthesis of poly(3HB) from acetyl-CoA requires only the three reactions described above, whereas in *Rhodospirillum rubrum*, two additional reactions are required to convert (*S*)-(+)-3-hydroxybutyryl-CoA to (*R*)-(−)-3-hydroxybutyryl-CoA to be used by the poly(3HB) polymerase (Steinbüchel, 1996). Formation of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) or poly(3HB-co-3HV) in *R. eutropha* requires the condensation of acetyl-CoA and propionyl-CoA to form 3-ketovaleryl-CoA; a 3-ketothiolase different from that catalyzing the formation of acetoacetyl-CoA is required for this reaction (Slater et al., 1998). In Pseudomonas oleovorans and *Pseudomonas aeruginosa*, which synthesize PHAs from medium-chain-length monomers (more than five carbon atoms), a multitude of reactions are involved in converting acetyl-CoA to (*R*)-(−)-3-hydroxyacyl-CoA or (*R*)-(−)-3-hydroxyacyl-ACP (acyl carrier protein) to serve as the substrate for the PHA polymerase (Steinbüchel, 1996).

The enzymes and their structural genes for the three key reactions for the synthesis of PHAs of short-chain-length monomers (three to five carbon atoms) have been extensively studied, and their salient features are summarized below.

3- OR β-KETOTHIOLASE. The thiolase for PHA synthesis is known as the biosynthetic thiolase because its physiological function is in the direction of combining two acyl-CoA molecules into a 3-ketoacyl-CoA molecule, instead of the thiolytic reaction occurring in β-oxidation of saturated fatty acids. Ralstonia eutropha has three or more biosynthetic 3-ketothiolases (enzyme A, enzyme B, and BktC) that differ in their substrate specificity and hence their role in PHA production (Haywood et al., 1988; Slater et al., 1998). The activity of enzyme A (encoded by the *phbA* gene) with 3-ketovaleryl-CoA (3-ketopentanoyl-CoA) is 3% of that with acetoacetyl-CoA and is hence responsible for the formation of poly(3HB). The activities of enzyme B (encoded by the bktB gene) with 3-ketoacyl-CoAs having 5 to 10 carbon atoms are 10–30% of that with acetoacetyl-CoA (Haywood et al., 1988). Enzyme B or BktB is responsible for the formation of the 3HV monomer for the production of

the copolymer poly(3HB-co-3HV) (Slater et al., 1998). Slater et al. (1998) reported the presence of another 3-ketothiolase (BktC) that may serve as a secondary route toward 3HV production. The BktC enzyme was detected in both the wild type and a double mutant for enzymes A and B.

The structural genes for 3-ketothiolase have been cloned and sequenced from over a dozen of PHA-producing bacteria, and they have been designated as either "*pha*" (for PHA metabolism) or "phb" (for poly[3HB] or PHB metabolism) genes (Madison and Huisman, 1999). The *phbA* gene encodes the 3-ketothiolase (enzyme A) for the synthesis of acetoacetyl-CoA, and the gene was first cloned from *Zoogloea ramigera* (Peoples et al., 1987) and then from *R. eutropha* (Peoples and Sinskey, 1989) and a number of other organisms. In *R. eutropha*, the *bktB* gene encodes the second 3-ketothiolase, also known as "enzyme B" or "BktB" (Slater et al., 1998). The *pha* genes are for the synthesis of medium-chain-length PHAs (PHAs composed of 3-hydroxy fatty acids with 6-16 carbon atoms), and at present there are fewer characterized *pha* than *phb* genes. Although the *pha* and *phb* genes were initially found to form operons, it now appears that these genes are not necessarily clustered and that the gene organization may vary from one species to another as these organisms have diverse PHA biosynthetic pathways (Madison and Huisman, 1999).

3- OR β-KETOACYL-COA REDUCTASE. The 3-ketoacyl-CoA reductase catalyzes a crucial reaction for the synthesis of PHA; it introduces the correct chirality into 3-hydroxyacyl-CoA to provide the substrate for PHA polymerase, and by consuming 3-ketoacyl-CoA, the reductase and PHA polymerase together make the 3-ketothiolase-catalyzed condensation reaction energetically favorable. Also, NADPH is the predominant coenzyme and reductant for the characterized 3-ketoacyl-CoA reductase. The availability of reducing power in the form of NADPH is thus considered the driving force for PHA formation (Madison and Huisman, 1999).

In *R. eutropha*, acetoacetyl-CoA reductase is encoded by the *phbB* gene (Peoples et al., 1987). The phb biosynthesis operon in *R. eutropha* consists of the *phbC* (encoding PHA polymerase), *phbA* (encoding 3-ketothiolase), and *phbB* genes in that order. The three enzymes encoded by the *R. eutropha phb* operon are sufficient for the synthesis of PHAs consisting of short-chain-length monomers (3-hydroxyacyl-CoAs with 3–5 carbon atoms).

PHA POLYMERASE OR SYNTHASE. The key enzyme for PHA biosynthesis is the PHA polymerase, also known as the "PHA synthase," which catalyzes the polymerization of the (*R*)-(−)-hydroxyacyl-CoA monomers to PHAs. To date, the structural genes for PHA polymerase have been cloned from about 30 bacteria. Based on the subunit composition and the substrate range, the PHA polymerases can be divided into three classes: the Class I represented by the enzyme of *R. eutropha*, the Class II represented by the enzyme of *Pseudomonas oleovorans*, and the Class III represented by the enzyme of *Chromatium vinosum*. The Class III PHA polymerases are two-component enzymes, encoded by the genes *phaC* and *phaE*, whereas the other PHA polymerases contain one polypeptide encoded by the *phaC* gene. A comparison of the PHA polymerases may be found in Hein et al. (1998) and Madison and Huisman (1999). The Class III PHA polymerases may be further divided into two classes, with the Class IV represented by the enzyme from *Thiocapsa pfennigii*, which has a broader substrate range (including CoA thioesters of medium-chain-length hydroxyalkanoic acids) than that of *C. vinosum* (Steinbüchel, 1996).

The genes for the two-component PHA polymerase of the cyanobacteria *Synechocystis* sp. strain PCC6803 were cloned and characterized (Hein et al., 1998). Because the genome of this organism has been sequenced, it also has allowed the identification of the genes for the PHA-specific 3-ketothiolase and acetoacetyl-CoA reductase in this cyanobacterium (Taroncher-Oldenburg et al., 2000). Transformation of *E. coli* or a PHA-negative mutant of *R. eutropha* with the PHA biosynthesis genes of *Synechocystis* sp. strain PCC6803 conferred the PHA-synthesizing ability to *E. coli* or *R. eutropha* and also allowed the positive identification of genes and enzymes for the complete PHA biosynthesis pathway of cyanobacteria.

In addition to the genes encoding the enzymes for the three key reactions of PHA synthesis, other identified *pha* genes play roles in the assembly of the PHA granules or in the conversion of metabolic intermediates leading to PHA synthesis. The *phaF* and *phaI* genes of *Pseudomonas oleovorans* encode major PHA granule-binding proteins (Prieto et al., 1999a). The *phaD* gene of *P. oleovorans* encodes a protein that is not a major granule-associated protein; however, the PhaD protein seems to be required for the presence of the PhaI protein (Klinke et al., 2000). Inactivation of the *phaD* gene caused the disappearance of the PhaI protein, a decrease in the PHA granule size, and an increase in the number of granules in the cell. The *phaG* gene encodes the 3-hydroxydecanoyl-ACP (acyl-carrier protein):CoA transacylase in *Pseudomonas putida*, which provides a metabolic link between the de novo biosynthesis of fatty acids and the biosynthesis of PHA consisting of C_6 to C_{14} monomers (Rehm et al., 1998).

Transfer of the *P. putida phaG* gene and the *P. aeruginosa phaC1* gene (encoding PHA polymerase) into *Pseudomonas fragi* (a PHA nonproducer) enables the transformant to synthesize PHAs containing primarily C_{10} monomers derived from gluconate, an unrelated carbon source (Fiedler et al., 2000).

Degradation Degradation of PHA is important for two practical reasons: it affects the yield and quality of PHA during commercial production, and it affects the disposal of consumer goods manufactured from PHA. Degradation of PHA can occur as a nonbiological process or as the result of extracellular and intracellular activities of PHA-degrading enzymes. This topic has been reviewed by Dawes and Senior (1973), Anderson and Dawes (1990), Hocking and Marchessault (1994), Steinbüchel (1996), and Jendrossek et al. (1996).

Nonbiological degradation of PHA is the result of spontaneous hydrolysis or thermal decomposition. Spontaneous hydrolysis occurs at a slow rate but is enhanced by acidic and alkaline conditions. There is an apparent initial increase in crystallinity, when the extent of hydrolysis increases, which is followed by a decrease in crystallinity and an elevated rate of hydrolysis. Thermal decomposition of many PHAs occurs at temperatures not far above the melting point, which necessitates the use of additives in the PHAs to minimize structural damages during the molding processes. If the molded articles will be subjected to prolonged heat exposure, cautionary measures are also necessary. The mechanisms of hydrolytic and thermal degradation have been reviewed by Hocking and Marchessault (1994).

Biodegradation in the environment normally proceeds via surface attack by bacteria, fungi and algae. Because the degradation process initiates on the surface, the rate of biodegradation depends in part on the ease of surface colonization. Therefore, the surface area and texture as well as the thickness of the article are important factors in affecting the rate of degradation. The supply of nutrients such as nitrogen and phosphorus as well as oxygen is the other environmental factor that controls microbial growth and hence the rate of biodegradation. Among the different environments, the anaerobe sewage allows the fastest rate of degradation, which is followed by well-watered soil, seawater sediments, aerobic sewage, the rumen of cattle, and seawater. A 1-mm thick molding resin test bar breaks down completely within 6 weeks in anaerobic sewage; this time increases to 60–70 weeks in aerobic sewage and soil at 25°C and to 350 weeks in seawater at 15°C. Packaging films 50 μm in thickness totally degrade in 1–2 weeks in anaerobic sewage, 7 weeks in aerobic sewage, 10 weeks in soil at 25°C and 15 weeks in seawater at 15°C. A molded shampoo bottle made of poly(3HB-co-3HV) is almost completely degraded after 15 weeks in a compost pile; significant degradation in a landfill occurs over 40 weeks. These results clearly indicate that the disposal of PHA-based consumer products must be properly managed or the time required for biodegradation may take years in some environments. The original studies yielding these results are referenced in the review by Hocking and Marchessault (1994).

Biodegradation in the environment involves extracellular enzymes secreted by microorganisms, and these enzymes have been collectively described as PHA depolymerases. Of the characterized depolymerases, most of them hydrolyze the PHA polymer into dimers, and the dimers are further hydrolyzed to the monomers by an extracellular or intracellular dimer hydrolase. The depolymerase from a *Comamonas* species, however, appears to hydrolyze poly(3HB) directly to 3-hydroxybutyrate (reviewed by Hocking and Marchessault, 1994). Not surprisingly, the degree of crystallinity significantly affects the rate of PHA degradation, and it was concluded that poly(3HB) molecules in the amorphous state are more easily hydrolyzed than poly(3HB) in the crystalline state (reviewed by Steinbüchel, 1996).

Extracellular PHA or oligomer depolymerases have been isolated from *Alcaligenes faecalis*, as reviewed by Anderson and Dawes (1990); *Pseudomonas lemoignei* (reviewed by Steinbüchel, 1996); and other organisms (reviewed by Steinbüchel, 1996). From each organism, the distinct depolymerases exhibit different substrate specificity and kinetic properties, and depending on the depolymerase, the dimeric product may be released from either the free hydroxy terminus or the carboxyl terminus of PHA or oligomers derived from the polymer. Structural genes for these depolymerases have been cloned and characterized. The deduced amino acid sequences of the depolymerases all contain the motif Gly-Leu-Ser-Xaa-Gly at the N-terminus, which corresponds to the Alipase box found in lipases and esterases (Jendrossek et al., 1995), and they also share a triad consisting of serine (from the Alipase box) plus histidine and aspartate, which corresponds to the active-site triad of bacterial lipases.

Intracellular degradation of PHAs in the PHA-producing organisms is a physiological process, and this activity can potentially influence the quality and yield of PHAs to be isolated for commercial applications. Research on this aspect of PHA metabolism has been reviewed by Anderson and Dawes (1990), Hocking and

Marchessault (1994), and Steinbüchel (1996), but less is understood about this process than extracellular biodegradation. In *R. eutropha*, biosynthesis and degradation of poly(3HB) have been reported to occur simultaneously, even when poly(3HB) is being accumulated, hence causing a constant turnover of poly(3HB) within the inclusion bodies (Doi et al., 1990b). It was also reported that high molecular weight poly(3HB) was produced in the first stage of accumulation (up to 10 h), and the molecular weight slowly decreased during the remaining period of accumulation (Kawaguchi and Doi, 1992).

Degradation of the native granules of poly(3HB) seems to involve different enzymes and accessory proteins in different organisms. In *R. rubrum* and *Azotobacter beijerinckii*, the native granules are self-hydrolyzing, whereas degradation of native granules isolated from *Bacillus megaterium* required a heat-labile factor associated with the granules together with three soluble components—a heat-stable protein activator, a heat-labile depolymerase and a hydrolase—prepared from the extracts of *R. rubrum* (reviewed by Anderson and Dawes, 1990; Hocking and Marchessault, 1994). Purified poly(3HB) or denatured granules did not serve as substrates. In *R. eutropha*, 3HB monomers are the direct product of depolymerase action, whereas in other organisms, the depolymerases degrade poly(3HB) to dimers and a dimer hydrolase completes the degradation to monomers. The 3HB monomers may be metabolized to acetyl-CoA through reactions formally resembling the reversal of the biosynthetic reactions.

Metabolic Engineering Metabolic engineering of PHAs was reviewed by Madison and Huisman (1999), and literature in this area is expanding at a fast rate because the use of molecular biology tools has been successful in improving the properties of organisms ranging from bacteria to higher plants for PHA production. The need to have better organisms for the production of PHAs became obvious after the commercial introduction of BIOPOL, which was produced by mutant strains of glucose-grown *R. eutropha* converting glucose and propionate into poly(3HB-co-3HV). *Ralstonia eutropha* can accumulate PHA to a high level in the cell; however, the bacterium has a relatively low optimal growth temperature and long generation time, the substrates are expensive, the cell is relatively hard to lyse, and it has enzymes for PHA degradation. Also, the genetics of *R. eutropha* were not well understood, which hindered genetic manipulations of this natural producer. Nevertheless, the *lac* and *gal* operons of *E. coli* were successfully transferred into *R. eutropha* and expressed, hence enabling *R. eutropha* to use lactose (the major carbohydrate in whey) as a less expensive carbon source (Pries et al., 1990). When the PHA-biosynthesis genes of *R. eutropha* were cloned and expressed in *E. coli*, it effectively started metabolic engineering of *E. coli* for PHA production (Schubert et al., 1988). Compared with *R. eutropha*, the recombinant *E. coli* strains have the desired properties of having a faster growth rate, ease of cell lysis when the cell is loaded with PHA, tolerance to propionic acid, and the feasibility to perform a variety of genetic manipulations.

Initially, the effect of added copies of PHA biosynthesis genes on PHA production was examined in several natural and non-natural PHA-producing bacteria. The studies found no dramatic increases in PHA production by introducing additional copies of *phb* or *pha* genes (reviewed by Madison and Huisman, 1999). The results are not surprising in light of the finding that other factors, such as the supply of NADPH (Lee et al., 1996c), limit PHA production. Supplementation of amino acids or oleate, both of which require substantial reducing power for their synthesis, increased poly(3HB) production (Lee et al., 1995c).

Different strains of *E. coli*, including K12, B, W, XL1-Blue, DH5α, HB101, JM 109 and C600, were similarly transformed with the stable high-copy-number plasmid pSYL105 containing the PHA biosynthesis genes of *R. eutropha*, and poly(3HB) production by the transformants was compared (Lee and Chang, 1995b). The rate of poly(3HB) synthesis, the extent of poly(3HB) accumulation, and the yield of poly(3HB) from glucose varied considerably from one strain to another. Strains XL1-Blue and B (harboring the plasmid) produced poly(3HB) at the highest rate. A poly(3HB) concentration of 81 g/liter could be obtained in 41 h from a pH-stat fed-batch culture of *E. coli* (pSYL105).

Following the transfer of the PHA biosynthesis genes from *R. eutropha* into *E. coli*, the recombinant *E. coli* strains were further altered by the introduction of other genes. The transfer of the *prpE* gene, encoding the propionyl-CoA synthetase, from *Salmonella enterica* serovar *typhimurium* enabled the strain to produce poly(3HP-co-3HB) (Valentin et al., 2000a). Inactivation of the ketoacyl-CoA degradation step of the β-oxidation enabled the strain to produce PHAs having different monomer compositions when grown on different alkanoates (Ren et al., 2000). Introduction of genes coding for the enzymes for the conversion of glutamate or 4-aminobutyrate to 4-hydroxybutyryl-CoA (see "Production of PHA Copolymers" in this Chapter) enabled the recombinant strain to produce poly(3HB-co-4HB) (Valentin et al., 2000b).

When the PHA polymerase genes of *Thiocapsa pfennigii* and the genes for butyrate kinase

and phosphotransbutyrylase of *Clostridium acetobutylicum* were transferred into *E. coli*, the recombinant strain produced copolymers and terpolymers of 3HB, 4HB and 4HV when provided with the corresponding hydroxy fatty acids (Liu and Steinbüchel, 2000b). Introduction of the PHA polymerase gene (*phaC1*) from *Pseudomonas oleovorans* into *E. coli* with a stable regulated expression system allowed the recombinant *E. coli* to produce chiral medium-chain-length PHAs (Prieto et al., 1999b). Other examples of expressing the PHA polymerase gene of pseudomonads in *E. coli* to produce medium-chain-length PHAs can be found in the review by Madison and Huisman (1999).

Chromobacterium violaceum produces poly(3HB) or poly(3HB-co-3HV) when grown on a fatty acid carbon source. When the PHA polymerase gene of *C. violaceum* was transferred into *E. coli* or *Pseudomonas putida* and expressed, the recombinants did not accumulate a significant level of PHA. However, similarly transformed *Klebsiella aerogenes* and *R. eutropha* accumulated poly(3HB), poly(3-HB-co-3HV) and poly(3-HB-co-3hydroxyhexanoate). Transfer of the *P. putida phaG* gene (encoding a transacylase) and the *P. aeruginosa phaC1* gene (encoding PHA polymerase) into *P. fragi* enables the recombinant strain to produce PHAs consisting of medium-chain-length monomers (Fiedler et al., 2000). These results illustrate the relationship between the availability of different metabolic intermediates as precursors for PHA synthesis and the composition of the PHA formed. They also show that a PHA polymerase can react with different hydroxyacyl-CoAs within a certain size range, and when metabolic engineering alters the pattern of hydroxyacyl-CoAs present in a cell, the composition of PHAs formed may be similarly altered.

When PHA-producing cells are lysed for processing, the released chromosomal DNA causes a dramatic increase in viscosity. The high viscosity of cell lysate hinders the isolation of PHA during industrial production. To reduce the viscosity, addition of commercial preparations of nuclease or heat treatment is practiced, which adds to the cost of PHA production. The viscosity of cell lysates of *Pseudomonas putida* was reduced by expressing a staphylococcal nuclease in this organism (Boynton et al., 1999). In this study, a nuclease gene from *Staphylococcus aureus* was integrated into the chromosome of several strains of *Pseudomonas putida* and *R. eutropha*, and the expressed nuclease was directed to the periplasm or to the growth medium. During downstream processing, the viscosity of the lysate from a nuclease-integrated *Pseudomonas* strain was reduced to a level similar to that obtained with the wild-type strain after treatment with commercial nuclease.

Metabolic engineering also was performed successfully in eukaryotes including yeast (Leaf et al., 1996) and higher plants (see "Production of PHAs by Transgenic Plants" in this Chapter) for the production of PHAs.

Commercial Applications

Production of BIOPOL After the discovery of the thermoplastic properties of poly(3HB), W. R. Grace and Co. in the United States produced poly(3HB) for the possible commercial applications in the early 1960s, but the process was discontinued because of a low production efficiency and the lack of suitable recovery methods (Lee and Chang, 1995a). In the 1970s, industrial interests in the production of PHB resumed at Imperial Chemical Industries (ICI, United Kingdom). Commercial production of PHA began at ICI Bio Products and Fine Chemicals, which later became Zeneca BioProducts, Billingham, United Kingdom, and was the only producer of PHA on a commercial scale (Byrom, 1992; Byrom, 1994; Lee and Chang, 1995b). The trade name BIOPOL was used to describe the family of PHA polymers manufactured. In 1996, Monsanto of St. Louis, in the United States, purchased the manufacturing technology from Zeneca BioProducts, but Monsanto ended its manufacturing of BIOPOL in 1998 (information provided by Monsanto).

Alcaligenes eutrophus (now *Ralstonia eutropha*) was the organism used in the commercial production of BIOPOL. The ICI process used a fed-batch system on a scale up to 200,000 liters in capacity and produced 300 tons per year. Nutrient limitation was used as the strategy to control the onset of the PHA accumulation phase that follows a period of normal growth to produce cells. The organism grows in a mineral salts medium with glucose as the sole carbon and energy source. At the time of inoculation, the medium in the production-stage fermentor contains a calculated amount of phosphate to allow production of a given amount of cell mass when the other nutrients are in excess. When the concentration of phosphate in the medium decreases to a threshold level so that it becomes limiting (about 60 h after inoculation), the cell begins to store PHA. At this point, more glucose is fed to the culture, if poly(3HB) is the desired product. The fermentation is continued for 40–60 h until the required PHA content is reached. Cell mass in excess of 100 g/liter (dry weight) and a poly(3HB) content of 70–80% are routinely obtained (Byrom, 1992; Byrom, 1994).

To produce the poly(3HB-co-3HV) copolymer, a mixture of glucose and propionic acid, instead of glucose alone, is fed to the culture when cells reach the PHA-accumulating stage under phosphate limitation. The 3HV content of the copolymer is controlled by adjusting the ratio of glucose to propionic acid in the feed. Normally, poly(3HB-co-3HV) copolymers containing 0–30 mol% of 3HV are produced. When the wild-type *R. eutropha* was used for poly(3HB-co-3HV) production, only about 30% of the propionic acid supplied was incorporated into the copolymer because the major proportion of the added propionate was metabolized to acetate, which resulted in poly(3HB), or to CO_2. The problem was solved by the isolation of a mutant that cannot convert propionate to acetate. Use of this mutant reduced the required amount of propionate in the feed for copolymer production, and it not only lowered the cost of production but also alleviated the toxicity problem caused by high concentrations of propionate in the medium (Byrom, 1994). Copolymers having a higher fraction (up to 90 mol%) of 3HV have been produced from butyric or pentanoic acid (Doi et al., 1990b; Lee and Chang, 1995a), but production on an industrial scale has not been reported.

The large-scale commercial production of PHA also required the development of procedures that were not necessary in the laboratory or in the pilot stages. In the laboratory, PHA is effectively extracted from the cell by using organic solvents such as chlorinated alkanes. Environmental considerations preclude the use of such organic solvents in the industrial process, and a water-based extraction procedure was successfully developed. The large size of the fermentor used in the industrial process also requires a much longer time for the harvesting of cells, which can lead to the degradation of the polymer during the harvesting and the extraction stages. These problems had to be solved before commercial production of PHA became feasible. The white powder produced by the industrial process is melted, extruded, and converted into chips, which are supplied to the fabricators. The aqueous effluent generated by the industrial process can be treated in a conventional activated-sludge plant.

Manufacturing BIOPOL-Based Consumer Products BIOPOL can be processed on conventional equipment for polyolefin or other plastics, such as injection molding, extrusion blow molding or fiber-spray gun molding, and therefore was used in the manufacturing of a wide range of consumer products. The first commercial product of PHA utilized BIOPOL and was introduced in 1990. It was a shampoo bottle for the German hair care company AWella, and it was available for some time on a limited market in Germany. Bottles for other cosmetics also came onto the market in Japan. Besides uses in the manufacturing of containers and bags, PHAs also can be used as coatings on items for food service or packaging. In the review by Steinbüchel (1996), many products manufactured from BIOPOL or other PHAs are described, and they include applications in agriculture, medicine, tobacco and foodstuff industry, chemical industry, fishing industry, and other areas.

Areas of Research and Development

Production of PHA Copolymers The homopolymer of 3-hydroxybutyric acid or poly(3HB) is the prototype biodegradable PHA that is naturally produced by many bacteria. Because poly(3HB) is a crystalline and relatively brittle substance, it is not a suitable substitute for the commonly used thermoplastics manufactured from petrochemicals. Copolymers of hydroxyalkanoic acids, on the other hand, are less brittle and more elastic. Therefore, a major area of research on PHA is to develop organisms that can produce PHA copolymers with better mechanical properties and biodegradability.

The three key enzymes for PHA synthesis, 3-ketothiolase, 3-ketoacyl-CoA reductase and PHA polymerase, can usually accommodate substrates with a slightly different chain length. Therefore, two or three kinds of hydroxyalkanoic acids can be incorporated during PHA synthesis to form a copolymer or terpolymer, if the additional kinds of CoA thioesters of hydroxyalkanoic acids can be made available to PHA polymerase through a special feeding regimen or metabolic engineering or a combination of both.

The first PHA copolymer to be produced commercially is poly(3HB-co-3HV), which was produced by *R. eutropha* when fed glucose and propionic acid (Byrom, 1992). The production strain is a glucose-utilizing mutant of *R. eutropha* (*A. eutrophus*) strain H16 that also can assimilate propionate more efficiently than the wild type. Propionic acid is converted to propionyl-CoA first. Condensation of propionyl-CoA and acetyl-CoA yields 3-ketovaleryl-CoA, which is then reduced to 3-hydroxyvaleryl-CoA to serve as a substrate for PHA polymerase. The concentration of poly(3HB-co-3HV) reached 70–80% of the dry weight of the cell. By varying the ratio of glucose and propionic acid, the 3HV content in the polymer can be controlled at particular values in the range of 5–30 mol%. The PHA polymerase of *R. eutropha* accepts CoA thioesters of 3-, 4-, or 5-hydroxyalkanoic acid

with 3 to 5 carbon atoms (Steinbüchel, 1996), and PHA copolymers consisting of these monomers may be produced.

Ralstonia eutropha can produce poly(3HB-co-3HV) to a high concentration. However, this bacterium has a slow growth rate, is sensitive to propionic acid, and is hard to lyse. In addition, it also contains PHA-degrading enzymes, which can lower the yield or quality of PHA. Organisms that do not have these shortcomings were thus developed for the production of copolymers. Through metabolic engineering (see "Metabolic Engineering" in this Chapter) and special feeding programs, numerous recombinant organisms have been constructed to produce a range of PHA copolymers. Progress in this area has been rapid in recent years.

Poly(3HB-co-3HV) can now be produced by recombinant *E. coli* that acquired the PHA biosynthesis genes from *R. eutropha* or *A. latus* (Choi and Lee, 1999b; Choi and Lee, 2000) or by the transgenic plants Arabidopsis and Brassica (Slater et al., 1999). Copolymers consisting of 3-HB and 3-hydroxyalkanoate units of 6–12 carbon atoms can be produced by recombinant Pseudomonas putida and *R. eutropha* that were PHA-negative (Matsusaki et al., 2000). Poly(3-HB-co-4HB) can be produced by a PHA-producing recombinant *E. coli* that also was conferred with the ability to form 4-hydroxybutyryl-CoA from 4-aminobutyrate or glutamate (Valentin et al., 2000a). The genes for four enzymes were introduced into *E. coli* to afford the transformant the capacity to form 4-HB; the four enzymes are glutamate decarboxylase, 4-aminobutyrate:2-ketoglutarate transaminase, 4-hydroxybutyrate dehydrogenase, and acetyl-CoA:4-hydroxybutyrate CoA-transferase. Various copolymers and terpolymers consisting of 3HB, 4HB and 4HV can be produced by recombinant *E. coli* that received the butyrate kinase and phosphotransbutyrylase genes (*buk* and *ptb*) from *Clostridium acetobutylicum* and the PHA polymerase genes (*phaE* and *phaC*) from *Thiocapsa pfennigii* and is fed 3HB, 4HB and 4HV (Liu and Steinbüchel, 2000b). The PHA polymerase of *T. pfennigii* has a broader substrate range than the polymerase of *R. eutropha*.

Poly(3-hydroxypropionate-co-3HB) or poly(3HP-co-3HB) can be produced by a recombinant *E. coli* that acquired the *prpE* gene, encoding a propionyl-CoA synthetase, from *Salmonella* and the PHA biosynthesis genes from *R. eutropha* (Valentin et al., 2000b). The PHAs with medium-chain-length hydroxyalkanoates can be produced by PHA-producing recombinant strains of *E. coli* that are blocked for the ketoacyl-CoA degradation step of the β-oxidation and are fed fatty acids ranging in size from 6 to 18 carbon atoms (Ren et al., 2000). The number of copolymer-producing organisms as well as the kind of copolymer produced can be expected to increase, and many new copolymers with better material properties should result from these efforts.

Production of Chiral Monomers Because PHAs are polymers composed of chiral building blocks, hydrolysis of PHAs leads to the release of the chiral monomers, which are valuable starting material for the synthesis of pharmaceuticals and specialty chemicals. An efficient method has been reported for the production of enantiomerically pure (*R*)-(-)-hydroxyalkanoic acids by in vivo depolymerization of PHAs (Lee et al., 1999c). Those (*R*)-(-)-3-hydroxyalkanoic acids of 4–12 carbon atoms and (*R*)-(-)-3-hydroxy-5-phenylvaleric acid were prepared by providing the environmental condition under which cells possess a high activity of intracellular PHA depolymerase and a low activity of a key enzyme that could convert the chiral monomer into another compound. Monomer (*R*)-(-)-3-hydroxybutyric acid could be produced at a high yield in 30 min by in vivo depolymerization of poly(3HB) accumulated in *Alcaligenes latus*.

In Vitro Biosynthesis of PHA Poly(3HB) is present as granules in bacterial cells and can reach sizes of up to 1 μm. When isolated by chloroform extraction, Poly(3HB) is highly crystalline and has molecular mass as large as 2×10^6 daltons (see "Physicochemical Properties" in this Chapter). However, nuclear magnetic resonance (NMR) and wide-angle X-ray diffraction studies showed that in vivo the poly(3HB) polymer is in a mobile, amorphous state (Barnard and Sanders, 1989; Kawaguchi and Doi, 1990). It was also suggested that additional agents, such as lipids and surfactants, may be needed as plasticizers if the poly(3HB) granule is assembled in vitro (Kawaguchi and Doi, 1990). Any insight into these fundamental aspects of PHA biosynthesis can influence the development of an innovative approach for improving the industrial production of PHA.

Using purified PHA polymerase from *R. eutropha* and synthetically prepared (*R*)-3-hydroxybutyryl-CoA, macroscopic poly(3HB) granules were produced in vitro, and the procedure established the minimal requirement for poly(3HB) granule formation (Gerngross and Martin, 1995). To make the in vitro biosynthesis practically useful, it is necessary to recycle coenzyme A, which is a product of the polymerization reaction. This has been accomplished by incorporating additional enzymes into the in vitro reaction (Jossek and Steinbüchel, 1998; Liu and Steinbüchel, 2000a). The use of butyrate kinase and phosphotransbutyrylase produced from

cloned genes of the anaerobic bacterium *Clostridium acetobutylicum* allowed not only the recycling of CoA but also the in vitro synthesis of 3- and 4-hydroxyacyl-CoA, which then are converted to homo- or copolymers of PHA by the PHA polymerase from *Chromatium vinosum* (Liu and Steinbüchel, 2000a). In this butyrate kinase-linked in vitro system, ATP is the energy-rich compound that must be replenished to sustain the reaction.

Production of PHAs by Genetically Engineered Bacteria In addition to the search for better PHA-producing organisms from nature, much effort has been devoted to the use of mutagenesis and recombinant DNA techniques to confer desired properties to selected bacteria for PHA production. These genetically engineered bacteria can produce PHAs of specific compositions for better material properties or can use less expensive substrates. The sections on metabolic engineering (see "Metabolic Engineering" in this Chapter) and production of PHA copolymers (see "Production of PHA Copolymers" in this Chapter) describe the construction of these recombinant bacteria and the novel PHAs produced by them. These recombinant organisms have been extensively used in fermentation studies (Table 23) to maximize their productivity.

At the Korea Advanced Institute of Science and Technology (Taejon, South Korea), Dr. S. Y. Lee and coworkers have conducted extensive studies with recombinant strains of *E. coli* engineered for PHA production (Lee and Chang, 1995b; Lee, 1996a; Lee, 1996b). The recombinant *E. coli* strain XL 1-Blue (pSYL107) harboring the PHA biosynthesis genes of *R. eutropha* and the *ftsZ* gene of *E. coli* on the high copy-number plasmid pSYL107 produced poly(3HB) to 77 g/liter, with a production rate of 2 g/liter · h, in a defined medium with glucose as the carbon source (Wang and Lee, 1998a). Overexpression of the cell-division protein FtsZ was crucial as it suppresses filamentation, which limits PHA production by *E. coli*. Further optimization of the fed-batch culture conditions increased the poly(3HB) concentration to 10^4 g/liter.

The recombinant *E. coli* strain XL1-Blue (pJC4) harboring the PHA biosynthesis genes of *A. latus* was used in pH-stat fed-batch cultures to produce poly(3HB-co-3HV) from glucose and propionic acid in a defined medium (Choi and Lee, 1999b). In the absence of an induction treatment with acetic acid to stimulate the uptake and utilization of propionic acid, the cells accumulated a relatively low level (42.5%) of poly(3HB-co-3HV). With the acetic acid induction treatment in the fed-batch culture, the recombinant *E. coli* produced a cell concentration of 141.9 g/liter, a poly(3HB-co-HV) concentration of 88.1 g/liter, a poly(3HB-co-3HV) content of 62.1%, and a 3HV fraction of 15.3 mol%. With the supplementation of oleic acid (to spare NADPH), the acetic acid induction, and an improved nutrient feeding strategy, the recombinant *E. coli* produced a cell concentration of 203.1 g/liter, a poly(3HB-co-3HV) concentration of 158.8 g/liter, a poly(3HB-co-3HV) content of 78.2%, and a 3HV fraction of 10.6 mol%, resulting in a high production rate of 2.88 g/liter · h.

The result of an economic analysis was reported for the production of the poly(3HB-co-3HV) copolymer by a recombinant *E. coli* strain (Choi and Lee, 2000). For this analysis, simple NaOH digestion was used for the release of PHA from the cell because *E. coli* cells accumulating large amounts of PHA become very fragile (Choi and Lee, 1999a). Because propionic acid is more expensive than glucose, the production cost for poly(3HB-co-3HV) increased linearly with the increase in the 3HV fraction in the copolymer. Not surprisingly, the production cost for the copolymer increased significantly when the 3HV content (increasing from 5 to 50 mol%) was high and the 3HV yield (g of 3HV produced from each g of propionic acid used) was low (decreasing from 0.5 to 0.2).

The recombinant *E. coli* strain CGSC 4401, harboring the PHA biosynthesis genes of *A. latus*, produced poly(3HB) from whey (concentrated to contain 280 g of lactose-equivalent per liter) in pH-stat fed-cultures and reached a final cell concentration of 119.5 g/liter and poly(3HB) concentration of 96.2 g/liter in 37.5 h, resulting in a poly(3HB) productivity of 2.57 g/liter · h (Ahn et al., 2000). This recombinant strain was then tested in a pH-stat cell-recycle membrane system to produce poly(3HB) from similarly concentrated whey as a feeding solution (Ahn et al., 2001). The cell-recycle system produced a final cell concentration of 194 g/liter, a poly(3HB) concentration of 168 g/liter, and a PHB content of 87% in 36.5 h, resulting in a poly(3HB) productivity of 4.6 g/liter · h. This level of PHA production is among the highest, other than the level produced by the methanol-grown bacteria (Table 23).

A recombinant strain of *E. coli* was used in the development of a quantitative spectroscopic method for the measurement of poly(3HB) content in the cell (Kansiz et al., 2000). Fourier transform infrared (FTIR) spectroscopy and multivariative statistical methods were performed to determine the cellular poly(3HB) content, and a correlation coefficient of 0.988, with a standard error of 1.49% poly(3HB), was obtained between the measured and the predicted values. The spectroscopic method circumvented the need to use solvents to extract the PHA before quantitative measurements.

Search for Inexpensive Carbon Substrates

Economic evaluation of the bacterial process for poly(3HB) production suggests that the cost of the carbon substrate accounts for up to 50% of the total cost of poly(3HB) production (Choi and Lee, 1997). A successful bacterial process for PHA production hence depends on the availability of a low-cost carbon substrate. Besides carbohydrates, organic acids and short-chain alcohols are also potential carbon substrates for the industrial production of PHAs. The cellular toxicity of the latter compounds, however, requires the isolation or identification of PHA-producers that are more tolerant to these compounds. Reported progress includes the production of PHAs from whey or acetic and butyric acids.

Whey is a major by-product in the manufacturing of cheese or casein from milk. Whey from bovine milk contains approximately 4.5% (wt/vol) lactose, 0.8% (wt/vol) protein, 0.1–0.8% (wt/vol) lactic acid, and 1% (wt/vol) salts (Wong and Lee, 1998). In the United States, only half of the whey is recycled into useful products such as food ingredients and animal feed (Ahn et al., 2000). Because of its high volume and high biological oxygen demand, the rest of the whey is regarded as a pollutant that is costly to manage. Using a concentrated whey solution (280 g of lactose equivalent per liter) and a recombinant *E. coli* strain harboring PHA biosynthesis genes, final cell and poly(3HB) concentration reached 119.5 and 96.2 g/liter, respectively, in 37.5 h, which resulted in a poly(3HB) productivity of 2.57 g/liter · h (Ahn et al., 2000). These investigators found that whey could actually be concentrated by evaporation to contain 280 g of lactose per liter, which exceeds the solubility of lactose in water (200–210 g/liter). By using the fed-batch culture with the highly concentrated whey solution and a stepwise decrease in the dissolved oxygen concentration from 40% to 15%, the highest poly(3HB) productivity was obtained. By using a cell-recycle membrane system, these investigators obtained cell concentration, poly(3HB) concentration, and poly(3HB) content of 194 g/liter, 168 g/liter and 87%, respectively (Ahn et al., 2001). In localities where the disposal of whey is a problem, the production of PHA from this carbon source should provide a higher economical return.

In an attempt to improve the economics of the anaerobic acetone-butanol (AB) fermentation, scientists in Vienna, Austria, are testing the by-products, acetate and butyrate, from the AB fermentation as substrates for PHA production (Parrer et al., 2000). A butanol-tolerant strain of *Alcaligenes* was isolated that could convert acetate (5 g/liter), butyrate (7 g/liter), and added valerate (3 g/liter) to poly(3HB-co-3HV) copolymers (37 mol% 3HV) that constituted 52% of the cellular dry matter (Parrer et al., 2000).

Production of PHAs by Transgenic Plants

The established commercial process for the production of BIOPOL used bacteria (see "Production of BIOPOL" in this Chapter). However, the high cost of the conventional carbon substrates has limited the usefulness of the bacterial process. Transgenic plants, which harbor the bacterial genes for the synthesis of PHAs, have been developed to couple directly the photosynthetic activity of the green plants to the production of PHAs (Poirier et al., 1992; Nawrath et al., 1994; John and Keller, 1996; Hahn et al., 1997; Slater et al., 1999).

Initially, the *R. eutropha* (*A. eutrophus*) genes for acetoacetyl-CoA reductase (*phbB*) and poly(3HB) polymerase (*phbC*) were introduced into *Arabidopsis thaliana* through Ti plasmid-mediated transformation and cross-pollination of homozygous transgenic lines harboring either the *phbB* or the *phbC* gene (Poirier et al., 1992). These two introduced genes are expressed in the cytoplasm of the transformed plant, where an endogenous 3-ketothiolase is present. Poly(3HB) is produced by the transgenic plant, but the expression of large amounts of acetoacetyl-CoA reductase in the cytoplasm of the transgenic plants caused a significant reduction in growth and seed production.

This problem was alleviated when the three *R. eutropha* genes (*phbA*, *phbB* and *phbC*) were properly fused to signal sequences to target the expression of these genes in plastids, where storage lipids are normally synthesized. These plants accumulated poly(3HB) up to 14% of the dry weight of leaves, as 0.2–0.7-μm granules within plastids (Nawrath et al., 1994). However, varying amounts of poly(3HB) were produced by different lines of the triple hybrids, and the variation appears to be related to the widely different levels of thiolase and acetoacetyl-CoA reductase activities that are expressed in the hybrid plants. Compared with starch synthesis, the average daily rate of poly(3HB) synthesis is relatively low, although poly(3HB) is not reutilized by the plant and hence acts as a terminal carbon sink.

The copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) or poly(3HB-co-3HV) was produced in transgenic *Arabidopsis thaliana* and in the seeds of transgenic *Brassica napus* (oilseed rape) by diverting metabolic pools of acetyl-CoA and threonine into PHA production through four introduced genes that are targeted for expression in the plastids (Slater et al., 1999). The four genes involved are:

1) the *E. coli ilvA* gene, which codes for the threonine deaminase, an enzyme converting

threonine to 2-ketobutyrate (the plant pyruvate dehydrogenase complex can convert 2-ketobutyrate to propionyl-CoA, which is the precursor for (*R*)-3-hydroxyvaleryl-CoA);

2) the *R. eutropha bktB* gene, which codes for the 3-ketothiolase that can produce both acetoacetyl-CoA (from acetyl-CoA) and 3-ketovaleryl-CoA (from acetyl-CoA and propionyl-CoA);

3) the *R. eutropha phbB* gene for acetoacetyl-CoA reductase; and

4) the *phbC* gene, for the poly(3HB) polymerase, from either *R. eutropha* or *Nocardia corallina*. This designed pathway allows the production of PHA copolymers by the transgenic plants, but the level of accumulated copolymer is low, less than 3% of plant tissue dry weight (*Arabidopsis*) or the seed weight (*Brassica*). These investigators expect that polymer concentrations in plants will need to reach at least 15% of dry weight for economical production to be feasible.

The *R. eutropha phbB* and *phbC* genes also have been introduced into cotton (*Gossypium hirsutum*) with the fiber cells as the target site of expression during early or late fiber development stages (John and Keller, 1996). The objective was to alter the characteristics of the cotton fibers. Although poly(3HB) was present at a low level in the fiber (3.4 mg/g of dry fiber), it already increased the heat capacity and hence improved the insulation properties of the purified cotton. Future goals for the effort are to improve cotton characteristics such as dyeability, warmth and wrinkle resistance.

Genes for poly(3HB) biosynthesis have been introduced into Black Mexican sweet corn (maize, *Zea mays*) and expressed in cultured cells (Hahn et al., 1997). During a two-year period in a bioreactor, the introduced *phbB* and *phbC* genes were unstable, whereas the thiolase activity was constant. Also, the transformed plant cells grew more slowly than the wild-type cells. The construction of suitable plant species for the production of PHAs in specific cellular locations and at the right time will depend on progress in knowledge and tools for genetic and metabolic engineering. The introduction of new characteristics into plant materials, such as the cotton fibers, by the incorporation of PHAs represents an excellent application for the biodegradable polymer.

Patent and Regulatory Issues

Many processes for the production and application of PHAs are under the protection of patents. Steinbüchel (1996) compiled 74 patents and patent applications in his review, and 16 of them were assigned to ICI. Among them, 33 patents or patent applications were for the production of PHA, encompassing the poly(3HB) homopolymer (e.g., EP 46344-A1, EP 144017-A1, JP 06251889-A2 and US 4910145), the poly(3HB-co-3HV) copolymer (e.g., EP 204442-A2, EP 288908-A2, EP 396289-A1, and EP 90304267.9), the poly(3HB-co-4HV) copolymer (EP466050-A1) and the poly(3HB-co-4HB) copolymer (e.g., JP 2234683-A2, JP 03216193-A2, and JP 03292889-A2), as well as the PHA biosynthesis genes (US 5229279, US 5245023, and US 5250430, all assigned to Metabolix); 23 were for the isolation of PHA, including those for the poly(3HB) homopolymer (e.g., EP 46335, EP 145233-A1, and JP 62205787-A2) and the poly(3HB-co-3HV) copolymer (EP 431883-A1 and WO 9118995-A1); 8 were for the processing or modification of PHA into films (US 3182036), 3HB monomer and oligomers (US 4365088-A1 and EP 320046-A1), and 3HB esters (EP 377260-A1 and US 5107016-A1); 10 were for the applications of PHA, which include microporous membranes (JP 60137402-A1), surgical devices for bone fractures (WO 8607250-A1), microcapsules for retard materials (DE 3428372-A1 and EP 315875-A1), cigarette filter tips (DE 4013293-C2 and DE 4013304-A1), toner and developer for photocopier (US 5004664-A1), flavor delivery systems (WO 9209210-A1), and cream substitute (WO 9209211-A1). The number of pertinent patents can be expected to increase when more recombinant organisms, novel production systems and applications are developed.

When the PHA-based consumer products or medical devices are proclaimed to be biodegradable, government regulations and standards become necessary to safeguard the quality of the environment and the welfare of patients. It is necessary to define biodegradability, standards, and the test methods. At the Third International Scientific Workshop on Biodegradable Plastics that was held in Osaka, Japan, in November 1993, a session was devoted to government policy, regulations and standards (Albertsson and Marchessault, 1994). Pertinent government policy and regulations of the United States (Narayan, 1994), Italy (Chiellini, 1994) and Korea (Chang et al., 1994) can be found in these publications.

Prospects

The commercialization of consumer goods manufactured from BIOPOL has confirmed the usefulness of PHAs as a substitute for petrochemical-derived thermoplastics. The discontinuation of the BIOPOL process, however, illustrates that the high cost of bacterial fermentation will limit the commercial potential of PHAs that are produced by fermentation-based

processes. The result of a case study casts doubt on the environmental benefits of producing PHAs from corn-derived glucose (Gerngross, 1999). Because a considerable amount of fossil fuels is needed to produce corn and then to convert corn starch into glucose for bacterial fermentation, a greater amount of fossil fuels (2.39 kg versus 2.26 kg) may actually be required to produce 1 kg of PHAs than to produce 1 kg of polystyrene from petrochemicals. To overcome this unfavorable situation, less expensive carbon substrates are being sought to replace glucose as the starting material. Whey has been identified as a promising alternative substrate (Ahn et al., 2001). In addition, phototrophic organisms are being tested as the primary producers of PHAs so that CO_2 can serve as the carbon substrate. Transgenic plants with introduced bacterial genes have been shown to produce PHAs, but the level of PHAs produced is so far lower than that in the bacteria. Another approach is to use unicellular phototrophic bacteria, which may be more efficient than the higher plants in the utilization of carbon and energy, to produce PHAs. The use of less expensive carbon substrates or phototrophic bacteria may allow the development of more economical and sustainable processes for the future commercial production of PHAs.

Besides uses as a commodity chemical for the manufacturing of consumer goods, PHAs also have other possible novel applications. For example, PHAs may be used in the delivery system for the long-term release of drugs, in open-heart surgery as non-woven patches for pericardium repair or in orthopedic medicine because the piezoelectric properties of PHAs stimulate bone growth (Steinbüchel, 1996). It has also been suggested that PHAs can serve as the source of a broad range of chiral hydroxy acids, which are valuable building blocks for the pharmaceutical industry (Lee et al., 1999c). These novel uses for PHAs will ensure a future for this class of biomaterial whether or not it will be produced by phototrophic or nonphototrophic organisms.

Literature Cited

Abbad-Andaloussi, S., C. Manginot-Durr, J. Amine, E. Petitdemange, and H. Petitdemange. 1995. Isolation and Characterization of Clostridium butyricum DSM 5431 mutants with increased resistance to 1,3-propanediol and altered production of acids. Appl. Environ. Microbiol. 61:4413–4417.

Abbad-Andaloussi, S., C. Durr, G. Raval, and H. Petitdemange. 1996. Carbon and electron flow in Clostridum butyricum grown in chemostat culture on glycerol and glucose. Microbiol. 142:1149–1158.

Abbad-Andaloussi, S., E. Guedon, E. Spiesser, and H. Petitdemange. 1996. Glycerol dehydratase activity: The limiting step for 1,3-propanediol production by Clostridium butyricum DSM 5431. Letts. Appl. Microbiol. 22:311–314.

Abrini, J., H. Naveau, and E.-J. Nyns. 1994. Clostridium autoethanogenum, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch. Microbiol. 16:345–351.

Adamse, A. D. 1980. New isolation of Clostridium aceticum, (Wieringa). Ant v. Leeuwenhoek 46:523–531.

Adler, H. I., and W. Crow. 1987. A technique for predicting the solvent-producing ability of Clostridium acetobutylicum. Appl. Environ. Microbiol. 53:2496–2499.

Afschar, A. S., K. H. Bellgardt, C. E. Vaz Rossell, A. Czok, and K. Schaller. 1991. The production of 2,3-butanediol by fermentation of high test molasses. Appl. Microbiol. Biotechnol. 34:582–585.

Afschar, A. S., C. E. Vas Rossell, R. Jonas, A. Quesada Chanto, and K. Schaller. 1993. Microbial production and downstream processing of 2,3-butanediol. J. Biotechnol. 27:317–329.

Agreda, V. H., and J. R. Zoeller. 1993. Acetic Acid and Its Derivatives. Dekker. New York, NY.

Ahn, W. K., S. J. Park, and S. Y. Lee. 2000. Production of poly(3-hydroxybutyrate) by fed-batch culture of recombinant Esherichia coli with a highly concentrated whey solution. Appl. Environ. Microbiol. 66:3624–3627.

Ahn, W. S., S. J. Park, and S. Y. Lee. 2001. Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant Esherichia coli. Biotechnol. Lett. 23:235–240.

Ahrens, K., K. Menzel, A. P. Zeng, and W. D. Deckwer. 1998. Kinetic, dynamic, and pathway studies of glycerol metabolism by Klebsiella pneumoniae in anaerobic continuous culture. III: Enzymes and fluxes of glycerol dissimilation and 1,3-propanediol formation. Biotechnol. Bioengin. 59:544–552.

Alam, S., D. Stevens, and R. Bajpai. 1988. Production of butyric acid by batch fermentation of cheese whey with Clostridum beijerinckii. J. Indust. Microbiol. 2:359–364.

Alam, S., F. Capit, W. A. Weigand, and J. Hong. 1990. Kinetics of 2,3-butanediol fermentation by Bacillus amyloliquefaciens: Effect of initial substrate concentration and aeration. J. Chem. Tech. Biotechnol. 47:71–84.

Albertsson, A.-C., and R. H. Marchessault. 1994. Governmental policy, regulations and standards. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 313–318.

Aldrich, H. C., L. McDowell, M. de. F. S. Barbosa, L. P. Yomano, R. K. Scopes, and L. O. Ingram. 1992. Immunocytochemical localization of glycolytic and fermentative enzymes in Zymomonas mobilis. J. Bacteriol. 174:4504–4508.

Allcock, E. R., and D. R. Woods. 1981a. Carboxymethyl cellulase and cellobiase production by Clostridium acetobutylicum in an industrial fermentation medium. Appl. Environ. Microbiol. 41:539–541.

Allcock, E. R., S. J. Reid, D. T. Jones, and D. R. Woods. 1981b. Autolytic activity and an autolysis-deficient mutant of Clostridium acetobutylicum. Appl. Environ. Microbiol. 42:929–935.

Allgeier, R. J., and F. M. Hildebrandt. 1960. Developments in vinegar manufacture. Adv. Appl. Microbiol. 8:163–182.

Alpers, J. 1999. Engineering metabolism for commercial gains. Science 283:1625–1626.

Altaras, N. E., and D. C. Cameron. 1999. Metabolic engineering of a 1,2-propanediol pathway in Escherichia coli. Appl. Environ. Microbiol. 65:1180–1185.

Altaras, N. E., and D. C. Cameron. 2000. Enhanced production of (R)-1,2-propanediol by metabolically engineered Escherichia coli. Biotechnol. Prog. 16:940–946.

Altaras, N. E., M. R. Etzel, and D. C. Cameron. 2001. Conversion of sugars to 1,2-propanediol by Thermoanaerobacterium thermosaccharolyticum HG-8. Biotechnol. Prog. 17:52–56.

Althouse, J. W., and L. L. Tavlarides. 1992. Analysis of organic extractant systems for acetic acid removal for calcium magnesium acetate production. Ind. Eng. Chem. Res. 31:1971–1981.

Aminabhavi, T. M., and R. H. Balundgi. 1990. A review of biodegradable plastics. Polym. Plast. Technol. Eng. 29:235–262.

Amrane, A., and Y. Prigent. 1996. A novel concept of bioreactor: Specialized function two-stage continuous reactor, and its application to lactose conversion into lactic acid. J. Biotechnol. 45:195–203.

Anastassiadis, S., A. Aivasidis, and C. Wandrey. 1999. Process for the production of gluconic acid with a strain of Aureobasidium pullulans (deBray) Arnaud. US Patent 5692286.

Van Andel, J. G., G. R. Zoutberg, P. M. Crabbendam, and A. M. Breure. 1985. Glucose fermentation by Clostridium butyricum grown under a self-generated gas atmosphere in chemostat culture. Appl. Microbiol. Biotechnol. 23:21–26.

Anderson, A. J., and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.

Andreesen, J. R., G. Gottschalk, and H. G. Schlegel. 1970. Clostridium formicoaceticum nov. spec. isolation description, and distinction from C. aceticum and C. thermoaceticum. Arch. Mikrobiol. 72:154–174.

Andrianova, Y. E., T. L. Bakuridze, V. G. Yargunov, I. V. Zhurov, and V. G. Vinter. 1998. Effects of succinate on the growth rates of potato, Rauwolfia, and ginseng in vitro. Prikl. Biokhim. Mikrobiol. 34:435–438.

Annous, B., and H. P. Blaschek. 1991. Isolation and characterization of Clostridium acetobutylicum mutants with enhanced amylolytic activity. Appl. Environ. Microbiol. 57:2544–2548.

Anonymous. 1956. Trends of world solvents production for the past ten years and the management of the Chiai (Taiwan) Solvents Works [in Chinese]. Petroleum Communications. China Petroleum Corporation. Taiwan, China. June, Issue 60:42–57.

Anonymous. 1958. New direction of development for the Chiai (Taiwan) Solvents Works [in Chinese]. Petroleum Communications. China Petroleum Corporation. Taiwan, China. May, Issue 83:6–7.

Anonymous. 1996a. Chemical profile: Propylene glycol. Chem. Mark. Rep. 249:37.

Anonymous. 1996b. Facts and figures for the chemical industry: Production by the US chemical industry. Chem. Eng. News June 24:41.

Anonymous. 1997. Facts and figures for the chemical industry—production: mixed in 1996. Chem. Eng. News June 23:41.

Anonymous. 1999. Shell Chemicals Research Team wins ACS 2000 award for innovation. Chem. Mark. Rep. 256.

Anonymous. 2001a. Chemical prices. Chem. Mark. Rep. 259:8–21.

Anonymous. 2001b. Chemical profile: Isopropanol. Chem. Mark. Rep. November 12:31.

Anonymous. 1979. Facts and figures for the U.S. chemical industry—production of organic chemicals. Chem. Eng. News June 11:37.

Anonymous. 1993. Chemical Marketing Reporter March 1:7.

Anonymous. 1999. All-microbial route yields chiral building blocks. Chem. Eng. News 77(8):57.

Anonymous. 2000. Chemical prices. Chemical Market Reporter 257(23):26–33.

Anonymous. 2001a. Chemical prices. Chem. Market. Rep. 160(17):23–26.

Anonymous. 2001b. Glycerol, propionic acid, butyric acid. Chem. Market. Rep. 259(13):30–33.

Arihara, K., and J. B. Luchansky. 1995. Dairy lactobacilli. *In:* Y. H. Hui and G. G. Khachatouriani (Eds.) Food Biotechnology: Microorganisms. VCH. New York, NY. 609–643.

Arzberger, C. F., W. H. Peterson, and E. B. Fred. 1920. Certain factors that influence acetone production by Bacillus acetoethylicum. J. Biol. Chem. 44:465–479.

Asai, T. 1968. Acetic Acid Bacteria: Classification and Biochemical Activities. University Press of Tokyo and University Park Press. Tokyo, Japan. Baltimore, MD.

Attwood, M. A., J. P. van Dijken, and J. T. Pronk. 1991. Glucose metabolism and gluconic acid production by Acetobacter diazotrophicus. J. Ferm. Bioeng. 72:101–105.

Auvray, F., M. Codderville, P. Ritzenthaler, and L. Dupont. 1997. Plasmid integration in a wide range of bacteria mediated by intgrase of Lactobacillus delbrueckii bacteriophage mv4. J. Bacteriol. 179:1837–1845.

Azeddoug, H., J. Hubert, and G. Reysset. 1992. Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of Clostridium acetobutylicum. J. Gen. Microbiol. 138:1371–1378.

Babb, B. L., H. J. Collett, S. J. Reid, and D. R. Woods. 1993. Transposon mutagenesis of Clostridium acetobutylicum P262: Isolation and characterization of solvent deficient and metronidazole resistant mutants. FEMS Microbiol. Lett. 114:343–348.

Babel, W., U. Iske, M. Jechorek, and D. Miethe. 1987. Method for production of products by fermentation of methanol. German Patent GDR 251,571. Chem. Abstr. 109:36639.

Babel, W., R. Mueller, D. Miethe, and U. Iske. 1988. Continuous microbial synthesis of products of incomplete oxidation, such as gluconic acid. German Patent DD 253,836. Chem. Abstr. 109:108918.

Babel, W., D. Miethe, U. Iske, K. Sattler, H. P. Richter, J. Schmidt, and Babel, W., N. Lofhagen, D. Miethe, R. Mueller, U. Iske, M. Jechorek, and R. Dueresch. 1991. Microbial manufacture of gluconic acid. German Patent DD 293, 135. Chem. Abstr. 115:278185.

Bahl, H., W. A. Andersch, K. Braun, and G. Gottschalk. 1982a. Effect of pH and butyrate concentration on the production of acetone and butanol by Clostridium acetobutylicum grown in continuous culture. Eur. J. Appl. Microbiol. Biotechnol. 14:17–20.

Bahl, H., W. Andersch, and G. Gottschalk. 1982b. Continuous production of acetone and butanol by Clostridium acetobutylicum in a two-stage phosphate limited chemostat. Appl. Microbiol. Biotechnol. 15:201–205.

Bahl, H., H. Müller, S. Behrens, H. Joseph, and F. Naberhaus. 1995. Expression of heat shock genes in Clostridium acetobutylicum. FEMS Microbiol. Rev. 17:341–348.

Bahner, B. 1994. Chemical Marketing Reporter March 21st:14.

Baker, R. C., and R. E. Kramer. 1999. Cytotoxicity of short-chain alcohols. Ann. Rev. Pharmocol. Toxicol. 39:127–150.

Balch, W. E., S. Schoberth, R. S. Tanner, and R. S. Wolfe. 1977. Acetobacterium, a new genus of hydrogen-oxidizing carbon dioxide-reducing, anaerobic bacteria. Int. J. Syst. Bacteriol. 27:355–361.

Baldus, J. M., B. D. Green, P. Youngman, and C. P. Morgan Jr. 1994. Phosphorylation of Bacillus subtilis transcription factor Spo0A stimulates transcription from the spoIIG promoter by enhancing binding to weak 0A boxes. J. Bacteriol. 176:296–306.

Ballerini, D., J. P. Desmarquest, J. Pourquie, F. Nativel, and M. Rebeller. 1994. Ethanol production from lignocellulosics: Large scale experimentation and economics. Bioresource Technol. 50:17–23.

Ballongue, J., J. Amine, E. Masion, H. Petitdemange, and R. Gay. 1985. Induction of acetoacetate decarboxylase in Clostridium acetobutylicum. FEMS Microbiol. Lett. 29:273–277.

Barber, J. M., F. T. Robb, J. R. Webster, and D. R. Woods. 1979. Bacteriocin production by Clostridium acetobutylicum in an industrial fermentation process. Appl. Environ. Microbiol. 37:433–437.

Barbirato, F., C. Camarasa-Claret, J. P. Grivet, and A. Bories. 1995. Glycerol fermentation by a new 1,3-propanediol-producing microorganism: Enterobacter agglomerans. Appl. Microbiol. Biotechnol. 43:786–793.

Barbirato, F., J. P. Grivet, P. Soucaille, and A. Bories. 1996a. 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by enterobacterial species. Appl. Environ. Microbiol. 62:1448–1451.

Barbirato, F., P. Soucaille, and A. Bories. 1996b. Physiologic mechanisms involved in accumulation of 3-hydroxypropionaldehyde during fermentation of glycerol by Enterobacter agglomerans. Appl. Environ. Microbiol. 62:4405–4409.

Barbirato, F., D. Chedaille, and A. Bories. 1997. Propionic acid fermentation from glycerol: Comparison with conventional substrates. Appl. Microbiol. Biotechnol. 47:441–446.

Barbirato, F., S. Astruc, P. Soucaille, C. Camarasa, J. M. Salmon, and A. Bories. 1997a. Anaerobic pathways of glycerol dissimilation by Enterobacter agglomerans CNCM 1210: Limitations and regulations. Microbiology 143:2423–2432.

Barbirato, F., A. Larguier, T. Conte, S. Astruc, and A. Bories. 1997b. Sensitivity to pH, product inhibition, and inhibition by NAD^+ of 1,3-propanediol dehydrogenase purified from Enterobacter agglomerans CNCM 1210. Arch. Microbiol. 168:160–163.

Barbirato, F., E. H. Himmi, T. Conte, and A. Bories. 1998. 1,3-propanediol production by fermentation: An interesting way to valorize glycerin from the ester and ethanol industries. Indust. Crops Prod. 7:281–289.

Barik, S., S. Prieto, S. B. Harrison, E. C. Clausen, and J. L. Gaddy. 1988. Biological production of alcohols from coal through indirect liquefaction: Scientific note. Appl. Biochem. Biotechnol. 18:363–378.

Barker, H. A. 1972. Co-enzyme B_{12}-dependent mutases causing carbon chain rearrangements. *In:* P. D. Boyer (Ed.) The Enzymes. Academic Press. New York, NY. 6:509–537.

Barnard, G. N., and J. K. M. Sanders. 1989. The poly-β-hydroxybutyrate granule in vivo: A new insight based on NMR spectroscopy of whole cells. J. Biol. Chem. 264:3286–3291.

Basu, R., J. S. Bershears, and E. C. Clausen. 1999. Calcium Magnesium Acetate at Lower Production Costs: Production of CMA Deicer from Biomass. US DOT. Publication No. FHWA-RD-98-055.

Bata, R. M., A. C. Elrod, and T. P. Lewandowski. 1991. Butanol as a blending agent with gasoline for I. C. engines. *In:* R. W. Hurn, W. F. Marshall, and J. R. Allsup (Eds.) Oxygenates in Motor Fuel Formulation. Society of Automotive Engineers. Warrendale, PA. 35–40.

Bates, E. E. M., H. J. Gilbert, G. P. Hazelwood, J. Huckle, J. I. Laurie, and S. P. Mann. 1989. Expression of a Clostridium thermocellum endoglucanase gene in Lactobacillus plantarum. Appl. Environ. Microbiol. 55:2095–2097.

Beall, D. S., and L. O. Ingram. 1993. Genetic engineering of soft-rot bacteria for ethanol production from lignocellulose. J. Ind. Microbiol. 11:151–155.

Beesch, S. C. 1952. Acetone-butanol fermentation of sugars. Ind. Engin. Chem. 44:1677–1682.

Beesch, S. C. 1953. Acetone-butanol fermentation of starches. Appl. Microbiol. 1:85–95.

Benda, I. 1982. Wine and brandy. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 293–402.

Bennett, G. N., and F. B. Rudolph. 1995. The central metabolic pathway from acetyl-CoA to butyryl-CoA in Clostridium acetobutylicum. FEMS Microbiol. Rev. 17:241–249.

Bennett, G. N., and K. Y. San. 2001. Microbial formation, biotechnological production and application of 1,2-propanediol. Appl. Microbiol. Biotechnol. 55:1–9.

Benninga, H. 1990. A History of Lactic Acid Making. Kluwer Academic Publishers. Dordrecht, Germany. Boston, MA.

Beppu, T. 1993. Genetic organization of Acetobacter for acetic acid fermentation. Ant. v. Leeuwenhoek 64:121–135.

Berglund, K. A., P. Elankovan, and D. A. Glassner. 1991. Carboxylic acid purification and crystallization process. US Patent. 5,034,105.

Berglund, K. A., S. Yedur, and D. D. Dunuwila. 1999. Succinic acid production and purification. US Patent. 5,958,744.

Bermejo, L. L., N. E. Welker, and E. T. Papoutsakis. 1998. Expression of Clostridium acetobutylicum ATCC 824 genes in Escherichia coli for acetone production and acetate detoxification. Appl. Environ. Microbiol. 64: 1079–1085.

Bernhauer, K. 1924. Zum Problem der Säurebildung durch A. niger. Biochem. Z. 153:517–521.

Bernhauer, K. 1928. Über die Characterisierung der Stämme von A. niger auf Grund ihres biochemischen Verhaltens. Biochem.Z 197:278–287.

Beronio Jr., P. B., and G. T. Tsao. 1993. Optimization of 2,3-butanediol production by Klebsiella oxytoca through oxygen transfer rate control. Biotechnol. Bioengin. 42:1263–1269.

Bertram, J., and P. Dü. 1989. Conjugal transfer and expression of streptococcal transposons in Clostridium acetobutylicum. Arch. Microbiol. 151:551–557.

Bertram, J., A. Kuhn, and P. Dü. 1990. Tn916-induced mutants of Clostridium acetobutylicum defective in regulation of solvent formation. Arch. Microbiol. 153:373–377.

Bhowmik, T., and J. L. Steele. 1994. Cloning, characterization, and insertional inactivation of Lactobacillus helveticus D(−)-lactate dehydrogenase. Appl. Microbiol. Biotechnol. 41:432–439.

Biebl, H. 1991. Glycerol fermentation of 1,3-propanediol by Clostridium butyricum: Measurement of product inhibition by use of a pH-auxostat. Appl. Microbiol. Biotechnol. 35:701–705.

Biebl, H., S. Marten, H. Hippe, and W. D. Deckwer. 1992. Glycerol conversion to 1,3-propanediol by newly isolated clostridia. Appl. Microbiol. Biotechnol. 36:592–597.

Biebl, H., and S. Marten. 1995. Fermentation of glycerol to 1,3-propanediol: Use of cosubstrates. Appl. Microbiol. Biotechnol. 44:15–19.

Biebl, H., A. P. Zeng, K. Menzel, and W. D. Deckwer. 1998. Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by Klebsiella pneumoniae. Appl. Microbiol. Biotechnol. 50:24–29.

Biebl, H., K. Menzel, A. P. Zeng, and W. D. Deckwer. 1999. Microbial production of 1,3-propanediol. Appl. Microbiol. Biotechnol. 52:289–297.

Bigelis, R., and S. P. Tsai. 1995. Microorganisms for organic acid production. *In:* Y. H. Hui and G. G. Khachatourians (Eds.) Food Biotechnology: Microorganisms. VCH. New York, NY. 239–280.

Billig, E., and D. R. Bryant. 1991. Oxoprocess. Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 17:903–919.

Billig, E. 1992. Butyl alcohols. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 4:691–700.

Birrer, G. A., W. R. Chesbro, and R. M. Zsigray. 1994. Electrotransformation of Clostridium beijerinckii NRRL B-592 with shuttle plasmid pHR106 and recombinant derivatives. Appl. Microbiol. Biotechnol. 41:32–38.

Blaschek, H., B. Annous, J. Formanek, and C.-K. Chen. 2002. Method of producing butanol using a mutant strain of Clostridium beijerinckii. US Patent. 6358717.

Blom, R. H., V. F. Pfeiffer, A. J. Moyer, D. H. Traufler, H. F. Conway, C. K. Crocker, R. E. Farison, and D. V. Hannibal. 1952. Sodium gluconate production-fermentation with A. niger. Ind. Eng. Chem. 44:435–440.

Blomqvist, K., M. Nikkola, P. Lehtovaara, M.-L. Suihko, U. Airaksinen, K. B. Straby, J. K. C. Knowles, and M. E. Penttila. 1993. Characterization of the genes of the 2,3-butanediol operons from Klebsiella terrigena and Enterobacter aerogenes. J. Bacteriol. 175:1392–1404.

Bock, A., and G. Sawers. 1996. "Fermentation" in Escherichia coli and Salmonella. *In:* F. C. Neidhardt (Eds.) Cellular and Molecular Biology. ASM Press. 262–282.

Boenigk, R., S. Bowien, and G. Gottschalk. 1993. Fermentation of glycerol to 1,3-propanediol in continuous cultures of Citrobacter freundii. Appl. Microbiol. Biotechnol. 38:453–457.

Bolt, J. A. 1980. A Survey of alcohol as a motor fuel. Alcohols as Motor Fuels. Society of Automotive Engineers. Warrendale, PA. 21–33.

Boutroux, L. 1880. Sur une fermentation nouvelle du glucose. CR Acad. Sci. 91:236–238.

Bouvet, O. M. M., P. Lenormand, J. P. Carlier, and P. A. D. Grimont. 1994. Phenotypic diversity of anaerobic glycerol dissimilation shown by seven enterobacterial species. Res. Microbiol. 145:129–139.

Bowles, L. K., and W. L. Ellefson. 1985. Effects of butanol on Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:1165–1170.

Bowring, S. N., and J. G. Morris. 1985. Mutagenesis of Clostridium acetobutylicum. J. Appl. Bacteriol. 58:577–584.

Boyaval, P., J. Seta, and C. Gavach. 1993. Concentrated propionic acid production by electrodialysis. Enzyme Microb. Technol. 15:683–686.

Boyaval, P., C. Corre, and M. N. Madec. 1994. Propionic acid production in a membrane bioreactor. Enzyme Microb. Technol. 16:883–886.

Boyaval, P., and C. Corre. 1995. Production of propionic acid. Lait 75:453–461.

Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1996a. Cloning, sequencing and expression of clustered genes encoding β-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from Clostridium acetobutylicum ATCC 824. J. Bacteriol. 178:3015–3024.

Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1996b. Cloning, sequencing, and expression of genes encoding phosphotransacetylase and acetate kinase from Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 62:2758–2766.

Boynton, Z. L., J. J. Koon, E. M. Brennan, J. D. Clouart, D. M. Horowitz, T. U. Gerngross, and G. W. Huisman. 1999. Reduction of cell lysate viscosity during processing of poly(3-hydroxyalkanoates) by chromosomal integration of the Staphylococcal nuclease gene in Pseudomonas putida. Appl. Environ. Microbiol. 65: 1524–1529.

Brandt, D. A. 1982. Distilled beverage alcohol. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 468–491.

Brau, B., and H. Sahm. 1986. Cloning and expression of the sturctural gene for pyruvate decarboxylase of Zymomonas mobilis in Escherichia coli. Arch. Microbiol. 144:296–301.

Braun, M., F. Mayer, and G. Gottschalk. 1981. Clostridium aceticum (Wieringa) a microorganism producing acetic acid from molecular hydrogen and carbon dioxide. Arch. Microbiol. 128:288–293.

Bringer-Meyer, S., K.-L. Schmiz, and H. Sahm. 1986. Pyruvate decarboxylase from Zymomonas mobilis: Isolation and partial characterization. Arch. Microbiol. 146:105–110.

Brown, A. T., and L. C. Breeding. 1980. Carbon dioxide metabolism by Actinomyces viscosus: Pathways for succinate and aspartate production. Infect. Immun. 28:82–91.

Brown, D. P., L. Ganova-Raevn, B. D. Green, S. R. Wilkinson, M. Young, and P. Youngman. 1994. Characterization of spoOA homologues in diverse Bacillus and Clostridium species identifies a probable DNA-binding domain. Molec. Microbiol. 14:411–426.

Bryant, F. O., J. Wiegel, and L. G. Ljungdahl. 1988. Purification and properties of primary and secondary alcohol dehydrogenases from Thermoanaerobacter ethanolicus. Appl. Environ. Microbiol. 54:460–465.

Bryant, R. S. 1990. Microbial enhanced oil recovery and compositions therefor. US Patent. 4905761.

Bryn, K., and F. C. Stormer. 1976. Decreased riboflavin formation in mutants of Aerobacter (Enterobacter) aerogenes deficient in the butanediol pathway. Biochim. Biophys. Acta 428:257–259.

Buchholz, S. E., M. M. Dooley, and D. E. Eveleigh. 1987. Zymomonas—an alcoholic enigma. Trends Biotechnol. 5:199–204.

Bucholz, K., and B. Gödelmann. 1978. Macrokinetics and operational stability of immobilized glucose oxidase and catalase. Biotechnol. Bioeng. 20:1201–1220.

Buchta, K. 1983. Lactic acid. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. Verlag Chemie. Weinheim, Germany. 409–417.

Bulthuis, B. A., A. A. Gatenby, S. L. Laynie, A. K. Hsu, and R. D. Lareau. 1998. Method for the production of glycerol by recombinant organisms. Patent Cooperation Treaty (PCT) Application WO 98/21340.

Burdette, D., and J. G. Zeikus. 1994. Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from Thermoanaerobacter ethanolicus 39E and characterization of the secondary-alcohol dehydrogenase (2°dh) as a bifunctional alcohol dehydrogenase-acetyl-CoA reductive thioesterase. Biochem. J. 302:163–170.

Burdette, D. S., C. Vieille, and J. G. Zeikus. 1996. Cloning and expression of the gene encoding the Thermoanaerobacter ethanolicus 39E secondary-alcohol dehydrogenase and biochemical charaterization of the enzyme. Biochem. J. 316:115–122.

Busche, R. M. 1985. Acetic acid manufacture—fermentation alternatives. *In:* P. N. Cherenisinoff and R. P. Ouellette (Eds.) Biotechnology Applications and Research. Technomic. Lancaster, PA. 88–102.

Busche, R. M. 1991. Extractive fermentation of acetic acid: Economic tradeoff between yield of Clostridium and concentration of Acetobacter. Appl. Biochem. Biotechnol. 28/29:605–621.

Buschhorn, H., P. Dü, and G. Gottschalk. 1989. Production and utilization of ethanol by homoacetogen Acetobacterium woodii. Appl. Environ. Microbiol. 55:1835–1840.

Byrom, D. 1992. Production of poly-β-hydroxybutyrate: Poly-β-hydroxyvalerate copolymers. FEMS Microbiol. Rev. 103:247–250.

Byrom, D. 1994. Polyhydroxyalkanoates. *In:* D. P. Mobley (Ed.) Plastics from Microbes. Hanser Publishers. Munich, Germany. 5–33.

Cameron, D. C., and C. L. Cooney. 1986. A novel fermentation: The production of R(−)-1,2-propanediol and acetol by Clostridium thermosaccharolyticum. Biotechnology 4:651–654.

Cameron, D. C., I. T. Tong, and F. A. Skraly. 1993. Metabolic engineering for the production of 1,3-propanediol. *In:* Chianelli, R. R. and Davison, B. H.ACS Symposium on Bioremediation and Bioprocessing. American Chemical Society. Denver, CO. 38:294–295.

Cameron, D. C., N. E. Altaras, M. L. Hoffman, and A. J. Shaw. 1998. Metabolic engineering of propanediol pathways. Biotechnol. Prog. 14:16–125.

Cameron, D. C., A. J. Shaw, and N. E. Altaras. 2000. Microbial production of 1,2-propanediol from sugar. US Patent 6087140.

Cann, I. K. O., P. G. Stroot, K. R. Mackie, B. A. White, and R. I. Mackie. 2001. Characterization of two novel saccharolytic, anaerobic thermophiles, Thermoanaerobacterium polysaccharolyticum sp. nov. and Thermoanaerobacterium zeae sp. nov., and emendation of the genus Thermoanaerobacterium. Int. J. Syst. Evol. Microbiol. 51:293–302.

Carr, N. G. 1966. The occurrence of poly-β-hydroxybutyrate in the blue-green alga, Chloroglea fritschii. Biochim. Biophys. Acta 120:308–310.

Cato, E. P., W. L. George, and S. M. Finegold. 1986. Genus Clostridium Prazmowski 1880. *In:* H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 2:1141–1200.

Cauvin, B., and J. B. Luchansky. 1992. Advances in electrotransformation of Gram-positive bacteria. Bio-rad Laboratories Bulletin 1350A.

Cayol, J.-L., B. Ollivier, B. K. C. Patel, G. Ravot, M. Magot, E. Ageron, P. A. D. Grimont, and J.-L. Garcia. 1995. Description of Thermoanaerobacter brockii subsp. lactiethylicus subsp. nov., isolated from a deep subsurface french oil well, a proposal to reclassify Thermoanaerobacter finnii as Thermoanaerobacter brockii subsp. finnii comb. nov., and an emended description of Thermoanaerobacter brockii. Int. J. Syst. Bacteriol. 45:783–789.

Champluvier, B., J. Decallonne, and P. G. Rouxhet. 1989. Influence of sugar source (lactose, glucose, galactose) on 2,3-butanediol production by Klebsiella oxytoca NRRL-B199. Arch. Microbiol. 152:411–414.

Chang, H. N., Y. C. Kim, S. Y. Lee, and B. S. Kim. 1994. Current status of biodegradable plastics in Korea: Research, commercial production and government policy. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 286–297.

Chang, D.-E., H.-C. Jung, J.-S. Rhee, and J.-G. Pan. 1999. Homofermentative production of D- or L-lactate in metabolically engineered Escherichia coli RR1. Appl. Environ. Microbiol. 65:1384–1389.

Chen, J.-S., and S. F. Hiu. 1986. Acetone-butanol-isopropanol production by Clostridium beijerinckii (synonym, Clostridium butylicum). Biotechnol. Lett. 8:371–376.

Chen, J.-S. 1987. Electron transport in anaerobes. *In:* T. J. Montville (Ed.) Concepts in Physiology and Metabolism. CRC Press. Boca Raton, FL. 1:61–101.

Chen, J.-S. 1993. Properties of acid- and solvent-froming enzymes of clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. Stoneham, MA. 51–76.

Chen, J. S. 1995. Alcohol dehydrogenase: Multiplicity and relatedness in the solvent-producing clostridia. FEMS Microbiol. Rev. 17:263–273.

Chen, C.-K., and H. P. Blaschek. 1999a. Acetate enhances solvent production and prevents degeneration in Clostridium beijerinckii BA101. Appl. Microbiol. Biotechnol. 52:170–173.

Chen, C.-K., and H. Blaschek. 1999b. Effect of acetate on molecular and physiological aspects of Clostridium beijerinckii NCIMB 8052 solvent production and strain degeneration. Appl. Environ. Microbiol. 65:499–505.

Cheng, P., R. E. Mueller, S. Jaeger, R. Bajpai, and E. L. Iannotti. 1991. Lactic acid production from enzyme-thinned corn starch using Lactobacillus amylovorus. J. Indust. Microbiol. 7:27–34.

Cheryan, M., S. Paretkh, M. Shah, and K. Witjitra. 1997. Production of acetic acid by Clostridium thermoaceticum. Adv. Appl. Microbiol. 43:1–33.

Cheryan, M. 1999. Acetic acid production. *In:* J. Lederberg (Ed.) Encyclopedia of Microbiology. Academic Press. San Diego, CA.

Chiellini, E. 1994. Status of government policy, regulation and standards on the issue of biodegradable plastic materials in Italy. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 273–285.

Choi, J., and S. Y. Lee. 1997. Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. Bioprocess Eng. 17:335–342.

Choi, J., and S. Y. Lee. 1999a. Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals. Biotechnol. Bioeng. 62:546–553.

Choi, J., and S. Y. Lee. 1999b. High-level production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fed-batch culture of recombinant Esherichia coli. Appl. Environ. Microbiol. 65:4363–4368.

Choi, J., and S. Y. Lee. 2000. Economic consideration in the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by bacterial fermentation. Appl. Microbiol. Biotechnol. 53:646–649.

Chotani, G., T. Dodge, A. Hsu, M. Kumar, R. LaDuca, D. Trimbur, W. Weyler, and K. Sanford. 2000. The commercial production of chemicals using pathway engineering. Biochim. Biophys. Acta 1543:434–455.

Chukwu, U. N., and M. Cheryan. 1996. Concentration of vinegar by electrodialysis. J. Food Sci. 61:1223–1226.

Chun, V. H., and P. L. Rogers. 1988. The simultaneous production of sorbitol from fructose and gluconic acid from glucose using an oxidoreductase of Zymomonas mobilis. Appl. Microbiol. Biotechnol. 29:19–24.

Clark, D. P., P. R. Cunningham, S. G. Reams, F. Mat-Jan, R. Mohammedkhani, and C. R. Williams. 1988. Mutants of Escherchia coli defective in acid fermentation. Appl. Biochem. Biotechnol. 17:163–173.

Clark, D. P. 1989. The fermentation pathways of Escherichia coli. FEMS Microbiol. Rev. 63:223–234.

Clark, S. W., G. N. Bennett, and F. B. Rudolph. 1989. Isolation and characterization of mutants of Clostridium acetobutylicum ATCC 824 deficient in acetoacetyl-coenzyme A: Acetate/butyrate:coenzyme A-transferase (EC 2.8.3.9) and in other solvent pathway enzymes. Appl. Environ. Microbiol. 55:970–976.

Clausen, E. C., and J. L. Gaddy. 1996. Ethanol from biomass by gasification/fermentation. *In:* M. R. Khan (Ed.) Conversion and Utilization of Waste Materials. Taylor & Francis. Washington DC, 157–167.

Cocconcelli, P. S., M. J. Gasson, L. Morelli, and V. Bottazzi. 1991. Single-stranded DNA plasmid vector construction and cloning of Bacillus stearothermophilus α-amylase in Lactobacillus. Res. Microbial. 142:643–652.

Cocks, G. T., J. Aguilar, and E. C. C. Lin. 1974. Evolution of L-1,2-propanediol catabolism in Escherichia coli by recruitment of enzymes for L-fucose and L-lactate metabolism. J. Bacteriol. 118:83–88.

Colby, G. D., and J.-S. Chen. 1992. Purification and properties of 3-hydroxybutyryl-coenzyme A dehydrogenase from Clostridium beijerinckii ("Clostridium butylicum") NRRL B593. Appl. Environ. Microbiol. 58:3297–3302.

Colby, G. D. 1993. CoA-transferase and 3-hydroxybutyryl-CoA Dehydrogenases: Acetoacetyl-CoA-reacting Enzymes from Clostridium beijerinckii NRRL B593 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Colin, T., A. Bories, and G. Moulin. 2000. Inhibition of Clostridium butyricum by 1,3-propanediol and diols during glycerol fermentation. Appl. Microbiol. Biotechnol. 54:201–205.

Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow. 1994. The phylogeny of the genus Clostridium: Proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44:812–826.

Colomban, A., L. Roger, and P. Boyaval. 1993. Production of propionic acid from whey permeate by sequential fermentation, ultrafiltration, and cell recycling. Biotechnol. Bioeng. 42:1091–1098.

Compere, A. L., and W. L. Griffith. 1979. Evaluation of substrates for butanol production. Devel. Ind. Microbiol. 20:509–517.

Compere, A. L., W. L. Griffith, and J. M. Googin. 1985. Solvents production by clostridia as a function of wood stream organic toxicant concentration. Devel. Ind. Microbiol. 26:535–541.

Conway, T., Y. A. Osman, J. I. Konnan, E. M. Hoffmann, and L. O. Ingram. 1987. Promoter and nucleotide sequences of the Zymomonas mobilis pyruvate decarboxylase. J. Bacteriol. 169:949–954.

Conway, T., and L. O. Ingram. 1989. Similarity of Escherichia coli propanediol oxidoreductase (fucO product) and an unusual alcohol dehydrogenase from Zymomonas mobilis and Saccharomyces cerevisiae. J. Bacteriol. 171:3754–3759.

Cook, G. M., F. A. Rainey, B. K. C. Patel, and H. W. Morgan. 1996. Characterization of a new obligately anaerobic thermophile, Thermanaerobacter wiegelii sp. nov. Int. J. Syst. Bacteriol. 46:123–127.

Cornillot, E., and P. Soucaille. 1996. Solvent-forming genes in clostridia. Nature 380:489.

Cornillot, E., C. Croux, and P. Soucaille. 1997a. Physical and genetic map of the Clostridium acetobutylicum ATCC 824 chromosome. J. Bacteriol. 179:7426–7434.

Cornillot, E., R. V. Nair, E. T. Papoutsakis, and P. Soucaille. 1997b. The genes for butanol and acetone formation in Clostridium acetobutylicum ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. J. Bacteriol. 179:5442–5447.

Croux, C., and J. L. Garcia. 1991. Sequence of the lyc gene encoding the autolytic lysozyme of Clostridium acetobutylicum ATCC 824: Comparison with other lytic enzymes. Gene 104:25–31.

Croux, C., and J. L. Garcia. 1992a. Reconstruction and expression of the autolytic gene from Clostridium acetobutylicum ATCC 824 in Escherichia coli. FEMS Microbiol. Lett. 95:13–20.

Croux, C., B. Canard, G. Goma, and P. Soucaille. 1992b. Autolysis of Clostridium acetobutylicum ATCC 824. J. Gen. Microbiol. 138:861–869.

Croux, C., B. Canard, G. Goma, and P. Soucaille. 1992c. Purification and characterization of an extracellular muramidase of Clostridium acetobutylicum ATCC 824 that acts on non-N-acetylated peptidoglycan. Appl. Environ. Microbiol. 58:1075–1081.

Crow, V. L. 1987. Citrate cycle intermediates in the metabolism of aspartate and lactate by Propionibacterium freudenreichii subsp. shermanii. Appl. Environ. Microbiol. 53:2600–2602.

Cueto, P. H., and B. S. Mendez. 1990. Direct selection of Clostridium acetobutylicum fermentation mutants by a protein suicide method. Appl. Environ. Microbiol. 56:578–580.

Cummins, C., and J. L. Johnson. 1971. Taxonomy of the clostridia: Wall composition and DNA homologies in Clostridium butyricum and other butyric acid-producing clostridia. J. Gen. Microbiol. 67:33–46.

Cummins, C. S., and J. L. Johnson. 1986. The genus Propionibacterium. *In:* P. H. A. Sneath, N. S. Mair, M. E. Sharpe,

and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 2:1346–1353.

Currie, J. N., and R. H. Carter (Charles Pfizer, and Co.). 1930. Gluconic Acid. US Patent 1896811. Chem. Abstr. 27:2757.

Currie, J. N., and A. Finlay. 1933. Fermentation such as the production of D-gluconic acid. US Patent 1908225. Chem. Abstr. 27:3774.

Dabrock, B., H. Bahl, and G. Gottschalk. 1992. Parameters affecting solvent production by Clostridium pasteurianum. Appl. Environ. Microbiol. 58:1233–1239.

Daniel, R., and G. Gottschalk. 1992. Growth temperature-dependent activity of glycerol dehydratase in Escherichia coli expressing the Citrobacter freundii dha regulon. FEMS Microbiol. Lett. 100:281–286.

Daniel, R., R. Boenigk, and G. Gottschalk. 1995a. Purification of 1,3-propanediol dehydrogenase from Citrobacter freundii and cloning, sequencing, and overexpression of the corresponding gene in Escherichia coli. J. Bacteriol. 177:2151–2156.

Daniel, R., K. Stuertz, and G. Gottschalk. 1995b. Biochemical and molecular characterization of the oxidative branch of glycerol utilization by Citrobacter freundii. J. Bacteriol. 177:4392–4401.

Daniel, R., Bobik, T. A., and G. Gottschalk. 1999. Biochemistry of coenzyme B12-dependent glycerol and diol dehydratases and organization of the encoding genes. FEMS Microbiol. Rev. 22:553–566.

Datta, R. 1989. Recovery and purification of lactate salts from whole fermentation broth by electrodialysis. US Patent to Michigan Biotech. Inst. 4,885,247.

Datta, R. 1992a. Process for the production of succinic acid by anaerobic fermentation. US Patent. 5,143,833.

Datta, R., D. A. Glassner, M. K. Jain, and J. R. Vick Roy. 1992b. Fermentation and purification process for succinic acid. US Patent. 5,168,055.

Datta, R., S.-P. Tsai, P. Bonsignore, S.-H. Moon, and J. R. Frank. 1995. Technological and economic potential of poly (lactic acid) and lactic acid derivitives. FEMS Microbiol. Rev. 16:221–231.

Davidson, B. E., R. M. Llanos, M. R. Cancilla, N. C. Redman, and A. J. Hillier. 1995. Current research on the genetics of lactic acid production in lactic acid bacteria. Int. Dairy J. 5:763– 784.

Dawes, E. A., and P. J. Senior. 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. 10:135–266.

Deckwer, W. D. 1995. Microbial conversion of glycerol to 1,3-propanediol. FEMS Microbiol. Rev. 16:143–149.

de F. S. Barbosa, M., and L. O. Ingram. 1994. Expression of the Zymomonas mobilis alcohol dehydrogenase II (adhB) and pyruvate decarboxylase (pdc) genes in Bacillus. Curr. Microbiol. 28:279–282.

De Koning, G., M. Kellerhals, C. van Meurs, and B. Witholt. 1997. *In:* G. Eggink, A. Steinbuchel, Y. Poirer, and B. Witholt (Eds.) Proceedings of the 1996 International Symposium on Bacterial Polyhdroxyalknoates. NRC Research Press. 137–142.

Demain, A. L., E. L. Rickes, D. Hendlin, and E. C. Barnes. 1961. Nutritional studies on Lactobacillus heterohiochi. J. Bacteriol. 81:147–153.

de Mas, C., N. B. Jansen, and G. T. Tsao. 1988. Production of optically active 2,3-butanediol by Bacillus polymyxa. Biotechnol. Bioengin. 31:366–377.

Demirci, A., and A. L. Pometto 3rd. 1992. Enhanced production of D(−) lactic acid by mutants of Lactobacillus delbrueckii ATCC 9649. J. Ind. Microbiol. 11:23–28.

Demirci, A., and A. L. Pometto 3rd. 1995. Repeated-batch fermentation in biofilm reactors with plastic composite supports for lactic acid production. Appl. Microbiol. Biotechnol. 43:585–589.

Dennis, D., and N. O. Kaplan. 1960. D- and L-lactic acid dehydrogenases in Lactobacillus plantarum. J. Biol. Chem. 235:810–818.

Depasse, E. 1945. Vue d'ensemble d'une production industrielle de cétones. Bull. Assoc. Chim. Sucr. Distill. Fr. 62:317–339.

Desai, R. P., and E. T. Papoutsakis. 1999. Antisense RNA stragies for metabolic engineering of Clostridium acetobytylicum. Appl. Environ. Microbiol. 65:936–945.

DeVos, W. M., and G. F. M. Simons. 1994. Gene cloning and expression systems in Lactococci. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 52–105.

DeWilt, H. G. J. 1972. Oxidation of glucose to gluconic acid. Ind. Eng. Chem. Prod. Res. Dev. 11:370–378.

Diaz-Torres, M., N. S. Dunn-Coleman, M. W. Chase, and D. Trimbur. 2000. Method for the recombinant production of 1,3-propanediol. US Patent 6136576.

Djordjevic, G. M., D. J. O'Sullivan, S. A. Walker, M. A. Conkling, and T. R. Kaenhammer. 1997. Triggered-suicide system designed for bacteriophage defense of Lactococcus lactis. J. Bacteriol. 179:6741–6748.

Doi, Y. 1990a. Microbial Polyesters. VCH. New York, NY.

Doi, Y., A. Segawa, Y. Kawaguchi, and M. Kunioka. 1990b. Cyclic nature of poly(3-hydroxyalkanoate) metabolism in Alcaligenes eutrophus. FEMS Microbiol. Lett. 67:165.

Doi, Y., and K. Fukuda (Eds.). 1994. Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands.

Donnelly, M. I., C. S. Millard, D. P. Clark, M. J. Chen, and J. W. Rathke. 1998a. A novel fermentation pathway in an Escherichia coli mutant producing succinic acid, acetic acid, and ethanol. Appl. Biochem. Biotechnol. 70–72:187–198.

Donnelly, M., C. S. Millard, and L. Stols. 1998b. Mutant *E. coli* strain with increased succinic acid production. US Patent. 5,770,435.

Doran, J. B., and L. O. Ingram. 1993. Fermentation of crystalline cellulose to ethanol by Klebsiella oxytoca containing chromosomally integrated Zymomonas mobilis genes. Biotechnol. Progr. 9:533–538.

Doran, J. B., H. C. Aldrich, and L. O. Ingram. 1994. Saccharification and fermentation of sugar cane bagasse by Klebsiella oxytoca P2 containing chromosomally integrated genes encoding the Zymomonas mobilis ethanol pathway. Biotechnol. Bioengin. 44:240–247.

Drake, H. L. 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: Past and current perspectives. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 3–60.

Dubos, R. J. 1988. Pasteur and Modern Science. Science Tech Publishers. Madison, WI.

Dueresch, R. 1986. Fermentative maufacture of gluconic acid. German Patent DD236, 754. Chem. Abstr. 106:31387.

Dumsday, G. J., B. Zhou, W. Yaqin, G. A. Stanley, and N. B. Pamment. 1999. Comaprative stability of ethanol pro-

duction by Escherichia coli KO11 in batch and chemostat culture. J. Ind. Microbiol. Biotechnol. 23:701–708.

Dü, P., A. Kuhn, M. Gottwald, and G. Gottschalk. 1987. Enzymatic investigations on butanol dehydrogenase and butyraldhyde dehydrogenase in extracts of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 26:268–272.

Dürre, P., R. J. Fischer, A. Kuhn, K. Lorenz, W. Schreiber, B. Stürzenhofecker, S. Ullmann, K. Winzer, and U. Sauer. 1995. Solventogenic enzymes of Clostridium acetobutylicum, catalytic properties, genetic organization and transcriptional regulation. FEMS Microbiol. Rev. 17:251–262.

Dürre, P., and H. Bahl. 1996. Microbial production of acetone/butanol/isopropanol. *In:* M. Roehr (Ed.) Products of Primary Metabolism, 2nd ed. VCH Publisher. Weinheim, Germany. 6:229–268.

Dürre, P. 1998. New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl. Microbiol. Biotechnol. 49:639–648.

Dürre, P., M. Bohringer, S. Nakotte, S. Schaffer, K. Thormann, and B. Zickner. 2002. Transcriptional regulation of solventogenesis in Clostridium acetobutylicum. J. Molec. Microbiol. Biotechnol. 4:295–300.

Ebner, H., and H. Follmann. 1983. Acetic acid. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. VCH. Weinheim, Germany. 3:387–407.

Ebner, H. 1985. Process for the production of vinegar with more than 12g/100ml acetic acid. US Patent. 4,503,078.

Ebner, H., S. Sellmer, and H. Follmann. 1996. Acetic acid. *In:* M. Roehr (Ed.) VCH. Weinheim, Germany. 6:381–401.

Eden, G., and G. Fuchs. 1982. Total synthesis of acetyl CoA involved in autotrophic CO_2 fixation in Acetobacterium woodii. Arch. Microbiol. 133:66–74.

Eden, G., and G. Fuchs. 1983. Autotrophic CO_2 fixation in Acetobacterium woodii. II: Demonstration of enzymes involved. Arch. Microbiol. 135:68–73.

Eiteman, M. A., and J. H. Miller. 1995. Effect of succinic acid on 2,3-butanediol production by Klebsiella oxytoca. Biotechnol. Lett. 17:1057–1062.

Ennis, B. M., and I. S. Maddox. 1985. Use of Clostridium acetobutylicum P262 for production of solvents from whey permeate. Biotechol. Lett. 7:601–606.

Entani, E., S. Ohmori, H. Masai, and K.-I. Suzuki. 1985. Acetobacter polyoxogenes, sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity. J. Gen. Appl. Microbiol. 31:475–490.

Evans, P. J., and H. Y. Wang. 1990. Effects of extractive fermentation on butyric acid production by Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 32:393–397.

Evans, J. D., and S. A. Martin. 1997. Factors affecting lactate and malate utilization by Selenomonas ruminantium. Appl. Environ. Microbiol. 63:4853–4858.

Evans, V. J., H. Liyanage, A. Ravagnani, M. Young, and E. R. Kashket. 1998. Truncation of peptide deformylase reduces the growth rate and stabilizes solvent production in Clostridium beijerinckii NCIMB 8052. Appl. Environ. Microbiol. 64:1780–1785.

Fayolle, F., R. Marchal, and D. Ballerini. 1990. Effect of controlled substrate feeding on butyric acid production by Clostridium tyrobutyricum. J. Indust. Microbiol. 6:179–183.

Feldmann, S., G. A. Sprenger, and H. Sahm. 1989. Ethanol production from xylose with a pyruvate-formate-lyase mutant of Klebsiella planticola carrying a pyruvate-decarboxylase gene from Zymomonas mobilis. Appl. Microbiol. Biotechnol. 31:152–157.

Fernbach, A., and E. H. Strange. 1911. Acetone and higher alcohols (amyl, butyl or ethyl alcohols and butyric, propionic or acetic acid) from starches, sugars and other carbohydrates. British Patent. 15203–15204.

Fiedler, S., A. Steinbüchel, and B. Rehm. 2000. PhaG-mediated synthesis of poly(3-hydroxyalkanoates) consisting of medium-chain-length constituents from nonrelated carbon sources in recombinant Pseudomonas fragi. Appl. Environ. Microbiol. 66:2117–2124.

Fischer, R. J., J. Helms, and P. Dü. 1993. Cloning, sequencing and molecular analysis of the sol operon of Clostridium acetobutylicum, a chromosomal locus involved in solventogenesis. J. Bacteriol. 175:6959–6969.

Fitz, A. 1878. Über spaltpilzgärungen, IV. Bericht der Deutsch. Chem. Ges. 11:1890.

Flores-Encarnacion, M., and M. Contreras-Zentella. 1999. The respiratory system and diazotrophic activity of Acetobacter diazotrophicus PAL5. J. Bacteriol. 181:6987–6995.

Fond, O., N. B. Jansen, and G. T. Tsao. 1985. A model of acetic acid and 2,3-butanediol inhibition of the growth and metabolism of Klebsiella oxytoca. Biotechnol. Lett. 7:727–732.

Fond, O., J.-M. Engasser, G. Matta-El-Amouri, and H. Petitdemange. 1986a. The acetone butanol fermentation on glucose and xylose. I: Regulation and kinetics in batch cultures. Biotechnol. Bioengin. 28:160–166.

Fond, O., J.-M. Engasser, G. Matta-El-Amouri, and H. Petitdemange. 1986b. The acetone butanol fermentation on glucose and xylose. II: Regulation and kinetics in fed-batch cultures. Biotechnol. Bioengin. 28:167–175.

Fontaine, F., W. H. Peterson, E. McCoy, M. J. Johnson, and G. Ritter. 1942. A new type of glucose fermentation by Clostridium thermoaceticum, n. sp. J. Bacteriol. 43:701–715.

Fontaine, L., I. Meynial-Salles, L. Girbal, X. Yang, C. Croux, and P. Soucaille. 2002. Molecular characterization and transcriptional analysis of adhE2, the gene encoding the NADH-dependent aldehyde/alcohol dehydrogenase responsible for butanol production in alcohologenic cultures of Clostridium acetobutylicum ATCC 824. J. Bacteriol. 184:821–830.

Forage, R. G., and M. A. Foster. 1982a. Glycerol fermentation in Klebsiella pneumoniae: Functions of the coenzyme B12-dependent glycerol and diol dehydratases. J. Bacteriol. 149:413–419.

Forage, R. G., and E. C. C. Lin. 1982b. Dha system mediating aerobic and anaerobic dissimilation of glycerol in Klebsiella pneumoniae NCIB 418. J. Bacteriol. 151:591–599.

Forde, A., C. Daly, and G. F. Fitzgerald. 1999. Identification of four phage resistance plasmids from Lactococcus lactis subsp. cremoris HO2. Appl. Environ. Microbiol. 65:1540–1547.

Frazer, F. R., and T. A. McCaskey. 1991. Effect of components of acid-hydrolyzed hardwood on conversion of D-xylose to 2,3-butanediol by Klebsiella pneumoniae. Enz. Microb. Technol. 13:110–115.

Freier-Schroeder, D., J. Wiegel, and G. Gottschalk. 1989. Butanol formation by Clostridium thermosaccharolyticum at neutral pH. Biotechnol. Lett. 11:831–836.

Fujimaki, T. 1998. Processability and properties of aliphatic polyesters, "BIONELLE," synthesized by polycondensation reaction. Polymer. Degrad. Stabil. 59:209–214.

Fukaya, M., H. Tagami, K. Tayama, H. Okumura, Y. Kawamura, and T. Beppu. 1989. Spheroplast fusion of Acetobacter aceti and its application to the breeding of strains for vinegar production. Agric. Biol. Chem. 53:2435–2440.

Fukaya, M., Y. S. Park, and K. Toda. 1992. Improvement of acetic acid fermentation by molecular breeding. J. Appl. Bacteriol. 73:447–454.

Gabriel, C. L. 1928. Butanol fermentation process. Indust. Engin. Chem. 20:1063–1067.

Gabriel, C. L., and F. M. Crawford. 1930. Development of the butyl-acetonic fermentation indistry. Indust. Engin. Chem. 22:1163–1165.

Gapes, J. R., D. Nimcevic, and A. Friedl. 1996. Long-term continuous cultivation of Clostridium beijerinckii in a two-stage chemostat with on-line solvent removal. Appl. Environ. Microbiol. 62:3210–3219.

Gapes, J. R. 2000a. The economics of the acetone-butanol fermentation: Theoretical and market considerations. J. Molec. Microbiol. Biotechnol. 2:27–32.

Gapes, J. R. 2000b. The history of the acetone-butanol project in Austria. J. Molec. Microbiol. Biotechnol. 2:5–8.

Garg, S. K., and A. Jain. 1995. Fermentative production of 2,3-butanediol: A review. Biores. Technol. 51:103–109.

Gasson, M. J., and W. M. DeVos (Eds.). 1994. Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY.

Gauss, W. F., S. Suzuki, and M. Takagi. 1976. US Patent. 3,990,944.

George, H. A., and J.-S. Chen. 1983a. Acidic conditions are not obligatory for onset of butanol formation by Clostridium beijerinckii (synonym, C. butylicum). Appl. Environ. Microbiol. 46:321–327.

George, H. A., J. L. Johnson, W. E. C. Moore, L. V. Holdeman, and J.-S. Chen. 1983b. Acetone, isopropanol, and butanol production by Clostridium beijerinckii (syn. Clostridium butylicum) and Clostridium aurantibutyricum. Appl. Environ. Microbiol. 45:1160–1163.

Gerischer, U., and P. Dü. 1990. Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from Clostridium acetobutylicum. J. Bacteriol. 172:6907–6918.

Gerngross, T. U., and D. P. Martin. 1995. Enzyme-catalyzed synthesis of poly[R-(-)-3-hydroxybutyrate]: Formation of macroscopic granules in vitro. Proc. Natl. Acad. Sci. USA 92:6279–6283.

Gerngross, T. U. 1999. Can biotechnology move us toward a sustainable society? Nature Biotechnol. 17:541–544.

Ghazvinizadeh, H., G. C. Turtura, and C. Zambonelli. 1972. The fermentation of L-rhamnose by clostridia. Ann. Microbiol. 22:155–158.

Ghommidh, C., J. M. Navarro, and G. Durand. 1981. Acetic acid production by immobilized Acetobacter cells. Biotechnol. Lett. 3:93–98.

Ghose, T. K., and A. Bhadra. 1985. Acetic acid. *In:* M. Moo-Young (Ed.) Biotechnology. Pergamon Press. New York, NY. 3:701–729.

Gibbs, D. F. 1983. The rise and fall (. . . and rise?) of acetone/butanol fermentations. Trends Biotechnol. 1:12–15.

Gilles, M., K. Kerstens, B. Hoste, D. Janssens, R. M. Kroppenstedt, M. P. Stephens, K. R. S. Teixeira, J. Dobereiner, and J. DeLey. 1989. Acetobacter diazotrophicus sp. nov. a nitrogen-fixing acetic acid bacterium associated with sugar cane. Int. J. Syst. Bacteriol. 39:361–364.

Girbal, L., and P. Soucaille. 1994. Regulation of Clostridium acetobutylicum metabolism as revealed by mixed-substrate steady-state continuous cultures: Rle of NADH/NAD ratio and ATP pool. J. Bacteriol. 176:6433–6438.

Girbal, L., C. Croux, I. Vasconcelos, and P. Soucaille. 1995a. Regulation of metabolic shifts in Clostridium acetobutylicum ATCC 824. FEMS Microbiol. Rev. 17:287–297.

Girbal, L., I. Vasoncelos, S. Saint-Amans, and P. Soucaille. 1995b. How neutral red modified carbon and electron flow in Clostridium acetobutylicum grown in chemostat culture at neutral pH. FEMS Microbiol. Rev. 16:151–162.

Girbal, L., and P. Soucaille. 1998. Regulation of solvent production in Clostridium acetobutylicum. Trends Biotechnol. 16:11–16.

Glassner, D. A., and R. Datta. 1992. Process for the production and purification of succinic acid. US Patent. 5,143, 834.

Godin, C., and J. M. Engasser. 1990. Two-stage continuous fermentation of Clostridium acetobutylicum: Effects of pH and dilution rate. Appl. Microbiol. Biotechnol. 33:269–273.

Gokarn, R. R., M. A. Eiteman, S. A. Martin, and K. E. L. Eriksson. 1997a. Production of succinate from glucose, cellobiose, and various cellulosic materials by the ruminal anaerobic bacteria Fibrobacter succinogenes and Ruminococcus flavefaciens. Appl. Biochem. Biotechnol. 68:69–80.

Gokarn, R. R., M. A. Eiteman, and J. Sridhar. 1997b. Production of succinate by anaerobic microorganisms. American Chemical Society Symposium Series 666:237–253.

Gokarn, R. R., M. A. Eiteman, and E. Altman. 1998. Expression of pyruvate carboxylase enhances succinate production in Escherichia coli without affecting glucose uptake. Biotechnol. Lett. 20:795–798.

Gokarn, R. R., M. A. Eiteman, and E. Altman. 1999. Pyruvate carboxylase overexpression for enhanced production of oxalacetate-derived biochemicals in microbial cells. PCT International Patent Application. WO 99/53035.

Goldberg, I., K. Lonberg-Holm, E. A. Bagley, and B. Stieglitz. 1983. Improved conversion of fumarate to succinate by Escherichia coli strains amplified for fumarate reductase. Appl. Env. Microbiol. 45:1838–1847.

Goodlove, P. E., P. R. Cunningham, J. Parker, and D. P. Clark. 1989. Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of Escherichia coli. Gene 85:209–214.

Gosalbes, M. J., C. D. Esteban, J. L. Galan, and G. Perez-Martinez. 2000. Integrative food-grade expression system based on the lactose regulon of Lactobacillus casei. Appl. Environ. Microbiol. 66:4822–4828.

Gottschalk, G. 1985. Bacterial Metabolism, 2nd ed. Springer-Verlag. New York, NY. 247.

Gottschalk, G. 1986. Bacterial Metabolism, 2nd ed. Springer-Verlag. New York, NY. 97–98.

Gottschalk, G., and B. Averhoff. 1992. Process for the microbiological preparation of 1,3-propane-diol from glycerol by Citrobacter. US Patent 5164309.

Gottwald, M., H. Hippe, and G. Gottschalk. 1984. Formation of n-butanol from D-gucose by strains of the "Clostridium tetanomorphum" group. Appl. Environ. Microbiol. 48:573–576.

Gottwald, M., and G. Gottschalk. 1985. The internal pH of Clostridium acetobutylicum and its effect on the shift

from acid to solvent formation. Arch. Microbiol. 143:42–46.

Goupil-Feuillerat, N., M. Cocaign-Bousquet, J.-J. Godon, S. D. Ehrlich, and P. Renault. 1997. Dual role of a-acetolactate decarboxylase in Lactococcus lactis subsp. lactis. J. Bacteriol. 179:6285–6293.

Green, E. M., and G. N. Bennett. 1996a. Inactivation of an aldehye/alcohol dehydrogenase gene from Clostridium acetobutylicum ATCC 824. Appl. Biochem. Biotechnol. 57/58:213–221.

Green, E. M., Z. L. Boynton, L. M. Harris, F. B. Rudolph, E. T. Papoutsakis, and G. N. Bennett. 1996b. Genetic manipulation of acid formation pathways by gene inactivation in Clostridium acetobutylicum ATCC 824. Microbiology 142:2079–2086.

Greenberg, K. 1999. New PDO technologies create opportunities. Chem. Mark. Rep. 255:4 and 9.

Grethlein, A. J., R. M. Worden, M. K. Jain, and R. Datta. 1990. Continuous production of mixed alcohols and acids from carbon monoxide. Appl. Biochem. Biotechnol. 24–25:875–884.

Grove, L. H. 1982. Process for the production of organic fuel. US Patent. 4326032.

Gruber, P. R., E. S. Hall, J. J. Kolstad, M. L. Iwen, R. D. Benson, and R. L. Borchardt. 1992. Continuous process for manufacture of lactide polymers with controlled optical purity. US Patent to Cargill, Inc. 5,142,023.

Gruber, P. R., E. S. Hall, J. J. Kolstad, M. L. Iwen, R. D. Benson, and R. L. Borchardt. 1994. Continuous process for manufacture of lactide polymers with purification by distillation. US Patent to Cargill, Inc. 5,357,035.

Grupe, H., and G. Gottschalk. 1992. Physiological events in Clostridium acetobutylicum during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Appl. Environ. Microbiol. 58:3896–3902.

Gu, Z., B. A. Glatz, and C. E. Glatz. 1998. Propionic acid production by extractive fermentation. I: Solvent consideration. Biotechnol. Bioeng. 57:454–461.

Gu, Z., D. A. Rickert, B. A. Glatz, and C. E. Glatz. 1999. Feasibility of propionic acid production by extractive fermentation. Lait 79:137–148.

Guettler, M. V., and M. K. Jain. 1996a. Method for making succinic acid, Anaerobiospirillum succiniciproducens variants for use in process and methods for obtaining variants. US Patent. 5,521,075.

Guettler, M. V., M. K. Jain, and D. Rumler. 1996b. Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. US Patent. 5,573, 931.

Guettler, M. V., M. K. Jain, and B. K. Soni. 1996c. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US Patent. 5,504,004.

Guettler, M. V., M. K. Jain, and B. K. Soni. 1998. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US Patent. 5,723,322.

Guettler, M. V., D. Rumler, and M. K. Jain. 1999. Actinobacillus succinogenes sp. nov., a novel succinic-acid-producing strain from the bovine rumen. Int. J. Syst. Bacteriol. 49:207–216.

Gunzel, B., S. Yonsel, and W. D. Deckwer. 1991. Fermentative production of 1,3-propanediol from glycerol by Clostridium butyricum up to a scale of 2 m^3. Appl. Microbiol. Biotechnol. 36:289–294.

Gupta, A., V. Verma, and G. N. Quazi. 1997. Transposon induced mutation in Gluconobacter oxydans with special reference to its direct glucose oxidation metabolism. FEMS Microbiol. Lett. 147:181–188.

Gupta, A., M. Felder, Y. Yerma, J. Cullum, and G. N. Qazi. 1999. A mutant of Gluconobacter oxydans deficient in gluconic acid dehydrogenase. FEMS Microbiol. Lett. 179:501–506.

Gutierrez, N. A., and I. S. Maddox. 1992. Product inhibition in a nonmotile mutant of Clostridium acetobutylicum. Enz. Microbiol. Technol. 14:101–105.

Hahn, J. J., A. C. Eschenlauer, M. H. Narrol, D. A. Somers, and F. Sriene. 1997. Growth kinetics, nutrient uptake, and expression of the Alcaligenes eutrophus poly(β-hydroxybutyrate) synthesis pathway in transgenic maize cell suspension cultures. Biotechnol. Prog. 13:347–354.

Hamilton, G. A., and F. H. Westheimer. 1959. A crystalline decarboxylase without biotin. J. Am. Chem. Soc. 8:2277.

Han, I. S., and M. Cheryan. 1996. Downstream processing of acetate fermentation broths by nanofiltration. Appl. Biochem. Biotechnol. 57/58:19–28.

Hancock, K. R., E. Rockman, C. A. Young, L. Pearce, I. S. Maddox, and D. B. Scott. 1991. Expression and nucleotide sequence of the Clostridium acetobutylicum β-galactosidase gene cloned in Escherichia coli. J. Bacteriol. 173:3084–3095.

Harlander, S. K. 1987. Transformation of Streptococcus lactis by electroporation. *In:* J. Ferretti and R. Curtiss (Eds.) Streptococcal Genetics. American Society for Microbiology. Washington DC, 229–233.

Harris, J., R. Mulder, D. B. Kell, R. P. Walter, and J. G. Morris. 1986. Solvent production by Clostridium pasteurianum in media of high sugar content. Biotechnol. Lett. 8:889–892.

Harris, L. M., R. P. Desai, N. E. Welker, and E. T. Papoutsakis. 2000. Characterization of recombinant strains of the Clostridium acetobutylicum butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition? Biotechnol. Bioengin. 67:1–11.

Harris, L. M., N. E. Welker, and E. T. Papoutsakis. 2002. Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in Clostridium acetobutylicum ATCC 824. J. Bacteriol. 184:3586–3597.

Hartmanis, M. G. N. 1987. Butyrate kinase from Clostridium acetobutylicum. J. Biol. Chem. 262:617–621.

Hastings, J. J. H. 1971. Development of the fermentation industries in Great Britain. Adv. Appl. Microbiol. 14:1–45.

Hastings, J. J. H. 1978. Acetone-butyl alcohol fermentation. *In:* A. H. Rose (Ed.) Primary Products of Metabolism. Academic Press. London, UK. 31–45.

Haynie, S. L., and L. W. Wagner. 1997. Process for making 1,3-propanediol from carbohydrates using mixed microbial cultures. US Patent 5599689.

Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes. 1988. The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism Alcaligenes eutrophus. FEMS Microbiol. Lett. 52:259–264.

Hein, S., H. Tran, and A. Steinbüchel. 1998. Synechocystis sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. Arch. Microbiol. 170:162–170.

Helbert, J. R. 1982. Beer. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 403–467.

Hermann, M., F. Fayolle, R. Marchal, L. Podvin, M. Sebald, and J.-P. Vandecasteele. 1985. Isolation and characterization of butanol-resistant mutants of Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:1238–1243.

Herrick, H. T., and O. E. May. 1928. The production of gluconic acid by the Penicillium luteum purpurogenum group II: Some optimal conditions for acid formation. J. Biol. Chem. 77:185–195.

Heyndrickx, M., P. De Vos, M. Vancanneyt, and J. De Ley. 1991. The fermentation of glycerol by Clostridium butyricum LMG 1212t2 and 1213t1 and C. pasteurianum LMG 3285. Appl. Microbiol. Biotechnol. 34:637–642.

Himmel, M. E., W. S. Adney, K. Grohmann, and M. P. Tucker. 1994. US Patent. 5,275,944.

Himmel, M. E., W. S. Adney, J. O. Baker, R. Elander, J. D. McMillan, R. A. Nieves, J. J. Sheehan, S. R. Thomas, T. B. Vinzant, and M. Zhang. 1997. Advanced bioethanol production technologies: A perspective. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:2–45.

Himmi, E. H., A. Bories, and F. Barbirato. 1999. Nutrient requirements for glycerol conversion to 1,3-propanediol by Clostridium butyricum. Biores. Technol. 67:123–138.

Himmi, E. H., A. Bories, A. Boussaid, and L. Hassani. 2000. Propionic acid fermentation of glycerol and glucose by Propionibacterium acidipropionici and Propionibacterium freudenreichii ssp. shermanii. Appl. Microbiol. Biotechnol. 53:435–440.

Hippe, H., J. R. Andresen, and G. Gottschalk. 1991. The genus Clostridium, non-medical. *In:* Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 1799–1866.

Hiu, S. F., C.-X. Zhu, R.-T. Yan, and J.-S. Chen. 1987. Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: Different alcohol dehydrogenases in two strains of Clostridium beijerinckii (Clostridium butylicum). Appl. Environ. Microbiol. 53:697–703.

Hlasiwetz, H., and J. Habermann. 1870. Zur Kenntnis einiger Zuckerarten (Glucose, Rhorzucker, Levulose, Sorbin, Phloroglucin). Ann Chem. Pharm. 155:128–144.

Ho, K.-L. G., A. L. Pometto 3rd, and P. N. Hinz. 1997a. Optimization of L-(+)-lactic acid production by ring and disc plastic composite supports through repeated-batch biofilm fermentation. Appl. Environ. Microbiol. 63: 2533–2542.

Ho, K.-L. G., A. L. Pometto 3rd, P. N. Hinz, and A. Demirci. 1997b. Nutrient leaching and end product accumutation in plastic composite supports for L-(+)-lactic acid biofilm fermentation. Appl. Environ. Microbiol. 63:2524–2532.

Ho, K.-L. G., A. L. Pometto 3rd, P. N. Hinz, J. S. Dickson, and A. Demirci. 1997c. Ingredients selection for plastic composite supports for L-(+)-lactic acid biofilm fermentation by Lactobacillus casei subsp. rhamnosus. Appl. Environ. Microbiol. 63:2516–2523.

Hocking, P. J., and R. H. Marchessault. 1994. Biopolymers. *In:* G. J. L. Griffin (Ed.) Chemistry and Technology of Biodegradable Polymers. Chapman and Hall. London, 48–96.

Hoffman, J. 2001. Butadiene hit hard by sharp drop in derivatives demand. Chem. Mark. Rep. 260:1 and 12.

Holland-Staley, C. A., K. Lee, D. P. Clark, and P. R. Cunningham. 2000. Aerobic activity of Escherichia coli alcohol dehydrogenase is determined by a single amino acid. J. Bacteriol. 18(2):6049–6054.

Holt, R. A., G. M. Stephens, and J. G. Morris. 1984. Production of solvents by Clostridium acetobutylicum cultures maintained at neutral pH. Appl. Environ. Microbiol. 48:1166–1170.

Holt, R. A., A. J. Cairns, and J. G. Morris. 1988. Production of butanol by Clostridium puniceum in batch and continuous culture. Appl. Microbiol. Biotechnol. 27:319–324.

Holten, C. M., A. Muller, and D. Rehbinder (Eds.). 1971. Lactic Acid. Weinheim/Bergstr. Germany.

Homann, T., C. Tag, H. Biebl, W. D. Deckwer, and B. Schink. 1990. Fermentation of glycerol to 1,3-propanediol by Klebsiella and Citrobacter strains. Appl. Microbiol. Biotechnol. 33:121–126.

Hong, R. 1999. The Cloning of a Putative Regulatory Gene and the sol Region from Clostridium beijerinckii M.S. thesis. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Hongo, M., and A. Murata. 1965a. Bacteriophages of Clostridium saccharoperbutylacetonicum. Part I: Some characteristics of the twelve phages obtained from the abnormally fermented broths. Agric. Biol. Chem. 29:1135–1139.

Hongo, M., and A. Murata. 1965b. Bacteriophages of Clostridium saccharoperbutylacetonicum. Part II: Enumeration of phages by the application of the plaque count technique and some factors influencing the plaque formation. Agric. Biol. Chem. 29:1140–1145.

Hoppner, T. C., and H. W. Doelle. 1983. Purification and kinetic characteristics of pyruvate decarboxylase and ethanol dehydrogenase from Zymomonas mobilis in relation to ethanol production. Eur. J. Appl. Microbiol. Biotechnol. 17:152–157.

Houben, M. C. M. 1995. Oxygenated blending components for gasoline-alcohols and ethers. *In:* E. L. Marshall and K. Owen (Ed.) Motor Gasoline: Critical Reports on Applied Chemistry. Royal Society of Chemistry. Cambridge, UK. 34:45–71.

Houge, C. 2000. Getting the MTBE out. Chem Eng. News 6.

Howaldt, M., A. Gottlob, K. D. Kulba, and H. Chmiel. 1988. Simultaneous conversion of glucose/fructose mixtures in a membrane reactor. Ann. NY Acad. Sci. 542:400–405.

Howaldt, M., K. D. Kulbe, and H. Chmiel. 1990. A continous enzyme membrane reactor retaining native nicotinamide cofactor NAD (H). Ann. NY Acad. Sci. 589:253–260.

Howard, W. L. 1991. Acetone. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 1:176–194.

Hrabak, O. 1992. Industrial production of poly-β-hydroxybutyrate. FEMS Microbiol. Rev. 103:251–256.

Hromatka, O., and H. Ebner. 1949. Investigations on vinegar fermentation: Generator for vinegar fermentation and aeration procedures. Enzymologia 13:369.

Hsu, S. T., and S. T. Yang. 1991. Propionic acid fermentation of lactose by Propionibacterium acidipropionici—effects of pH. Biotechnol. Bioeng. 38:571–578.

Huang, Y., and S. T. Yang. 1998a. Acetate production from whey lactose using co-immobilized cells of homolactic and homoacetic bacteria in a fibrous-bed bioreactor. Biotechnol. Bioeng. 60:498–507.

Huang, Y. L., K. Mann, and J. M. Mavak. 1998b. Acetic acid production from fructose by Clostridium formicaceticum immobilized in a fibrous bed bioreactor. Biotechnol. Prog. 800–806.

Huang, K.-X., F. B. Rudolph, and G. N. Bennett. 1999. Characterization of methylglyoxal synthase from Clostridium acetobutylicum ATCC 824 and its use in the formation of 1,2-propanediol. Appl. Environ. Microbiol. 65:3244–3247.

Huang, K.-X., S. Huang, F. B. Rudolph, and G. N. Bennett. 2000. Identification and characterization of a second butyrate kinase from Clostridium acetobutylicum ATCC 824. J. Molec. Microbiol. Biotechnol. 2:33–38.

Huff, G. F., and N. Yata. 1976. US Patent. 3,990,945.

Husemann, M., and E. T. Papoutsakis. 1986. Effect of acetoacetate, butyrate, and uncoupling ionophores on growth and product formation of Clostridium acetobutylicum. Biotechnol. Lett. 8:37–42.

Husemann, M. H. W., and E. T. Papoutsakis. 1988. Solventoggenesis in Clostridium acetobutylicum fermentations related to carboxylic acid and proton concentrations. Biotechnol. Bioengin. 32:843–852.

Husemann, M. H. W., and E. T. Papoutsakis. 1989. Comparison between in vivo and in vitro enzyme activities in continuous and batch fermentations of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 30:585–595.

Ingram, L. O., and T. M. Buttke. 1984. Effects of alcohols on micro-organisms. Adv. Microb. Physiol. 25:253–300.

Ingram, L. O. 1986. Microbial tolerance to alcohols: Role of the cell membrane. Trends Biotechnol. 4:40–44.

Ingram, L. O., T. Conway, D. P. Clark, G. W. Sewell, and J. F. Preston. 1987. Genetic engineering of ethanol production in Escherichia coli. Appl. Environ. Microbiol. 53: 2420–2425.

Ingram, L. O., and T. Conway. 1988. Expression of different levels of ethanologenic enzymes from Zymomonas mobilis in recombinant strains of Escherichia coli. Appl. Environ. Microbiol. 54:397–404.

Ingram, L. O., and K. M. Dombek. 1989. Effects of ethanol on Escherichia coli. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 227–237.

Ingram, L. O. 1990. Ethanol tolerance in bacteria. Crit. Rev. Biotechnol. 9:305–319.

Ingram, L. O., F. Alterthum, and T. Conway. 1991. US Patent. 5,000,000.

Ingram, L. O., and D. C. Clark. 1992. US Patent. 5,028,539.

Ingram, L. O., and J. B. Doran. 1995a. Conversion of cellulosic materials to ethanol. FEMS Microbiol. Rev. 16:235–241.

Ingram, L. O., D. S. Beall, G. F. H. Burchhardt, W. V. Guimaraes, K. Ohta, B. E. Wood, K. T. Shanmugam, D. E. Fowler, and B.-B. A. 1995b. US Patent. 5,424,202.

Ingram, L. O., and M. D. F. Barbosa-Alleyne. 1996. US Patent. 5,482,846.

Ingram, L. O., and K. W. Ohta, B. E. 1998a. US Patent. 5821093.

Ingram, L. O., P. F. Gomez, X. Lai, M. Moniruzzaman, B. E. Wood, L. P. Yamano, and S. W. York. 1998b. Metabolic engineering of bacteria for ethanol production. Biotechnol. Bioengin. 58:204–214.

Ingram, L. O., H. C. Aldrich, A. C. C. Borges, T. B. Causey, A. Martinez, F. Morales, A. Saleh, S. A. Underwood, L. P. Yomano, S. W. York, J. Zaldivar, and S. Zhou. 1999. Enteric bacterial catalysts for fuel ethanol production. Biotechnol. Progr. 15:855–866.

Inoue, Y., and A. Kimura. 1995. Methylglyoxal and regulation of its metabolism in microorganisms. Adv. Microb. Physiol. 37:177–227.

Inskeep, G. C., G. G. Taylor, and W. C. Breitzke. 1952. Lactic acid from corn sugar. Ind. Eng. Chem. 44:1955–1966.

Isbell, H. S., H. L. Frush, and F. J. Bates. 1932. Manufacture of calcium gluconate by electrolytic oxidation of glucose. Ind. Eng. Chem. 24:375–378.

Ishizaki, A., N. Taga, T. Takeshita, T. Sugimoto, T. Tsuge, and K. Tanaka. 1997. Microbial production of biodegradable plastics from carbon dioxide and agricultural waste material. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, 295–306.

Ismaiel, A. A., C.-X. Zhu, G. D. Colby, and J.-S. Chen. 1993. Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of Clostridium beijerinckii. J. Bacteriol. 175:5097–5105.

Ismaiel, A. A., and J. S. Chen. 1998. Purification of the aldehyde-alcohol dehydrogenase encoded by the aad gene from Clostridium acetobutylicum ATCC 824. *In:* Abstracts of the 98th General Meeting of the American Society of Microbiology. American Society for Microbiology. Washington, D.C., O-40:400.

Iversen, T.-G., R. Standal, T. Pederson, and D. H. Caucheron. 1994. IS 1032 from Acetobacter xylinum, a new mobile insertion sequence. Plasmid 32:46–54.

Jabalquinto, A. M., M. Laivenieks, J. G. Zeikus, and E. Cardemil. 1999. Characterization of the oxaloacetate decarboxylase and pyruvate kinase-like activities of Saccharomyces cerevisiae and Anaerobiospirillum succiniciproducens phosphoenolpyruvate carboxykinases. J. Prot. Chem. 18:659–664.

Jackson, M. D., and C. B. Moyer. 1991. Alcohol fuels. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 1:826–864.

Jan, G., A. Rouault, and J. L. Maubois. 2000. Acid stress susceptibility and acid adaptation of Propionibacterium freudenreichii subsp. shermanii. Lait 80:325–336.

Jan, G., P. Leverrier, V. Pichereau, and P. Boyaval. 2001. Changes in protein synthesis and morphology during acid adaptation of Propionibacterium freudenreichii. Appl. Environ. Microbiol. 67:2029–2036.

Jansen, N. B., and G. T. Tsao. 1983. Bioconversion of pentoses to 2,3-butanediol by Klebsiella pneumoniae. *In:* A. Fiechter and T. W. Jeffries (Eds.) Advances in Biochemical Engineering/Biotechnology. Springer-Verlag. New York, NY. 27:85–99.

Jansen, N. B., M. C. Flickinger, and G. T. Tsao. 1984. Production of 2,3-butanediol from D-xylose by Klebsiella oxytoca ATCC 8724. Biotechnol. Bioengin. 26:362–369.

Jarvis, L. 2001. Lactic acid outlook up as polylactide nears market. Chemical Market Reporter 259(9):5, 14.

Jendrossek, D., M. Backhaus, and M. Andermann. 1995. Characterization of the Comamonas sp. poly(3-hydroxybutyrate) (PHB) depolymerase and of its structural gene. Can. J. Microbiol. 41:160–169.

Jendrossek, D., A. Schirmer, and H. G. Schlegel. 1996. Biodegradation of polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 46:451–463.

Jin, Z. W., and S. T. Yang. 1998. Extractive fermentation for enhanced propionic acid production from lactose by Propionibacterium acidipropionici. Biotechnol. Prog. 14:457–465.

Joachimsthal, E., K. D. Haggett, J.-H. Jang, and P. L. Rogers. 1998. A mutant of Zymomonas mobilis ZM4 capable of ethanol production from glucose in the presence of high acetate concentrations. Biotechnol. Lett. 20:137–142.

Johansen, L., Bryn, K., and F. C. Stormer. 1975. Physiological and biochemical role of the butanediol pathway in Aerobacter (Enterobacter) aerogenes. J. Bacteriol. 123:1124–1130.

John, M. E., and G. Keller. 1996. Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. USA 93:12768–12773.

Johnson, J. L. 1984. Bacterial classification. III: Nucleic acids in bacterial classification. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:8–11.

Johnson, J. L., and J.-S. Chen. 1995. Taxonomic relationships among strains of Clostridium acetobutylicum and other phenotypically similar organisms. FEMS Microbiol. Rev. 17:233–240.

Johnson, J. L., J. Toth, S. Santiwatanakul, and J.-S. Chen. 1997. Cultures of "Clostridium acetobutylicum" from various collections comprise Clostridium acetobutylicum, Clostridium beijerinckii, and two other distinct types based on DNA-DNA reassociation. Int. J. Syst. Bacteriol. 47:420–424.

Jones, R. P., and P. Greenfield. 1982. Effect of carbon dioxide on yeast growth and fermentation. Enz. Microbiol. Technol. 4:210–223.

Jones, D. T., W. A. Jones, and D. R. Woods. 1985. Production of recombinants after protoplast fusion in Clostridium acetobutylicum P262. J. Gen. Microbiol. 131:1213–1216.

Jones, D. T., and D. R. Woods. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50:484–524.

Jones. D. T. 1993. Mutagenesis and its application to biotechnology. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 77–98.

Jones, D. T., and S. Keis. 1995. Origins and relationships of industrial solvent-producing clostridial strains. FEMS Microbiol. Rev. 17:223–232.

Jones, D. T., M. Shirley, X. Wu, and S. Keis. 2000. Bacteriophage infections in the industrial AB fermentation process. J. Molec. Microbiol. Biotechnol. 2:21–26.

Jossek, R., and A. Steinbüchel. 1998. In vitro synthesis of poly-(3-hydroxybutyric acid) by using an enzymatic coenzyme A recycling system. FEMS Microbiol. Lett. 168:319–324.

Junelles, A.-M., R. Janati-Idrissi, A. El Kanouni, H. Petitdemange, and R. Gay. 1987. Acetone-butanol fermentation by mutants selected for resistance to acetate and butyrate halogen analogues. Biotechnol. Lett. 9:175–178.

Juni, E., and G. A. Heym. 1956. A cyclic pathway for the bacterial dissimilation of 2,3-butanediol, acetylmethylcarbinol, and diacetyl. I: General aspects of the 2,3-butanediol cycle. J. Bacteriol. 71:425–432.

Kandler, O., and N. Weiss. 1986. *In:* P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 2:1208–1234.

Kaneuchi, C., M. Seki, and K. Komagata. 1988. Production of succinic acid from citric acid and related acids by Lactobacillus strains. Appl. Environ. Microbiol. 54:3053–3056.

Kansiz, M., H. Billman-Jacobe, and D. McNaughton. 2000. Quantitative determination of the biodegradable polymer poly(β-hydroxybutyrate) in a recombinant Escherichia coli strain by use of mid-infrared spectroscopy and multivariative statistics. Appl. Environ. Microbiol. 66:3415–3420.

Kashket, E. R., and Z. Y. Cao. 1995. Clostridial strain degeneration. FEMS Microbiol. Rev. 17:307–315.

Kawaguchi, Y., and Y. Doi. 1990. Structure of native poly(3-hydroxybutyrate) granules characterized by X-ray diffraction. FEMS Microbiol. Lett. 70:151–156.

Kawaguchi, Y., and Y. Doi. 1992. Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in Alcaligenes eutrophus. Macromolecules 25:2324–2329.

Keis, S., C. F. Bennett, V. K. Ward, and D. T. Jones. 1995. Taxonomy and phylogeny of industrial solvent-producing clostridia. Int. J. Syst. Bacteriol. 45:693–705.

Keis, S., R. Shaheen, and D. T. Jones. 2001a. Emended description of Clostridium acetobutylicum and Clostridium beijerinckii and descriptions of Clostridium saccharoperbutylacetonicum sp. nov. and Clostridium saccharobutylicum sp. nov. Int. J. Syst. Evol. Microbiol. 51:2095–2103.

Keis, S., J. T. Sullivan, and D. T. Jones. 2001b. Physical and genetic map of the Clostridium saccharobutylicum (formerly Clostridium acetobutylicum) NCP 262 chromosome. Microbiology 147:1909–1922.

Kelly, F. C. 1936. One Thing Leads To Another. Houghton Mifflin. Boston, MA.

Kennedy, J. F., J. D. Humphreys, S. A. Barker, and R. N. Greenshields. 1980. Application of living immobilized cells to the acceleration of the continuous conversions of ethanol (wort) to acetic acid (vinegar) by hydrous titanium (IV) oxide-immobilized Acetobacter species. Enz. Microbiol. Technol. 2:209–216.

Keshav, K. F., L. P. Yomano, H. An, and L. O. Ingram. 1990. Cloning of the Zymomonas mobilis structural gene encoding alcohol dehydrogenase I (adhA): Sequence comparison and expression in Escherichia coli. J. Bacteriol. 172:2491–2497.

Kessler, D., I. Leibrecht, and J. Knappe. 1991. Pyruvate-formate-lyase-deactivase and acetyl-CoA reductase activities of Escherichia coli reside on a polymeric protein particle encoded by adhE. FEBS Lett. 281:59–63.

Kessler, D., W. Herth, and J. Knappe. 1992. Ultrastructure and pyruvate formate-lyase radical quenching property of the multienzymic AdhE protein of Escherichia coli. J. Biol. Chem. 267:18073–18079.

Kiatpapan, P., Y. Hashimoto, H. Nakamura, Y.-Z. Piao, H. Ono, M. Yamashita, and Y. Murooka. 2000. Characterization of pRG01, a plasmid from Propionibacterium acidipropionici, and its use for development of a host-vector system in propionibacteria. Appl. Environ. Microbiol. 66:4688–4695.

Killeffer, D. H. 1927. Butanol and acetone from corn. Indust. Engin. Chem. 19:46–50.

Kim, D. M., and H. S. Kim. 1992a. Continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an oxido reductase from Zymomonas mobilis and inulinase. Biotechnol. Bioeng. 39:336–342.

Kim, B. S., S. Y. Lee, and H. N. Chang. 1992b. Production of poly-beta-hydroxybutyrate by fed-batch culture of recombinant Escherichia coli. Biotechnol. Lett. 14:811–816.

Kim, B. S., S. C. Lee, S. Y. Lee, H. N. Chang, Y. K. Chang, and S. I. Woo. 1994. Production of poly(3-

hydroxybutyric acid) by fed-batch culture of Alcaligenes eutrophus with glucose concentration control. Biotechnol. Bioeng. 43:892–898.

Kim, S. W., P. Kim, H. S. Lee, and J. H. Kim. 1996. High production of poly-beta-hydroxybutyrate (PHB) from Methylobacterium organophilum under potassium limitation. Biotechnol. Lett. 18:25–30.

Kim, G. J., I. Y. Lee, S. C. Yoon, Y. C. Shih, and Y. H. Park. 1997. Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of Pseudomonas putida by combined use of glucose and octanoate. Enz. Microb. Technol. 20:500–505.

Kinoshita, S., T. Kakizono, K. Kadota, K. Das, and H. Taguchi. 1985. Purification of two alcohol dehydrogenases from Zymomonas mobilis and their properties. Appl. Microbiol. Biotechnol. 22:249–254.

Kirk Othmer. 1983. Succinic acid and succinic anhydride. Kirk-Othmer Encyclopedia of Chemical Technology, 3rd ed. 21:848–864.

Kirschner, E. M. 1996. Growth of top 50 chemicals slowed from very high 1994 rate. Chem. Eng. News 74(15):16–19.

Kitahara, K., T. Kaneko, and O. Goto. 1957. Taxonomic studies on the hiochi-bacteria specific saprophytes of sake. I: Isolation and grouping of bacterial strains. J. Gen. Appl. Microbiol. (Japan) 3:102–110.

Klaenhammer, T. R., and G. F. Fitzgerald. 1994. Bacteriophages and bacteriophage resistance. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 106–168.

Klapatch, R. R., M. L. Guerinot, and L. R. Lynd. 1996. Electrotransformation of Clostridium thermosaccharolyticum. J. Ind. Microbiol. 16:342–347.

Klasson, K. T., M. D. Ackerson, E. C. Clausen, and J. L. Gaddy. 1992. Bioconversion of synthesis gas into liquid or gaseous fuels. Enzyme Microbiol. Technol. 14:602–608.

Klinke, S., G. de Roo, B. Witholt, and B. Kessler. 2000. Role of phaD in accumulation of medium-chain-length poly(3-hydroxyalkanoates) in Pseudomonas olevorans. Appl. Environ. Microbiol. 66:3705–3710.

Knauf, H. J., R. F. Vogel, and W. P. Hammes. 1992. Cloning, sequence, and phenotypic expression of KatA which encodes the catalase of Lactobacillus sake LTH677. Appl. Environ. Microbiol. 58:832–839.

Kok, J., and J. M. DeVos. 1994. The proteolytic system of lactic acid bacteria. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 169–210.

Konda, T., and M. Konda. 1996. Efficient production of acetic acid from glucose in a mixed culture of Zymomonas mobilisand Acetobacter sp. J. Ferment. Bioeng. 81:42–46.

Kondo, K., and S. Horinouchi. 1997. A new insertion sequence IS 1452 from Acetobacter pasteurianus. Microbiology 143:539–546.

Korkhin, Y., A. J. Kalb(Gilboa), M. Peretz, O. Bogin, Y. Burstein, and F. Frolow. 1998. NADP-dependent bacterial alcohol dehydrogenases: Crystal structure, cofactor-binding and cofactor specificity of the ADHs of Clostridium beijerinckii and Thermoanaerobacter brockii. J. Molec. Biol. 278:967–981.

Kosaric, N. 1996. Ethanol-potential source of energy and chemical products. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. VCH. New York, NY. 6:123–203.

Kusano, K., H. Yamada, M. Niwa, and K. Yamasoto. 1997. Propionibacterium cyclohexanicum sp nov., a new acid-tolerant omega-cyclohexy/fatty acid-containing propionibacterium isolated from spoiled orange juice. Int. J. Syst. Bacteriol. 47:825–831.

Kutzenok, A., and M. Aschner. 1952. Degenerative processes in a strain of Clostridium butylicum. J. Bacteriol. 64:829–836.

Kutzing, F. T. 1837. Prakt. Chem. J. 11:385.

Kwon, Y. J., R. Kaol, and B. Mattiasson. 1996. Extractive lactic acid fermentation in Poly (ethyleneimine)-based aqueous two-phase system. Biotechnol. Bioeng. 50:280–290.

Ladisch, M. R. 1991. Fermentation-derived butanol and scenarios for its uses in energy-related applications. Enz. Microbiol. Technol. 13:280–283.

Laffend, L. A., V. Nagarajan, and C. E. Nakamura. 1997. Bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism. US Patent. 5686276.

Laffend, L. A., V. Nagarajan, and C. E. Nakamura. 2000. Bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism. US Patent. 6025184.

Laivenieks, M., C. Vielle, and J. G. Zeikus. 1997. Cloning, sequencing, and overexpression of the Anaerobiospirillum succiniciproducens phosphoenolpyruvate carboxykinase (pckA) gene. Appl. Env. Microbiol. 63:2272–2280.

Lamed, R. J., and J. G. Zeikus. 1981. Novel NADP-linked alcohol-aldehyde/ketone oxidoreductase in thermophilic ethanologenic bacteria. Biochem. J. 195:183–190.

Landucci, R., B. Goodman, and C. Wyman. 1994. Methodology for evaluating the economics of biologically producing chemicals and materials from alternative feedstocks. Appl. Biochem. Biotechnol. 45/46:677–696.

Largier, S. T., S. Long, J. D. Santangelo, D. T. Jones, and D. R. Woods. 1985. Immobilized Clostridium acetobutylicum P 262 mutants for solvent production. Appl. Environ. Microbiol. 50:477–481.

Larsen, S. H., and F. C. Stormer. 1973. Diacetyl (Acetoin) reductase from Aerobacter aerogenes. Eur. J. Biochem. 34:100–106.

Larsen, L., P. Nielsen, and B. K. Ahring. 1997. Thermoanaerobacter mathranii sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. Arch. Microbiol. 168:114–119.

Laube, V. M., D. Groleau, and S. M. Martin. 1984. 2,3-butanediol production from xylose and other hemicellulosic components by Bacillus polymyxa. Biotechnol. Lett. 6:257–262.

Layman, P. 1998. Acetic acid industry gears up for possible building boom. Chem. Eng. News 76(31):17–18.

Leaf, T. A., M. S. Peterson, S. K. Stoup, D. Somers, and F. Srienc. 1996. Saccharomyces cerevisiae expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. Microbiol. 142:1169–1180.

Lee, S. F., C. W. Forsberg, and L. N. Gibbins. 1985. Cellulolytic activity of Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:220–228.

Lee, H. K., and I. S. Maddox. 1986. Continuous production of 2,3-butanediolfrom whey permeate using Klebsiella

pneumoniae immobilized in calcium alginate. Enz. Microb. Technol. 8:409–411.

Lee, S. Y., G. N. Bennett, and E. T. Papoutsakis. 1992. Construction of Escherichia coli-Clostridium acetobutylicum shuttle vectors and transfromation of Clostridium acetobutylicum strains. Biotechnol. Lett. 14:427–432.

Lee, Y.-E., M. K. Jain, C. Lee, S. E. Lowe, and J. G. Zeikus. 1993. Taxonomic distinction of saccharolytic thermophilic anaerobes: Description of Thermoanaerobacterium xylanolyticum gen. nov., sp. nov., and Thermoanaerobacterium saccharolyticum gen. nov., sp. nov.; reclassification of Thermoanaerobium brockii, Clostridium thermosulfurogenes, and Clostridium thermohydrosulfuricum E100-69 as Thermoanaerobacter brockii comb. nov., and Thermoanaerobacter thermosulfurigenes comb. nov., and Thermoanaerobacter thermohydrosulfuricus comb. nov., respectively; and transfer of Clostridium thermohydrosulfuricum 39E to Thermoanaerobacter ethanolicus. Int. J. Syst. Bacteriol. 43:41–51.

Lee, S. Y., and H. N. Chang. 1995a. Production of poly(hydroxyalkanoic acid). Adv. Biochem. Eng. Biotechnol. 52:27–58.

Lee, S. Y., and H. N. Chang. 1995b. Production of poly(3-hydroxybutyric acid) by recombinant Escherichia coli strains: Genetic and fermentation studies. Can. J. Microbiol. 41, Suppl. 1:207–215.

Lee, S. Y., Y. K. Lee, and H. N. Chang. 1995c. Stimulatory effects of amino acids and oleic acid on poly(3-hydroxybutyric acid) synthesis by recombinant Escherichia coli. J. Ferment. Bioeng. 79:177–180.

Lee, S. Y. 1996a. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49:1–14.

Lee, S. Y. 1996b. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol. 14:431–438.

Lee, I. Y., M. K. Kim, Y. H. Park, and S. Y. Lee. 1996c. Regulatory effects of cellular nicotinamide nucleotides and enzyme activities on poly(3-hydroxybutyrate) synthesis in recombinant Escherichia coli. Biotechnol. Bioeng. 52:707–712.

Lee, S. Y. 1997. E. coli moves into the plastic age. Nature Biotechnol. 15:17–18.

Lee, P. C., W. G. Lee, S. Kwon, S. Y. Lee, and H. N. Chang. 1999a. Succinic acid production by Anaerobiospirillum succiniciproducens: Effects of the H_2/CO_2 supply and glucose concentration. Enz. Microbiol. Technol. 24:549–554.

Lee, P. C., W. G. Lee, S. Y. Lee, and H. N. Chang. 1999b. Effects of medium components on the growth of Anaerobiospirillum succiniciproducens and succinic acid production. Proc. Biochem. 35:49–55.

Lee, S. Y., Y. Lee, and F. L. Wang. 1999c. Chiral compounds from bacterial polyesters: Sugars to plastics to fine chemicals. Biotechnol. Bioeng. 65:363–368.

Lefranc, L. et Cie. 1923. A process for the manufacture of butyric acid and other fatty acids with recovery of the gases of fermentation. British Patent 186-572. Chem Abstr. 17:324.

Leh, M. B., and M. Charles. 1989. The effect of whey protein hydrolyzate average molecular weight on the lactic acid fermentation. J. Ind. Microbiol. 4:77–80.

Leigh, J. A., F. Mayer, and R. S. Wolfe. 1981. Acetogenium kivui a new thermophilic hydrogen-oxidizing, acetogenic bacterium. Arch. Microbiol. 129:275–280.

Lemme, C. J., and J. R. Frankiewicz. 1985. Strains of Clostridium acetobutylicum and process for its preparation. US Patent. 4521516.

Lemmel, S. A. 1985. Mutagenesis in Clostridium acetobutylicum. Biotechnol. Lett. 7:711–716.

Lemmel, S. A., R. Datta, and J. R. Frankiewicz. 1986. Fermentation of xylan by Clostridium acetobutylicum. Enz. Microbiol. Technol. 8:217–221.

Lemoigne, M. 1925. Etudes sur l'autolyze microbienne acidification par formation d'acide β-oxybutyrique. Ann. Inst. Pasteur 39:144–173.

Lemoigne, M. 1926. Produits de déshydration et de polymérisation de l'acide β-oxybutyrique. Bull. Soc. Chim. Biol. 8:770–782.

Lemoigne, M., and H. Girard. 1943. Réserves lipidiques β-hydroxybutyriques chez Azotobacter chroococcum. Comp. Rend. Acad. Sci. 217:557–558.

Lenz, T. G., and A. R. Moreira. 1980. Economic evaluation of the acetone-butanol fermentation. Indust. Engin. Chem. Prod. Res. Devel. 19:478–483.

Lepage, C., F. Fayolle, M. Hermann, and J.-P. Vandecasteele. 1987. Changes in membrane liquid composition of Clostridium acetobutylicum during acetone-butanol fermentation: Effects of solvents, growth temperature and pH. J. Gen. Microbiol. 133:103–110.

Lewis, V. P., and S. T. Yang. 1992. Continuous propionic acid fermentation by immobilized Propionibacterium acidipropionici in a novel packed bed bioreactor. Biotechnol. Bioeng. 40:465–474.

Li, G. 1998. Development of a Reporter System for the Study of Gene Expression for Solvent Production in Clostridium beijerinckii NRRL B582 and Clostridium acetobutylicum ATCC 824 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Liebergesell, M., E. Hustede, A. Timm, A. Steinbüchel, R. C. Fuller, R. W. Lenz, and H. G. Schlegel. 1991. Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. Arch. Microbiol. 155:415–421.

Lin, Y.-L., and H. P. Blaschek. 1984. Transformation of heat-treated Clostridium acetobutylicum protoplasts with pUB110 plasmid DNA. Appl. Environ. Microbiol. 48:737–742.

Lin, R. W., E. E. Atkinson, and D. E. Balhoff. 1996. Process for producing (S,S)-ethylenediamine-N,N′-disuccinic acid. US Patent. 5554791.

Lin, M.-Y., S. Harlander, and D. Saviano. 1999. Construction of an integrative food-grade cloning vector for Lactobacillus acidophilus. Appl. Microb. Biotechnol. 45:484–489.

Linden, J. C., and R. H. Kuhn. 1989. Biochemistry of alcohol effects on Clostridia. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 271–291.

Lindsay, S. E., R. J. Bothast, and L. O. Ingram. 1995. Improved strains of recombinant Escherichia coli for ethanol production from sugar mixture. Appl. Microbiol. Biotechnol. 43:70–75.

Lipinsky, E. S., and R. G. Sinclair. 1986. Is lactic acid a commodity chemical? Chem. Eng. Prog. 82(8):26–32.

Litchfield, J. H. 1996. Microbial production of lactic acid. Adv. Appl. Microbiol. 42:45–95.

Liu, S.-Y., F. A. Rainey, H. W. Morgan, F. Mayer, and J. Wiegel. 1996. Thermoanaerobacterium aotearoense sp. nov., a slightly acidophilic, anaerobic thermophile isolated from various hot springs in New Zealand, and

emendation of the genus Thermoanaerobacterium. Int. J. Syst. Bacteriol. 46:388–396.

Liu, S.-J., and A. Steinbüchel. 2000a. Exploitation of butyrate kinase and phosphotransbutyrylase from Clostridium acetobutylicum for the in vitro biosynthesis of poly(hydroxyalkanoic acid). Appl. Microbiol. Biotechnol. 53:545–552.

Liu, S.-J., and A. Steinbüchel. 2000b. A novel genetically engineered pathway for synthesis of poly(hydroxyalkanoic acids) in Escherichia coli. Appl. Environ. Microbiol. 66:739–743.

Ljungdahl, L. G. 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Ann. Rev. Microbiol. 40:415–450.

Ljungdahl, L. G. 1994. The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 63–85.

Lockwood, L. B., D. E. Yoder, and M. Zienty. 1965. Lactic acid. Ann. NY Acad. Sci. 119:854–867.

Logsdon, J. E. 1994. Ethanol. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 9:812–860.

Logsdon, J. E., and R. A. Loke. 1996. Isopropyl alcohol. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 20:216–240.

Logsdon, J. E., and R. A. Loke. 1999. Isopropyl alcohols. *In:* J. I. Kroschwitz (Ed.) Kirk-Othmer Concise Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 1654–1656.

Loos, H., R. Krämer, H. Sahm, and G. A. Sprenger. 1994. Sorbitol promotes growth of Zymomonas mobilis in environment with high concentrations of sugar: Evidence for a physiological function of glucose-fructose oxidoreductase in osmoprotection. J. Bacteriol. 176: 7688–7693.

Lorowitz, W. H., and M. P. Bryant. 1984. Peptostreptococcus productusstrain that grows rapidly with CO as an energy source. Appl. Environ. Microbiol. 47:961–964.

Lowe, S. E., M. K. Jain, and J. G. Zeikus. 1993. Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. Microbiol. Rev. 57:451–509.

Lowenheim, F. A., and M. K. Moran. 1975. Industrial Chemicals, 4th ed. John Wiley & Sons. New York, NY.

Luchansky, J. B., P. M. Muriana, and T. R. Klaenhammer. 1988. Application of electroporation for transfer of plasmid DNA to Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Bacillus, Staphylococcus, Enterococcus, and Propionibacterium. Molec. Microbiol. 2:637–646.

Luers, F. M., R. Seyfried, R. Daniel, and G. Gottschalk. 1997. Glycerol conversion to 1,3-propanediol by Clostridium pasteurianum: Cloning and expression of the gene encoding 1,3-propanediol dehydrogenase. FEMS Microbiol. Lett. 154:337–345.

Lugar, R. G., and R. J. Woolsey. 1999. The new petroleum. Foreign Affairs 78:88–102.

Lund, B. M., T. F. Brocklehurst, and G. M. Wyatt. 1981. Characterization of strains of Clostridium puniceum sp. nov., a pink-pigmented, pectolytic bacterium. J. Gen. Microbiol. 122:17–26.

Luttik, M. A. H., R. Van Spanning, D. Schipper, J. P. Van Dijken, and J. T. Pronk. 1997. The low biomass yields of the acetic acid bacterium Acetobacter pasteurianus are due to a low stoichiometry of respiration-coupled proton translocation. Appl. Envron. Microbiol. 63:3345–3351.

Lynd, L. R. 1989. Ethanol production from lignocellulosic substrates using thermophilic bacteria. Adv. Biochem. Eng. Biotechnol. 38:1–52.

Lynd, L. R., J. H. Cushman, R. J. Nichols, and C. E. Wyman. 1991. Fuel ethanol from cellulosic biomass. Science 251:1318–1323.

Lynd, L. R., S. Baskaran, and S. Casten. 2001. Salt accumulation resulting from base added for pH control, and not ethanol, limits growth of Thermoanaerobacterium thermosaccharolyticum HG-8 at elevated feed xylose concentrations in continuous culture. Biotechnol. Progr. 17:118–125.

Lyon, W. J., and B. A. Glatz. 1995. Propionibacteria. *In:* Y. H. Hui and G. G. Khachatourians (Eds.) Food Biotechnology, Microorganisms. VCH Publishers. New York, NY. 703–719.

Macis, L., R. Daniel, and G. Gottschalk. 1998. Properties and sequence of the coenzyme B12-dependent glycerol dehydratase of Clostridium pasteurianum. FEMS Microbiol. Lett. 164:21–28.

Mackenzie, K. F., C. K. Eddy, and L. O. Ingram. 1989. Modulation of alcohol dehydrogenase isoenzyme levels in Zymomonas mobilis by iron and zinc. J. Bacteriol. 171:1063–1067.

Maddox, I. S. 1980. Production of n-butanol from whey filtrate using Clostridium acetobutylicum NCIB 2951. Biotechnol. Lett. 2:493–498.

Maddox, I. S. 1996. Microbial production of 2, 3-butanediol. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology, 2nd ed. VCH. New York, NY. 6:270–291.

Maddox, I. S., E. Steiner, S. Hirsch, S. Wessner, N. A. Gutierrez, J. R. Gapes, and K. C. Schuster. 2000. The cause of "acid crash" and "acidogenic fermentations" during the batch acetone-butanol-ethanol (ABE-) fermentation process. J. Molec. Microbiol. Biotechnol. 2:95–100.

Madison, L. L., and G. W. Huisman. 1999. Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic. Microbiol. Molec. Biol. Rev. 63:21–53.

Mai, V., W. W. Lorenz, and J. Wiegel. 1997. Transformation of Thermoanaerobacterium sp. strain JW/SL-YS485 with plasmid pIKM1 conferring kanamycin resistance. FEMS Microbiol. Lett. 148:163–167.

Mai, V., and J. Wiegel. 2000. Advances in development of a genetic system for Thermoanaerobacterium spp.: Expression of genes encoding hydrolytic enzymes, development of a second shuttle vector, and integration of genes into chromosome. Appl. Environ. Microbiol. 66:4817–4821.

Malinowski, J. J. 1999. Evaluation of liquid extraction potentials for downstream separation of 1,3-propanediol. Biotechnol. Tech. 13:127–130.

Malinowski, J. J. 2000. Reactive extraction for downstream separation of 1,3-propanediol. Biotechnol. Prog. 16:76–79.

Marchal, R., M. Ropars, J. Pourquie, F. Fayolle, and J. P. Vandecasteele. 1992. Large-scale enzymatic hydrolysis of agricultural lignocellulosic biomass. Part II: Conversion into acetone-butanol. Biores. Technol. 42:205–217.

Martin, M. E., M. Wayman, and G. Graf. 1961. Fermentation of sulfite waste liquor to produce organic acids. Can. J. Microbiol. 7:341–346.

Martin, M. C., J. C. Alonso, J. E. Suarez, and M. A. Alvarez. 2000. Generation of food-grade recombinant lactic acid bacterium strains by site-specific recombination. Appl. Environ. Microbiol. 66:2599–2604.

Mat-Jan, F., Alam, K. Y., and D. P. Clark. 1989. Mutants of Escherichia coli deficient in the fermentative lactate dehydrogenase. J. Bacteriol. 171:342–348.

Matsusaki, H., H. Abe, K. Taguchi, T. Fukui, and Y. Doi. 2000. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene phaC1 from Pseudomonas sp. 61-3. Appl. Microbiol. Biotechnol. 53:401–409.

Matsushita, K., H. Toyama, and O. Adachi. 1994. Respiratory chains and bioenergetics of acetic acid bacteria. Adv. Microb. Physiol. 36:247–301.

Mattsson, D. M., and P. Rogers. 1994. Analysis of Tn916-induced mutants of Clostridium acetobutylicum altered in solventogenesis and sporulation. J. Ind. Microbiol. 13:258–268.

May, O. E., H. T. Herrick, A. J. Moyer, and P. A. Wells. 1934. Gluconic acid-production by submerged mold growths under increased air pressure. Ind. Eng. Chem. 26:575–578.

McCoy, E., E. B. Fred, W. H. Peterson, and E. G. Hastings. 1926. A cultural study of the acetone butyl alcohol organism. J. Infect. Dis. 39:457–483.

McCoy, E., and L. S. McClung. 1935. Studies on anaerobic bacteria. J. Infect. Dis. 56:333–346.

McCoy, E., and E. B. Fred. 1941. The stability of a culture for industrial fermentation. J. Bacteriol. 41:90–91.

McCoy, M. 1998. Biomass ethanol inches forward. Chem. Eng. News 76:29–32.

McCutchan, W. N., and R. J. Hickey. 1954. The butanol-acetone fermentations. *In:* L. A. Underkofler and R. J. Hickey (Eds.) Industrial Fermentations. Chemical Publishing. New York, NY. 1:347–388.

McGee, H. 1984. On Food and Cooking: The Science and Lore of the Kitchen. Scribner's. New York, NY. 36.

McLellan, P. J., A. J. Daugulis, and J. Li. 1999. The incidence of oscillatory behavior in the continuous fermentation of Zymomonas mobilis. Biotechnol. Progr. 15:667–680.

McNeil, B., and B. Kristiansen. 1986. The acetone butanol fermentation. Adv. Appl. Microbiol. 31:61–92.

Meinecke, B., H. Bahl, and G. Gottschalk. 1984. Selection of an asporogenous strain of Clostridium acetobutylicum in continuous culture under phosphate limitation. Appl. Environ. Microbiol. 48:1064–1065.

Menzel, K., A. P. Zeng, and W. D. Deckwer. 1997. High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by Klebsiella pneumoniae. Enz. Microbiol. Technol. 20:82–86.

Mercenier, A., P. H. Pouwels, and B. M. Chassy. 1994. Genetic engineering of lactobacilli, leuconostocs, and Streptococcus thermophilus. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 252–293.

Mercier, P., L. Yerushalmi, D. Rouleau, and D. Dochain. 1992. Kinetics of lactic acid fermentation on glucose and corn by Lactobacillus amylophilus. J. Chem. Tech. Biotechnol. 55:111–121.

Mermelstein, L. D., N. E. Welker, G. N. Bennett, and E. T. Papoutsakis. 1992. Expression of cloned homologous fermentative genes in Clostridium acetobutylicum ATCC 824. Biotechnology 10:190–195.

Mermelstein, L. D., and E. T. Papoutsakis. 1993a. Evaluation of macrolide and lincosamide antibiotics for plasmid maintenance in low pH Clostridium acetobutylicum ATCC 824 fermentations. FEMS Microbiol. Lett. 113:71–76.

Mermelstein, L. D., and E. T. Papoutsakis. 1993b. In vivo methylation in Escherichia coli by the Bacillus subtilis phage 3T I methyltransferase to protect plasmids from restriction upon transformation of Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 59:1077–1081.

Mermelstein, L. D., E. T. Papoutsakis, D. J. Petersen, and G. N. Bennett. 1993c. Metabolic engineering of Clostridium acetobutylicum ATCC 824 for increased solvent production by enhancement of acetone formation enzyme activities using a synthetic acetone operon. Biotechnol. Bioengin. 42:1053–1060.

Mermelstein, L. D., N. E. Welker, D. J. Petersen, G. N. Bennett, and E. T. Papoutsakis. 1994. Genetic and metabolic engineering of Clostridum acetobutylicom. ATCC 824. Ann. NY Acad. Sci. 721:54–68.

Mesa, M. M., I. Caro, and D. Cantero. 1996. Viability reduction of Acetobacter aceti due to the absence of oxygen in submerged culture. Biotech. Progress 12:709–712.

Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990a. Control of the selectivity of butyric acid production and improvement of fermentation performance with Clostridium tyrobutyricum. Appl. Microbiol. Biotechnol. 32:387–392.

Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990b. Butyrate production in continuous culture of Clostridum tyrobutyricum. Appl. Microbiol. Biotechnol. 33:127–131.

Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990c. Butyric fermentation: Metabolic behavior and production performance of Clostridium tryobutyricum in a continuous culture with cell recycle. Appl. Microbiol. Biotechnol. 34:172–177.

Mickelson, M. N., and C. H. Werkman. 1940. Formation of trimethyleneglycol from glycerol by Aerobacter. Enzymologia 8:252–256.

Millard, C. S., Y. P. Chao, J. C. Liao, and M. I. Donnelly. 1996. Enhanced production of succinic acid by overexpression of phosphoenolpyruvate caboxylase in Escherichia coli. Appl. Env. Microbiol. 62:1808–1810.

Milson, P. E., and J. L. Meers. 1985. Gluconic and itaconic acids. *In:* M. Moo-Young (Ed.) Comprehensive Biotechnology. Pergamon Press. Oxford, 3:681–700.

Minton, N. P., and J. D. Oultram. 1988. Host:vector systems for gene cloning in Clostridium. Microbiol. Sci. 5:310–315.

Minton, N. P., J. K. Brehm, T. J. Swinfield, S. W. Whelan, M. L. Mauchline, N. Bodsworth, and J. D. Oultrum. 1993. Clostridial cloning vectors. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 120–150.

Mishra, P., and S. Kaur. 1991. Lipids as modulators of ethanol tolerance in yeast. Appl. Microbiol. Biotechnol. 34:697–702.

Misoph, M., and H. L. Drake. 1996. Effect of CO_2 on the fermentation capacities of the acetogen Peptostreptococcus productus U-1. J. Bacteriol. 178:3140–3145.

Mitchell, W. J. 1998. Physiology of carbohydrate to solvent conversion by clostridia. Adv. Microb. Physiol. 39:31–130.

Mobley, D. P. (Ed.). 1994. Plastics from Microbes. Hanser. Munich, Germany.

Möller, B., R. Ossmer, B. H. Howard, G. Gottschalk, and H. Hippe. 1984. Sporomusa, a new genus of Gram negative anaerobic bacteria including Sporomusa sphaeroides spec. nov. and Sporomusa ovata spec. nov. Arch. Microbiol. 139:388–396.

Molliard, M. 1922. Survene nouvelle fermentation acide produite par le Sterigmatcystis nigra. C.R. Hebd. Seances Acad. Sci. 174:881–883.

Monot, F., J.-M. Engasser, and H. Petitdemange. 1984. Influence of pH and undissociated butyric acid in the production of acetone and butanol in batch cultures of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 19:422–426.

Montoya, D., C. Arevalo, S. Gonzales, F. Aristizabal, and W. H. Schwarz. 2001. New solvent-producing Clostridium sp. strains, hydrolyzing a wide range of polysaccharides, are closely related to Clostridium butyricum. J. Ind. Microbiol. Biotechnol. 27:329–335.

Moore, S. K. 1999. DuPont, Genencor close in on biological propanediol route. Chem. Week 161:17.

Moreira, A. R., D. C. Ulmer, and J. C. Linden. 1981. Butanol toxicity in butylic fermentation. Biotechnol. Bioengin. Symp. 11:567–579.

Mori, A., and G. Tervi. 1972. Kinetic studies on submerged acetic acid fermentation. III: Efficiency of energy metabolism in acetic acid fermentation using Acetobacter rancens. J. Ferment. Technol. 50:510–517.

Mori, A. 1993. Vinegar production in a fluidized-bed reactor with immobilized bacteria. *In:* A. Tanaka, T. Tosa, and T. Kobayashi (Eds.) Industrial Application of Immobilized Biocatalysts. Dekker. New York, NY. 291–313.

Morris, J. G. 1993. History and future potential of the clostridia in biotechnology. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 1–23.

Moyer, A. J., P. A. Wells, J. J. Stubbs, H. T. Herrick, and O. E. May. 1937. Gluconic acid production-development of innoculum and composition of fermentation solution for gluconic acid production by submerged mold growths under increased air pressure. Ind. Eng. Chem. 29:777–781.

Muller, H. M., and D. Seebach. 1993. Poly(hydroxyfettsaureester) eine funfte Klasse von physiologisch bedeutsamen organischen Biopolymeren? Agnew. Chem. 105:483–459.

Müller, V., and G. Gottschalk. 1994. The sodium ion cycle in acetogenic and methanogenic bacteria: Generation and utilization of a primary electrochemical sodium ion gradient. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 125–156.

Muraoka, H., Y. Watabe, and N. Ogasawara. 1982. Effect of oxygen deficiency on acid production and morphology of bacterial cells in submerged acetic fermentation by Acetobacter aceti. J. Ferment. Technol. 60:171–180.

Muriana, P. M., and T. R. Klaenhammer. 1991. Cloning, phenotypic expression, and DNA sequence of the gene for lactacin F, an antimicrobial peptide produced by Lactobacillus spp. J. Bacteriol. 173:1779–1788.

Nagarajan, V., and C. E. Nakamura. 1998. Production of 1,3-propanediol from glycerol by recombinant bacteria expressing recombinant diol dehydratase. US Patent 5821092.

Nair, R. V., G. N. Bennett, and E. T. Papoutsakis. 1994. Molecular characterization of an alcohol/aldehyde dehydrogenase gene of Clostridium acetobutylicum ATCC 824. J. Bacteriol. 176:871–885.

Nakamura, C. E., A. A. Gatenby, A. K. Hsu, R. D. LaReau, S. L. Haynie, R. Diaz-Torres, D. E. Trimbur, G. M. Whited, A. Nagarajan, M. S. Payne, S. K. Picataggio, and R. V. Nair. 2000. Method for the production of 1,3-propanediol by recombinant microorganisms. US Patent 6013494.

Nakano, M. M., Y. P. Dailly, P. Zuber, and D. P. Clark. 1997. Characterization of anaerobic fermentative growth of Bacillus subtilis: Identification of fermentation end products and genes required for growth. J. Bacteriol. 179:6749–6755.

Nakas, J. P., M. Schaedle, C. M. Parkinson, C. E. Coonley, and S. W. Tanenbaum. 1983. System development for linked-fermentation production of solvents from algal biomass. Appl. Environ. Microbiol. 46:1017–1023.

Nakashimada, Y., K. Kanai, and N. Nishio. 1998. Optimization of dilution rate, pH and oxygen supply on optical purity of 2,3-butanediol produced by Paenibacillus polymyxa in chemostat culture. Biotechnol. Lett. 20:113–1138.

Nakashimada, Y., B. Marwoto, T. Kashiwamura, T. Kakizono, and N. Nishio. 2000. Enhanced 2,3-butanediol production by addition of acetic acid in Paenibacillus polymyxa. J. Biosci. Bioeng. 90:661–664.

Nakotte, S., S. Schaffer, M. Bohringer, and P. Dü. 1998. Electroporation of, plasmid isolation from and plasmid conservation in Clostridium acetobutylicum DSM 792. Appl. Microbiol. Biotechnol. 50:564–567.

Narayan, R. 1994. Impact of governmental policies, regulations, and standards activities on an emerging biodegradable plastics industry. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 261–272.

Nativel, F., J. Pourquie, D. Ballerini, J. P. Vandecasteele, and P. Renault. 1992. The biotechnology facilities at Soustons for biomass conversion. Int. J. Solar Energy 11:219–229.

Nawrath, C., Y. Poirier, and C. Somerville. 1994. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of Arabidopsis thaliana results in high levels of polymer accumulation. Proc. Natl. Acad. Sci. USA 91:12760–12764.

Neale, A. D., R. K. Scopes, J. M. Kelly, and R. E. H. Wettenhall. 1986. The two alcohol dehyrogenases of Zymomonas mobilis: Purification by differential dye ligand chromatography, molecular characterisation and physiological roles. Eur. J. Biochem. 154:119–124.

Neale, A. D., R. K. Scopes, R. E. H. Wettenhall, and N. J. Hoogenraad. 1987. Nucleotide sequence of the pyruvate decarboxylase gene from Zymomonas mobilis. Nucleic Acids Res. 15:1753–1761.

Neale, A. D., R. K. Scopes, and J. M. Kelly. 1988. Alcohol production from glucose and xylose using Escherichia coli containing Zymomonas mobilis genes. Appl. Microbiol. Biotechnol. 29:162–167.

Nghiem, N. P., B. H. Davison, J. E. Thompson, B. E. Suttle, and G. R. Richardson. 1996. The effect of biotin on the production of succinic acid by Anaerobiospirillum succiniciproducens. Appl. Biochem. Biotechnol. 57/58:633–638.

Nghiem, N. P., B. H. Davison, B. E. Suttle, and G. R. Richardson. 1997. Production of succinic acid by Anaerobiospirillum succiniciproducens. Appl. Biochem. Biotechnol. 63–65:565–576.

Nghiem, N. P., M. I. Donnelly, and C. S. Millard. 1999. Method for the production of dicarboxylic acids. US Patent. 5,869,301.

Nickol, G. B. 1979. Vinegar. *In:* H. J. Peppler and D. Pearlman (Eds.) Microbial Technology. Academic Press. New York, NY. 2:155–172.

Nidetzky, B., M. Furlinger, and D. Gollhofer. 1997. Improved operational stability of cell-free glucose-fructose oxidoreductase from Zymomonas mobilis for the efficient synthesis of gluconic acid and sorbitol, in acoutin ultra filtration membrane reactor. Biotechnol. Bioeng. 53:623–629.

Nilegaonkar, S., S. B. Bhosale, D. C. Kshirsagar, and A. H. Kapadi. 1992. Production of 2,3-butanediol from glucose by Bacillus licheniformis. World J. Microbiol. Biotechnol. 8:378–381.

Nimcevic, D., M. Schuster, and J. R. Gapes. 1998. Solvent production by Clostridium beijerinckii NRRL B592 growing on different potato media. Appl. Microbiol. Biotechnol. 50:426–428.

Nimcevic, D., and J. R. Gapes. 2000. The acetone-butanol fermentation in pilot plant and pre-industrial scale. J. Molec. Microbiol. Biotechnol. 2:15–20.

Nishikawa, M., R. M. R. Brancon, K. L. Pinder, and G. A. Strasdine. 1970. Fermentation of spent sulfite liquor to produce acetic acid, propionic acid and vitamin B12. Pulp and Paper Magazine of Canada 71(3):T59–T64.

Nishioka, M., T. Tuzuki, Y. Wanajyo, H. Oonami, and T. Horiuchi. 1994. Biodegradation of Bionolle. Stud. Polym. Sci. 12:584–590.

Nölling, J., G. Breton, M. V. Omelchenko, K. S. Markarova, Q. Zeng, R. Gibson, H. M. Lee, J. DuBois, D. Qiu, J. Hitti, GTC Sequencing Center Production, Finishing, and Bioinformatics Teams, Y. I. Wolf, R. L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M. J. Daly, G. N. Bennett, E. V. Koonin, and D. R. Smith. 2001. Genome sequence and comparitive analysis of the solvent-producing bacterium Clostridium acetobutylicum. J. Bacteriol. 183:4823–4838.

Noon, R. 1982. Power-grade butanol. Chemtech 12:681–683.

Northrop, J. H., L. H. Ashe, and J. K. Senior. 1919. Biochemistry of Bacillus acetoethylicum with reference to the formation of acetone. J. Biol. Chem. 39:1–21.

Noury, and N. V. van der Lande. 1962. Process for preparation of gluconic acid monohydrate. British Patent 902609. Chem. Abstr. 57:13020g.

Oben, J. M., M. J. Amagro, and A. Manjon. 1996. Continuous retention of native NADP(H) in an enzyme membrane reactor for gluconate and glutamate production. J. Biotechnol. 50:27–36.

Ogata, S., and M. Hongo. 1979. Bacteriophages of the genus Clostridium. Adv. Appl. Microbiol. 25:241–273.

Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991a. Genetic improvement of Escherichia coli for ethanol production: Chromosomal integration of Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. 57:893–900.

Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991b. Metabolic engineering of Klebsilla oxytoca M5A1 for ethanol production from xylose and glucose. Appl. Environ. Microbiol. 57:2810–2815.

Oiwa, H., M. Naganuma, and S.-I. Ohnuma. 1987. Acetone-butanol production from dahlia inulin by Clostridium pasteurianum var. I-53. Agric. Biol. Chem. 51:2819–2820.

Okumura, H., H. Tagami, M. Fukaya, H. Masai, Y. Kawamura, S. Horinouchi, and T. Beppu. 1988. Cloning of the β-isopropylmalate dehydrogenase gene from Acetobacter aceti and its use for construction of a new host vector system for Acetobacter. Agric. Biol. Chem. 52:3125–3129.

Olijve, W., and J. J. Kok. 1979a. An analysis of growth of Gluconobacter oxydans in chemostat cultures. Arch. Microbiol. 121:291–297.

Olijve, W., and J. J. Kok. 1979b. Analysis of growth of Gluconobacter oxydans in glucose containing medium. Arch. Microbiol. 121:283–290.

O'Mullan, P. J., S. E. Buchholz, T. Chase Jr., and D. E. Eveleigh. 1995. Roles of alcohol dehydrogenases of Zymomonas mobilis (ZADH): Characterization of a ZADH-2-negative mutant. Appl. Microbiol. Biotechnol. 43:675–678.

O'Neill, H., S. G. Mayhew, and G. Butler. 1998. Cloning and analysis of the genes for a novel electron-transferring flavoprotein from Megasphaera elsdenii. J. Biol. Chem. 273:21015–21024.

Osuga, J., A. Mori, and J. Kato. 1984. Acetic acid production by immobilized Acetobacter aceti cells entrapped in a kappa-carrageenan gel. J. Ferment. Technol. 62:139–149.

Osuna, J., X. Soberon, and E. Morett. 1997. A proposed architecture for the central domain of the bacterial enhancer-binding proteins based on secondary structure prediction and fold recognition. Protein Sci. 6:543–555.

Oude Elferink, S. J. W. H., J. Krooneman, J. C. Gorrschal, S. F. Spoelstra, F. Faber, and F. Driehuis. 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by Lactobacillus buchneri. Appl. Environ. Microbiol. 67:125–132.

Ouellette, J. 2000. PO and PG markets face tight margins. Chem. Mark. Rep. 257:5 and 14.

Oultram, J. D., and M. Young. 1985. Conjugal transfer of plasmid pAMβ1 from Streptococcus lactis and Bacillus subtilis to Clostridium acetobutylicum. FEMS Microbiol. Lett. 27:129–134.

Oultram, J. D., A. Davies, and M. Young. 1987. Conjugal transfer of a small plasmid from Bacillus subtilis to Clostridium acetobutylicum by cointegrate formation with plasmid pAMβ1. FEMS Microbiol. Lett. 42:113–119.

Oultram, J. D., M. Loughlin, T.-J. Swinfield, J. K. Brehm, D. E. Thompson, and N. P. Minton. 1988. Introduction of plasmids into whole cells of Clostridium acetobutylicum by electroporation. FEMS Microbiol. Lett. 56:83–88.

Oultram, J. D., I. D. Burr, M. J. Elmore, and N. P. Minton. 1993. Cloning and sequence analysis of the genes encoding phosphotransbutyrylase and butyrate kinase from Clostridium acetobutylicum NCIMB 8052. Gene 131:107–112.

Owen, K., and T. Coley. 1990. Oxygenated blend components for gasoline. Automotive Fuels Handbook. Society of Automotive Engineers. Warrendale, PA. 221–259.

Page, W. J., and A. Cornish. 1993. Growth of Azotobacter vinelandii UWD in fish peptone medium and simplified extraction of poly-beta-hydroxybutyrate. Appl. Environ. Microbiol. 59:4236–4244.

Paik, H. D., and B. A. Glatz. 1994. Propionic acid production by immobilized cells of a propionate-tolerant strain of Propionibacterium acidipropionici. Appl. Microbiol. Biotechnol. 42:22–27.

Palosaari, N. R., and P. Rogers. 1988. Purification and properties of the inducible coenzyme A-linked butyralde-

hyde dehydrogenase from Clostridium acetobutylicum. J. Bacteriol. 170:2971–2976.

Pan, J. G., S. A. Soo, C. K. Park, P. Kim, D. E. Chang, and J. E. Kim. 1999. A pta ldhA double mutant Escherichia coli SS373 and the method of producing succinic acid therefrom. PCT Application. WO 99/06532.

Papanikolaou, S., P. Ruiz-Sanchez, B. Pariset, F. Blanchard, and M. Fick. 2000. High production of 1,3-propanediol from industrial glycerol by a newly isolated Clostridium butyricum strain. J. Biotechnol. 77:191–208.

Papoutsakis, E. T., and C. L. Meyer. 1985. Equations and calculations of product yields and preferred pathways for butanediol and mixed-acid fermentations. Biotechol. Bioengin. 27:50–66.

Papoutsakis, E. T., and G. N. Bennett. 1993. Cloning, structure, and expression of acid and solvent pathway genes of Clostridium acetobutylicum. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 157–200.

Papoutsakis, E. T., and G. N. Bennett. 1999. Molecular regulation and metabolic engineering of solvent production by Clostridium acetobutylicum. *In:* E. T. Papoutsakis and S. Y. Lee (Eds.) Bioprocess Technology. Marcel Dekker. New York, NY. 24:253–279.

Paquet, V., C. Croux, G. Goma, and P. Soucaille. 1991. Purification and characterization of the extracellular α-amylase from Clostridium acetobutylicum. Appl. Environ. Microbiol. 57:212–218.

Parekh, S. R., and M. Cheryan. 1991. Production of acetate by mutant strains of Clostridium thermoaceticum. Appl. Microbiol. Biotechnol. 36:384–387.

Parekh, S. R., and M. Cheryan. 1994a. Continuous production of acetate by Clostridium thermoaceticum in a cell-recycle membrane bioreactor. Enz. Microb. Technol. 16:104–109.

Parekh, S. R., and M. Cheryan. 1994b. High concentrations of acetate with a mutant strain of Clostridium thermoaceticum. Biotechnol. Lett. 16:139–142.

Park, Y. S., H. Ohtake, M. Fukaya, H. Okumura, Y. Kawamura, and K. Toda. 1989. Acetic acid production using a fermenter equipped with a hollow fiber filter module. Biotechnol. Bioeng. 33:918–923.

Park, Y. S., H. Ohtake, M. Fukaya, H. Kawamura, and K. Toda. 1991. Production of high concentration of acetic acid by Acetobacter aceti using a repeated fed-batch culture with cell recycling. Appl. Microbiol. Biotechnol. 35:149–153.

Park, D. H., and G. K. Zeikus. 1999. Utilization of electrically reduced neutral red by Actinobacillus succinogenes: Physiological function of netural red in membrane-driven fumarate reduction and energy conservation. J. Bacteriol. 181:2403–2410.

Parrer, G., G. Schroll, J. R. Gapes, W. Lubitz, and K. C. Schuster. 2000. Conversion of solvent evaporation residues from the AB-(acetone-butanol) bioprocess into bacterial cells accumulating thermoplastic polyesters. J. Molec. Microbiol. Biotechnol. 2:81–86.

Pasteur, L. 1861a. Animalcules infusoires vivant sans gaz oxygène libre et déterminant des fermentations. Comptes Rendus 52:344–347.

Pasteur, L. 1861b. Mémoire sur les corpuscles organisés qui éxistent dans l'atmosphere: Esamen de la doctrine des générations spontanées. Annales Sci. Natur., 4th series 16:5–98.

Pasteur, L. 1868. Etudes sur le vinaigre, sa fabrication se maladres, moyens de les préveair nouvelles observations sur la conservation des vinc par la châleur. Gauthier-Villars. Paris, FR.

Pasteur, L. 1879. Studies on Fermentation. London.

Pedroni, P., A. Volpe, G. Galli, G. M. Mura, C. Pratesi, and G. Grandi. 1995. Characterization of the locus encoding the [Ni-Fe] sulfhydrogenase from the archaeon Pyrococcus furiosus: Evidence for a relationship to bacterial sulfite reductases. Microbiology 141:449–458.

Peguin, S., G. Goma, P. Delorme, and P. Soucaille. 1994. Metabolic flexibility of Clostridium acetobutylicum in response to methyl viologen addition. Appl. Microbiol. Biotechnol. 42:611–616.

Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey. 1987. Biosynthetic thiolase from Zoogloea ramigera. III: Isolation and characterization of the structural gene. J. Biol. Chem. 262:97–102.

Peoples, O. P., and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16: Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. 264:15293–15297.

Peretz, M., O. Bogin, S. Tel-Or, A. Cohen, G. Li, J.-S. Chen, and Y. Burstein. 1997. Molecular cloning, nucleotide sequencing, and expression of genes encoding alcohol dehydrogenase from the thermophile Thermoanaerobacter brockii and the mesophile Clostridium beijerinckii. Anaerobe 3:259–270.

Perozich, J., H. Nicholas, B. C. Wang, R. Lindahl, and J. Hempel. 1999. Relationships within the adehyde dehydrogenase extended family. Protein Sci. 8:137–146.

Petersen, D. J., R. W. Welch, F. B. Rudolph, and G. N. Bennett. 1991. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from Clostridium acetobutylicum ATCC 824. J. Bacteriol. 173:1831–1834.

Petersen, D. J., J. W. Cary, J. Vanderleyden, and G. N. Bennett. 1993. Sequence and arrangement of genes encoding enzymes of the acetone-production pathway of Clostridium acetobutylicum ATCC 824. Gene 123:93–97.

Petitdemange, H., C. Cherrier, G. Raval, and R. Gay. 1976. Regulation of NADH and NADPH ferredoxin oxidoreductases in clostridia of the butyric group. Biochem. Biophys. Acta 421:334–347.

Petitdemange, E., C. Durr, S. Abbad-Andalousi, and G. Raval. 1995. Fermentation of raw glycerol to 1,3-propanediol by new strains of Clostridium butyricum. J. Indust. Microbiol. 15:498–502.

Pflugmacher, U., and G. Gottschalk. 1994. Development of an immobilized cell reactor for the production of 1,3-propanediol by Citrobacter freundii. Appl. Microbiol. Biotechnol. 41:313–316.

Philips, J. R., E. C. Clausen, and J. L. Gaddy. 1994. Synthesis gas as substrate for the biological production of fuels and chemicals. Appl. Biochem. Biotechnol. 45–46:145–157.

Phillips, M. A. 1963. Catalytic hydrogenation of glucose to sorbitol using a highly active catalyst. Br. Chem. Eng. 8(11):767–769.

Pierce, S. M., and M. Wayman. 1983. Diesel fuel by fermentation of wastes. US Patent. 4368056.

Piveteau, P. 1999. Metabolism of lactate and sugars by dairy propionibacteria: A review. Lait 79:23–41.

Planas, J., P. Radström, F. Tjerneld, and B. Hahn-Hägerdal. 1996. Enhanced production of lactic acid through the use of a novel aqueous two-phase system as an extractive fermentation system. Appl. Microbiol. Biotechnol. 45:737–743.

Playne, M. J. 1985. Propionic acid and butyric acid. *In:* Moo-Young (Ed.) Comprehensive Biotechnology. Pergamon. New York, NY. 3:731–755.

Podkovyrov, S. M., and J. G. Zeikus. 1993. Purification and characterization of phosphoenolpyruvate carboxykinase, a catabolic CO_2-fixing enzyme, from Anaerobiospirillum succiniciproducens. J. Gen. Microbiol. 139: 223–228.

Poehland, H. D., V. Schierz, and R. Schumann. 1993. Optimization of gluconic acid synthesis by removing limitations and inhibitions. Acta Biotechnol. 13:257–268.

Poirier, Y., D. E. Dennis, K. Klomparens, and C. Somerville. 1992. Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science 256:520–522.

Poirier, Y., C. Nawrath, and C. Somerville. 1995. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Bio/technology 13:142–150.

Posno, M., P. T. H. M. Heuvelmans, M. J. F. van Giezen, B. C. Lokman, R. J. Leer, and P. H. Pouwels. 1991. Complimentation of the inability of Lactobacillus strains to utilize D-xylose with D-xylose catabolism-encoding genes of Lactobacillus pentosus. Appl. Environ. Microbiol. 57:2764–2766.

Potera, C. 1997. Genencor & DuPont create "green" polyester. Genet. Eng. News 17:17.

Prescott, S. C., and C. G. Dunn. 1949. The acetone-butanol fermentation. Industrial Microbiology, 2nd ed. McGraw-Hill. New York, NY. 312–351.

Prescott, S. C., and C. G. Dunn. 1949. The acetone-butanol fermentation. Industrial Microbiology, 2nd ed. McGraw-Hill. New York, NY. 477–486.

Prescott, S. C., and C. G. Dunn. 1959a. The acetone-butanol fermentation. *In:* Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 250–284.

Prescott, S. C., and C. G. Dunn. 1959b. The acetone-ethanol fermentation. *In:* Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 295–298.

Prescott, S. C., and C. G. Dunn. 1959c. The butanol-isopropanol fermentation. Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 285–294.

Pries, A., A. Steinbüchel, and H. G. Schlegel. 1990. Lactose-utilizing and galactose-utilizing strains of poly(hydroxyalkanoic acid)-accumulating Alcaligenes eutrophus and Pseudomonas saccharophila obtained by recombinant DNA technology. Appl. Microbiol. Biotechnol. 33:410–417.

Prieto, M. A., B. Buhler, K. Jung, B. Witholt, and B. Kessler. 1999a. PhaE, a polyhydroxyalkanoate-granule-associated protein of Pseudomonas oleovorans GPo1 involved in the regulatory expression system for pha genes. J. Bacteriol. 181:858–868.

Prieto, M. A., M. B. Kellerhals, G. B. Bozzato, D. Radnovic, B. Witholt, and B. Kessler. 1999b. Engineering of stable recombinant bacteria for production of chiral medium-chain-length poly-3-hydroxyalkanoates. Appl. Environ. Microbiol. 65:3265–3271.

Pronk, J. T., P. R. Levering, W. Olijve, and J. P. Van Dijken. 1989. The role of NADP-dependent and quino protein glucose dehydrogenase in gluconic acid production by Gluconobacter oxydans. Enz. Microb. Technol. 11:160–164.

Quesada-Chanto, A., A. S. Afschar, and F. Wagner. 1994a. Microbial production of propionic acid and vitamin B-12 using molasses or sugar. Appl. Microbiol. Biotechnol. 41:378–383.

Quesada-Chanto, A., A. S. Afschar, and F. Wagner. 1994b. Optimization of a Propionibacterium acidipropionici continuous culture utilization of sucrose. Appl. Microbiol. Biotechnol. 42:16–21.

Quesada-Chanto, A., J. P. C. L. da Costa, M. M. Silveira, A. G. Schroeder, A. C. Schmidt-Meyer, and R. Jona. 1998a. Influence of different vitamin-nitrogen sources on cell growth and propionic acid production from sucrose by Propionibacterium shermanii. Acta Biotechnologia 18:267–274.

Quesada-Chanto, A., M. M. Silveira, A. C. Schmidt-Meyer, A. G. Schroeder, J. P. C. L. da Costa, J. Lopez, M. F. Carvalho-Jonas, M. J. Artolozaga, and R. Jona. 1998b. Effect of oxygen supply on pattern of growth and corrinoid and organic acid production of Propionibacterium shermanii. Appl. Microbiol. Biotechnol. 49:732–736.

Qureshi, N., and H. P. Blaschek. 2001a. ABE production from corn: A recent economic evaluation. J. Indust. Microbiol. Biotechnol. 27:292–297.

Qureshi, N., A. Lolas, and H. P. Blaschek. 2001b. Soy molasses as fermentation substrate for production of butanol using Clostridium beijerinckii BA101. J. Indust. Microbiol. Biotechnol. 26:290–295.

Ragsdale, S. W. 1994. CO dehydrogenase and the central role of this enzyme in fixation of carbon-dioxide by anaerobic bacteria. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 88–126.

Ragsdale, S. W., and C. G. Riordan. 1996. The role of nickel in acetyl-CoA synthesis by the bifunctional enzyme CO dehydrogenase/acetyl-CoA synthase: Enzymology and model chemistry. J. Biol. Inorg. Chem. 1:489–493.

Rakhley, G., Z. H. Zhou, M. W. W. Adams, and K. L. Kovacs. 1999. Biochemical and molecular characterization of the [NiFe] hydrogenase from the hyperthermophilic archaeon, Thermococcus litoralis. Eur. J. Biochem. 266:1158–1165.

Ramachandran, K. B., and G. Goma. 1988. 2,3-Butanediol production from glucose by Klebsiella pneumoniae in a cell recycle system. J. Biotechnol. 9:39–46.

Rani, K. S., and G. Seenayya. 1999. High ethanol tolerance of new isolates of Clostridium thermocellum strains SS21 and SS22. World J. Microbiol. Biotechnol. 15:173–178.

Rao, G., and R. Mutharasan. 1986. Alcohol production by Clostridium acetobutylicum induced by methyl viologen. Biotechnol. Lett. 8:893–896.

Rao, G., and R. Mutharasan. 1987. Altered electron flow in continuous cultures of Clostridium acetobutylicum induced by viologen dyes. Appl. Environ. Microbiol. 53:1232–1235.

Rao, G., and R. Mutharasan. 1988. Altered electron flow in a reduced environment in Clostridium acetobutylicum. Biotechnol. Lett. 10:129–132.

Raspoet, D., B. Pot, D. De Deyn, P. De Vos, K. Kersters, and J. De Ley. 1991. Differentiation between 2,3-butanediol producing Bacillus licheniformis and B. polymyxa strains by fermentation product profiles and whole-cell protein electrophoretic patterns. Syst. Appl. Microbiol. 14:1–7.

Ravagnani, A., K. C. B. Jennert, E. Steiner, R. Grunberg, J. R. Jefferies, S. R. Wilkinson, D. I. Young, E. C. Tidswell, D. P. Brown, P. Youngman, J. G. Morris, and M. Young. 2000. Spo0A directly controls the switch from

acid to solvent production in solvent-forming clostridia. Molec. Microbiol. 37:1172–1185.

Rayner, A. 1926. The occurrence, properties, and uses of trimethylene glycol, and the fermentation of glycerin lyes. J. Soc. Chem. Indust. 45:265T–266T.

Reed, G. 1982. Production of fermentation alcohol as a fuel source. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 835–859.

Rehberger, J. L., and B. A. Glatz. 1998. Response of cultures of Propionibacterium to acid and low pH tolerance and inhibition. J. Food Protect. 61:211–216.

Rehm, B. H. A., N. Kruger, and A. Steinbüchel. 1998. A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. J. Biol. Chem. 237:24044–24051.

Rehr, B., C. Wilhelm, and H. Sahm. 1991. The production of sorbitol and gluconic acid by permeablized cells of Zymomonas mobilis. Appl. Microbiol. Biotechnol. 35:144–148.

Reid, S. J., E. R. Allcock, D. T. Jones, and D. R. Woods. 1983. Transformation of Clostridium acetobutylicum protoplasts with bacteriophage DNA. Appl. Environ. Microbiol. 45:305–307.

Reid, M. F., and C. A. Fewson. 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. 20:13–56.

Reimann, A., and H. Biebl. 1996. Production of 1,3-propanediol by Clostridium butyricum DSM 5431 and product tolerant mutants in fedbatch culture: Feeding strategy for glycerol and ammonium. Biotechnol. Lett. 18:827–832.

Reimann, A., S. Abbad-Andaloussi, H. Biebl, and H. Petitdemange. 1998a. 1,3-propanediol formation with product-tolerant mutants of Clostridium butyricum DSM 5431 in continuous culture: Productivity, carbon and electron flow. J. Appl. Microbiol. 84:1125–1130.

Reimann, A., H. Biebl, and W. D. Deckwer. 1998b. Production of 1,3-propanediol by Clostridium butyricum in continuous culture with cell recycling. Appl. Microbiol. Biotechnol. 49:359–363.

Reisch, M. S. 1999. Vision 2020 comes into focus. Chem. Eng. News 77(32):10–12.

Ren, Q., N. Sierro, M. Kellerhals, B. Kessler, and B. Witholt. 2000. Properties of engineered poly-3-hydroxyalkanoates produced in recombinant Esherichia coli strains. Appl. Environ. Microbiol. 66:1311–1320.

Reusch, R. N., and H. L. Sadoff. 1988. Putative structure and functions of a poly-β-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc. Natl. Acad. Sci. USA 85:4176–4180.

Reusch, R. N. 1992a. Biological complexes of poly-β-hydroxybutyrate. FEMS Microbiol. Rev. 103:119–130.

Reusch, R. N., and A. Sparrow. 1992b. Transport of poly-β-hydroxybutyrate in human plasma. Biochim. Biophys. Acta 1123:33–40.

Reynen, M., and H. Sahm. 1988. Comparison of the structural genes for pyruvate decarboxylase in different Zymomonas mobilis strains. J. Bacteriol. 170:3310–3313.

Reysset, G., and M. Sebald. 1985. Conjugal transfer of plasmid-mediated antibiotic resistance from streptococci to Clostridium acetobutylicum. Ann. Inst. Pasteur/Microbiol. 136B:275–282.

Reysset, G., and M. Sebald. 1993. Transformation/electrotransformation of clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 151–158.

Rickert, D. A., C. E. Glatz, and B. A. Glatz. 1998. Improved organic acid production by calcium alginate-immobilized propionibacteria. Enzyme Microb. Technol. 22:409–414.

Ro, H. S., and H. S. Kim. 1991. Continuous production of gluconic acid and sorbitol from sucrose using invertase and an oxidoreductase from Zymomonas mobilis. Enz. Microb. Technol. 13:920–924.

Roehr, M., C. P. Kubicek, and J. Kominek. 1996. Gluconic acid. *In:* M. Roehr (Ed.) Biotechnology, 2nd ed. VCH. New York, NY. 348–362.

Rogers, P. L., K. J. Lee, M. L. Skotnicki, and D. E. Tribe. 1982. Ethanol production by Zymomonas mobilis. Adv. Biochem. Engin. 23:37–84.

Rogers, P. 1986. Genetics and biochemistry of Clostridium relevant to development of fermentation processes. Adv. Appl. Microbiol. 31:1–60.

Rogers, P., and N. Palosaari. 1987. Clostridium acetobutylicum mutants that produce butyraldehyde and altered quantities of solvents. Appl. Environ. Microbiol. 53:2761–2766.

Rogers, P. L., K. J. Lee, G. M. Smith, and K. D. Barrow. 1989. Ethanol tolerance of Zymomonas mobilis. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 239–256.

Rogers, P., and G. Gottschalk. 1993. Biochemistry and regulation of acid and solvent production in clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. Stoneham, MA. 25–50.

Rogers, P. 1999. Clostridia: Solvent formation. *In:* M. C. Flickinger and S. W. Drew (Eds.) Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation. John Wiley. New York, NY. 2:670–687.

Roos, J. W., J. K. McLaughlin, and E. T. Papoutsakis. 1985. The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of Clostridium acetobutylicum. Biotechnol. Bioengin. 27:681–694.

Rose, N. 1986. Chaim Weizmann. Penguin Books. New York, NY.

Rosenberg, S. L. 1980. Fermentation of pentose sugars to ethanol and other neutral products by microorganisms. Enz. Microb. Technol. 2:185–193.

Ross, D. 1961. The acetone-butanol fermentation. Progr. Indust. Microbiol. 3:71–90.

Rudolph, F. B., D. L. Purich, and H. J. Fromm. 1968. Coenzyme A-linked aldehyde dehydrogenase from Escherichia coli. I: Partial purification, properties, and kinetic studies of the enzyme. J. Biol. Chem. 243:5539–5545.

Ryden, R. 1958. Development of anaerobic fermentation processes: Acetone-butanol. *In:* R. Steel (Ed.) Biochemical Engineering. Macmillan. New York, NY. 125–148.

Sablayrolles, J. M., and G. Goma. 1984. Butanediol production by Aerobacter aerogenes NRRL B199: Effects of initial substrate concentration and aeration agitation. Biotechnol. Bioengin. 26:148–155.

Saeki, A., M. Taniguchi, K. Matsushita, H. Yoyama, G. Theeragol, N. Lotong, and O. Adachi. 1997a. Microbial aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. Biosci. Biotech. Biochem. 61:317–323.

Saeki, A., G. Theeragol, and K. Matsushita. 1997b. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at high temperatures. Biosci. Biotech. Biochem. 61:138–145.

Saha, B. C., and R. J. Bothast. 1999. Production of 2,3-butanediol by newly isolated Enterobacter cloacae. Appl. Microbiol. Biotechnol. 52:321–326.

Saint-Amans, S., P. Perlot, G. Goma, and P. Soucaille. 1994. High production of 1,3-propanediol from glycerol by Clostridium butyricum VPI 3266 in a simply controlled fed-batch system. Biotechnol. Lett. 16:831–836.

Saint-Amans, S., L. Girbal, J. Andrade, K. Ahrens, and P. Soucaille. 2001. Regulation of carbon and electron flow in Clostridium butyricum VP 13266g grown on glucose-glycerol mixtures. J. Bacteriol. 183:1748–1754.

Sakura, H., H. W. Lee, S. Sato, S. Mukataka, and J. Takahashi. 1989. Gluconic acid production by Aspergillus niger immobilized on nonwoven fabric. J. Ferment. Bioeng. 67:404–408.

Samuelov, N. S., R. Lamed, S. Lowe, and J. G. Zeikus. 1991. Influence of CO_2-HCO_3-levels and pH on growth, succinate production, and enzyme activities of Anaerobiospirillum succiniciproducens. Appl. Environ. Microbiol. 57:3013–3019.

Samuelov, N. S., R. Datta, M. K. Jain, and J. G. Zeikus. 1999. Whey fermentation by Anaerobiospirillum succiniciproducens for production of a succinate-based animal feed additive. Appl. Env. Microbiol. 65:2260–2263.

Sanchez-Riera, F., D. C. Cameron, and C. L. Cooney. 1987. Influence of environmental factors in the production of R(-)-1,2-propanediol by Clostridium thermosaccharolyticum. Biotechnol. Lett. 9:449–454.

Sass, C., J. Walter, and G. N. Bennett. 1993. Isolation of mutants of Clostridium acetobutylicum ATCC 824. Curr. Microbiol. 26:151–154.

Sauer, E. T. 1991. Carboxylic acids (economic aspects). *In:* Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 5:179–187.

Sauer, U., and P. Dürre. 1995. Differential induction of genes related to solvent formation during the shift from acidogenesis to solventogenesis in continuous culture of Clostridium acetobutylicum. FEMS Microbiol. Lett. 125:115–120.

Sauer, U., J. D. Santangelo, A. Trever, M. Bucholz, and P. Dürre. 1995. Sigma factor and sporulation genes in Clostridium. FEMS Microbiol. Rev. 17:331–340.

Scheirlinck, T., J. Mahillon, H. Joos, P. Dahaese, and F. Michiels. 1989. Integration and expression of α? amylase and endoglucanase genes in the Lactobacillus plantarum chromosome. Appl. Environ. Microbiol. 55:2130–2137.

Schilling, L. B. 1995. Chemicals from alternative feedstocks in the United States. FEMS Microbiol. Rev. 16:101–110.

Schlegel, H. G., G. Gottschalk, and R. Von Vartha. 1961. Formation and utilization of poly-β-hydroxybutyric acid by Knallgas bacteria (Hydrogenomonas). Nature 191:463–465.

Schneider, Z., E. G. Larsen, G. Jacobson, B. C. Johnson, and J. Pawelkiewicz. 1970. Purification and properties of glycerol dehydrase. J. Biol. Chem. 245:3388–3396.

Schoutens, G. H., M. C. H. Nieuwenhuizen, and N. W. F. Kossen. 1984. Butanol from whey ultrafiltrate: Batch experiments with Clostridium beyerinckii LMD 27.6. Appl. Microbiol. Biotechnol. 19:203–206.

Schoutens, G. H., and W. J. Groot. 1985a. Economic feasibility of the production of iso-propanol-butanol-ethanol fuels from whey permeate. Process Biochem. 20:117–121.

Schoutens, G. H., M. C. H. Nieuwenhuizen, and N. W. F. Kossen. 1985b. Continuous butanol production from whey permeate with immobilized Clostridium beyerinckii LMD 27.6. Appl. Microbiol. Biotechnol. 21:282–286.

Schubert, P., A. Steinbüchel, and H. G. Schlegel. 1988. Cloning of the Alcaligenes eutrophus gene for synthesis of poly-β-hydroxybutyric acid and synthesis of PHB in Escherichia coli. J. Bacteriol. 170:5837–5847.

Schuster, K. C., R. Goodacre, J. R. Gapes, and M. Young. 2001. Degeneration of solventogenic Clostridium strains monitored by Fourier transform infrared spectroscopy of bacterial cells. J. Ind. Microbiol. Biotechnol. 27:314–321.

Scopes, R. K. 1983. An iron-activated alcohol dehydrogenase. FEBS Lett. 156:303–306.

Seifert, C., S. Bowien, G. Gottschalk, and R. Daniel. 2001. Identification and expression of the genes and purification nd characterization of the gene products involved in reactivation of coenzyme B12-dependent glycerol dehydratase of Citrobacter freundii. Eur. J. Biochem. 268:2369–2378.

Senthuran, A., V. Senthuran, B. Mathasson, and R. Kaul. 1997. Lactic acid fermentation in a recycle batch reactor using immobilized Lactobacillus casei. Biotechnol. Bioeng. 55:841– 853.

Seyfried, M., R. Daniel, and G. Gottschalk. 1996. Cloning, sequencing, and overexpression of the genes encoding coenzyme B12-dependent glycerol dehydratase of Citrobacter freundii. J. Bacteriol. 178:5793–5796.

Shah, M. M., and M. Cheryan. 1995a. Acetate production by Clostridium thermoaceticum in corn steep liquor media. J. Ind. Microbiol. 15:424–428.

Shah, M. M., and M. Cheryan. 1995b. Improvement of productivity in acetic acid fermentation with Clostridium thermoaceticum. Appl. Biochem. Biotechnol. 51/52: 418–422.

Sheehan, J. 2000. The road to bioethanol: A strategic perspective of the U.S. Department of Energy's national ethanol program. *In:* H. M. E., J. O. Baker, and J. N. Saddler (Ed.) Glycosyl Hydrolases for Biomass Conversion. American Chemical Society. Washington DC, ACS Symposium Series 769:2–25.

Shelley, S., and R. L. D'Aquino. 1999. Three routes vie for the 1,3-propanediol market. Chem. Engin. 106:56.

Shen, G. J., B. A. Annons, R. W. Lovitt, M. K. Jain, and J. G. Zeikus. 1996. Biochemical route and control if butyrate synthesis in Butyribacterium methylotropicum. Appl. Microbiol. Biotechnol. 45:355–362.

Sherman, J. M., and R. H. Shaw. 1923. Process for the production of propionates and propionic acid. US Patent 1,470,885. Chem Abstr. 18:146.

Shiraishi, F., K. Kawakami, S. Kono, and A. Tamura. 1989a. Continous production of free gluconic acid by Gluconobacter suboxydans IFO 3290 immobilized by adsorption on ceramic honeycomb monolith: Effect of reactor configuration on further oxidation of gluconic acid to ketogluconic acid. Appl. Microbiol. Biotechnol. 31:445–447.

Shiraishi, F., K. Kawakami, S. Kono, A. Tamura, S. Tsuruta, and K. Kosmoki. 1989b. Characterization of production of free gluconic acid by Gluconobacter suboxydans absorbed on ceramic honeycomb monolith. Biotechnol. Bioeng. 33:1413–1418.

Shoham, Y., R. Lamed, and E. A. Bayer. 1999. The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol. 7:275–281.

Shone, C. C., and H. J. Fromm. 1981. Steady-state and pre-steady-state kinetics of coenzyme A linked aldehyde

dehydrogenase from Escherichia coli. Biochemistry 20:7494–7501.

Sievers, M., S. Sellmer, and M. Teuber. 1992. Acetobacter europaeus sp. nov., a main component of industrial vinegar fermenters in central Europe. Syst. Appl. Microbiol. 15:386–392.

Sievers, M., W. Ludwig, and M. Teuber. 1994a. Phylogenetic positioning of Acetobacter, Gluconobacter, Rhodophila, and Acidiphilium species as a branch of acidophilic bacteria in the α-subclass of proteobacteria based on 16S ribosomal DNA sequences. Syst. Appl. Microbiol. 17:189–196.

Sievers, M., W. Ludwig, and M. Teuber. 1994b. Phylogenetic positioning of Acetobacter, Gluconobacter, Rhodophila and Acidiphilium species as a branch of acidophilic bacteria in the α subclass of Proteobacteria based on 16S RNA sequences. Syst. Appl. Microbiol. 17:352–354.

Sievers, M., W. Ludwig, and M. Teuber. 1994c. Revival of the species Acetobacter methanolicus (ex: Uhlig et al., 1986) nom. rev. Syst. Appl. Microbiol. 17:352–354.

Silva-Martinez, M., D. Haltrich, S. Novalic, K. D. Kobe, and B. Nidetzky. 1998. Simultaneous enzymatic syntheses of gluconic acid and sorbitol. Appl. Biochem. Biotechnol. 70–72:863–875.

Silveira, M. M., E. Wisbeck, and C. Lemmel. 1999. Bioconversion of glucose and fructose to sorbitol and gluconic acid by untreated cells of Zymomonas mobilis. J. Biotechnol. 75:99–103.

Silveira, M. M., E. Wisbeck, I. Hoch, and R. Jonas. 2001. Production of glucose-fructose oxidoreductase and ethanol by Zymomonas mobilis ATCC 29191 in medium containing corn steep liquor as a source of vitamins. Appl. Microbiol. Biotechnol. 55:442–445.

Sinskey, A. J., M. Akedo, and C. L. Cooney. 1981. Acrylate fermentations. *In:* A. Hollaender (Ed.) Trends in the Biology of Fermentations. Plenum Press. New York, NY. 473–492.

Skraly, F. A., B. L. Lytle, and D. C. Cameron. 1998. Construction and characterization of a 1,3-propanediol operon. Appl. Env. Microbiol. 64:98–105.

Slapack, G. E., I. Russell, and G. G. Stewart. 1987. Thermophilic Microbes in Ethanol Production. CRC Press. Boca Raton, FL.

Slater, S., K. L. Houmiel, M. Tran, T. A. Mitsky, N. B. Taylor, S. R. Padgette, and K. J. Gruys. 1998. Multiple β-ketothiolase mediate poly(β-hydroxyalkanoate) copolymer synthesis in Ralstonia eutropha. J. Bacteriol. 180:1979–1987.

Slater, S., T. A. Mitsky, K. L. Houmiel, M. Hao, S. E. Reiser, N. B. Taylor, M. Tran, H. E. Valentin, J. Rodriguez, D. A. Stone, S. R. Padgette, G. Kishore, and K. J. Gruys. 1999. Metabolic engineering of Arabidopsis and Brassica for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production. Nature Biotechnol. 17:1011–1016.

Smith, W. P. 1993. Soap/Cosmetic Specialties 9:54–58.

Société Lefranc et Cie. 1925. An improved process for the manufacture of dipropylketone. British Patent 216-120. Chem. Abstr. 19:77.

Sokollek, S. J., and W. P. Hammes. 1997. Description of a starter culture preparation for vinegar fermentation. Syst. Appl. Microbiol. 20:481–491.

Sokollek, S. J., C. Hertel, and W. P. Hammes. 1998. Cultivation and preservation of vinegar bacteria. J. Biotechnol. 60:195–206.

Solichien, M. S., D. O'Brien, E. G. Hammond, and C. E. Glatz. 1995. Membrane-based extractive fermentation to produce propionic and acetic acids: toxicity and mass transfer considerations. Enzyme Microb. Technol. 17:23–31.

Solomon, B. O., A. P. Zeng, H. Biebl, A. O. Ejiofor, C. Posten, and W. D. Deckwer. 1994. Effects of substrate limitation on product distribution and H_2/CO_2 ratio in Klebsiella pneumoniae during anaerobic fermentation of glycerol. Appl. Microbiol. Biotechnol. 42:222–226.

Solomon, B. O., A.-P. Zeng, H. Biebl, H. Schlieker, C. Posten, W.-D. Deckwer. 1995. Comparison of the energetic efficiencies of hydrogen and oxychemicals formation in Klebsiella pneumoniae and Clostridium butyricum during anaerobic growth on glycerol. J. Biotechnol. 39:107–117.

Solomons, G. L. 1976. Solvents from carbohydrates: Some economic considerations. Process Biochem. 11:32–37.

Somrutai, W., M. Takagi, and T. Yoshida. 1996. Acetone-butanol fermentation by Clostridium aurantibutyricum ATCC 17777 from a model medium for palm oil mill effluent. J. Ferment. Bioengin. 81:543–547.

Spivey, M. J. 1978. The acetone/butanol/ethanol fermentation. Process Biochem. 13:2–25.

Sprenger, G. A., B. A. Hammer, E. A. Johnson, and E. C. Lin. 1989. Anaerobic growth of Escherichia coli on glycerol by importing genes of the dha regulon from Klebsiella pneumoniae. J. Gen. Microbiol. 135:1255–1262.

Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846–849.

Steinbüchel, A. 1991. Polyhydroxyalkanoic acids. *In:* D. Byrom (Ed.) Biomaterials: Novel Materials from Biological Sources. Macmillan.

Steinbüchel, A., and G. Schmack. 1995. Large-scale production of poly(3-hydroxyvaleric acid) by fermentation of Chromobacterium violaceleum, processing, and characterization of the homopolyester. J. Environ. Polym. Degrad. 3:243–258.

Steinbüchel, A. 1996. PHB and other polyhydroxyalkanoic acids. *In:* M. Roehr (Ed.) Biotechnology, 2nd edn. VCH. Weinheim, Germany. 6:403–464.

Steinbüchel, A., and V. Gorenflo. 1997. Biosynthetic and biodegradable polyesters from renewable resources: Current state and prospects. Macromol. Symp. 123:61–66.

Steinbüchel, A., and B. Füchtenbusch. 1998. Bacterial and other biological systems for polyester production. Trends Biotechnol. 16:419–427.

Stephens, G. M., R. A. Holt, J. C. Gottschal, and J. G. Morris. 1985. Studies on the stability of solvent production by Clostridium acetobutylicum in continuous culture. J. Appl. Bacteriol. 59:597–605.

Stevens, D., S. Alam, and R. Bajpai. 1988. Fermentation of cheese whey by a mixed culture of Clostridium beijerinckii and Bacillus cereus. J. Ind. Microbiol. 3:15–19.

Stieb, M., and B. Schink. 1984. A new 3-hydroxybutyrate fermenting anaerobe, Ilyobacter polytropus, gen. nov.sp.nov., possessing various fermentation pathways. Arch Microbiol. 140:139–146.

Stim-Herndon, K. P., D. J. Petersen, and G. N. Bennett. 1995. Molecular characterization of the acetyl coenzyme A acetyltransferase (thiolase) from Clostridium acetobutylicum ATCC 824. Gene 154:81–85.

St. Martin, E. J., W. B. Freedberg, and E. C. C. Lin. 1977. Kinase replaceent by a dehydrogenase for Escherichia coli glycerol utilization. J. Bacteriol. 131:1026–1028.

Stols, L., and M. I. Donnelly. 1997a. Production of succinic acid through overexpression of NAD^+-dependent malic enzyme in an Escherichia coli mutant. Appl. Env. Microbiol. 63:2695–2701.

Stols, L., G. Kulkarni, B. G. Harris, and M. I. Donnelly. 1997b. Expression of Ascaris suum malic enzyme in a mutant Escherichia coli allows production of succinic acid from glucose. Appl. Biochem. Biotechnol. 63–65:153–158.

Stormer, F. C. 1975. 2,3-Butanediol biosynthetic system in Aerobacter aerogenes. *In* W. A. Wood (Eds.) Methods in Enzymology. Academic Press. New York, NY. 41:518–533.

Strecker, A. 1854. Über eine eigentümliche Bildungsweise der Propionsaüre und einige Salze derselben. Ann. Chem. 92:80.

Streekstra, H., M. J. Teixera de Mattos, O. M. Neijssel, and D. W. Tempest. 1987. Overflow metabolism during anaerobic growth of Klebsiella aerogenes NCTC 418 on glycerol and dihydroxyacetone in chemostat culture. Arch. Microbiol. 147:268–275.

Suzuki, T., and H. Onishi. 1968. Aerobic dissimilation of L-rhamnose and the production of L-rhamnonic acid and 1,2-propanediol by yeasts. Agric. Biol. Chem. 32:888–893.

Suzuki, T., T. Yamane, and S. Shimizu. 1986. Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. Appl. Microbiol. Biotechnol. 24:370–374.

Swick, R. W., and H. G. Wood. 1960. The role of transcarboxylation in propionic acid fermentation. Proc. Natl. Acad. Sci. USA 46:28–41.

Swings, J., and J. De Ley. 1977. The biology of Zymomonas. Bacteriol. Rev. 41:1–46.

Swings, J., and J. De Ley. 1984. Genus Zymomonas. *In:* N. R. Krieg and J. G. Holt (Ed.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:576–580.

Swings, J. 1992. The genera Acetobacter and Gluconobacter. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 3:2268–2286.

Syu, M. J. 2001. Biological production of 2,3-butanediol. Appl. Microbiol. Biotechnol. 55:10–18.

Szmant, H. H. 1989. Organic Building Blocks of the Chemical Industry. John Wiley. New York, NY. 347–348.

Taguchi, H., and T. Ohata. 1991. D-Lactate dehydrogenase is a member of the D-isomer- specific 2-hydrox acid dehydrogenase family. J. Biol. Chem. 266:12588–12594.

Takahashi, R., N. Fujimoto, M. Suzuki, and T. Endo. 1997. Biodegradabilities of ethylenediamine-N,N′-disuccinic acid (EDDS) and other chelating agents. Biosci. Biotech. Biochem. 61:1957–1959.

Takemura, H., S. Horinouchi, and T. Beppu. 1991. Novel insertion sequence IS1380 from Acetobacter pasteurianus is involved in loss of ethanol-oxidizing ability. J. Bacteriol. 173:7070–7076.

Takiyama, E., and T. Fujimaki. 1994. "BIONELLE" biodegradable plastic through chemical synthesis. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 150–174.

Tanaka, K., A. Ishizaki, T. Kanamaru, and T. Kawano. 1995. Production of poly(D-3-hydroxybutyrate) from CO_2, H_2, and O_2 by high cell-density autotrophic cultivation of Alcaligenes eutrophus. Biotechnol. Bioeng. 45:268–275.

Tang, J. C. T., F. E. Ruch, and E. C. C. Lin. 1979. Purification and properties of a nicotinamide adenine dinucleotide-linked dehydrogenase that serves an Escherichia coli mutant for glyerol catabolism. J. Bacteriol. 140:182–187.

Tang, J. C. T., R. G. Forage, and E. C. C. Lin. 1982. Immunochemical properties of NAD^+-linked glycerol dehydrogenases from Escherichia coli and Klebsiella pneumoniae. J. Bacteriol. 152:1169–1174.

Tanner, R. S., L. M. Miller, and D. Yang. 1993. Clostrium ljungdahlii sp. nov., an acetogenic species in clostridial rRNA homology group I. Int. J. Syst. Bacteriol. 43:232–236.

Taroncher-Oldenburg, G., K. Nishia, and G. Stephanopoulos. 2000. Identification and analysis of polyhydroxyalkanoate-specific β-ketothiolase and acetoacetyl coenzyme A reductase genes in cyanobacterium Synechocystis sp. strain PCC6803. Appl. Environ. Microbiol. 66:4440–4448.

Taylor, M. B., and E. Juni. 1960. Stereoisomeric specificities of 2,3-butanediol dehydrogenases. Biochim. Biophys. Acta 39:448–457.

Terracciano, J. S., and E. R. Kashket. 1986. Intracellular conditions required of initiation of solvent production by Clostridium acetobutylicum. Appl. Environ. Microbiol. 52:86–91.

Thauer, R. K., K. Jungerman, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100–180.

Thauer, R. K. 1989. Biochemistry of acetic acid metabolism in anaerobic chemotropic bacteria. Ann. Rev. Microbiol. 43:43–67.

Thayer, A. 2000. Challenges of a biobased economy. C & EN May 29:40.

Thomas, S. R., W. S. Adney, J. O. Baker, Y.-C. Chou, and M. E. Himmel. 1997. US Patent. 5,712,142.

Thompson, D. K., and J.-S. Chen. 1990. Purification and properties of an acetoacetyl coenzyme A-reacting phosphotransbutyrylase from Clostridium beijerinckii ("Clostridium butylicum") NRRL B593. Appl. Environ. Microbiol. 56:607–613.

Thomson, A. W., J. G. O'Neill, and J. F. Wilkinson. 1976. Acetone production by methlobacteria. Arch. Microbiol. 109:243–246.

Thormann, K., L. Feustel, K. Lorenz, S. Nakotte, and P. Dü. 2002. Control of butanol formation in Clostridium acetobutylicum by transcriptional activation. J. Bacteriol. 184:1966–1973.

Tobimatsu, T., T. Hara, M. Sakaguchi, Y. Kishimoto, Y. Wada, M. Isoda, T. Sakai, and T. Toraya. 1995. Molecular cloning, sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of Klebsiella oxytoca. J. Biol. Chem. 270:712–7148.

Tobimatsu, T., M. Azuma, H. Matsubara, H. Takatori, T. Niida, K. Nishinoto, H. Satoh, R. Hayashi, and T. Toraya. 1996. Cloning, sequencing, and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydrase of Klebsiella pneumoniae. J. Biol. Chem. 271:22352–22357.

Tobimatsu, T., H. Kjiura, M. Yunoki, M. Azuma, and T. Toraya. 1999. Identification and expression of the genes encoding a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. J. Bacteriol. 181:4110–4113.

Toda, K., Y. S. Park, T. Asakura, C. Y. Cheng, and H. Ohtake. 1989. High rate acetic acid production in a shallow flow bioreacter. Appl. Microbiol. Biotechnol. 30:559–563.

Tolan, J. S., and R. K. Finn. 1987a. Fermentation of D-xylose and L-arabinose to ethanol by Erwinia chrysanthemi. Appl. Environ. Microbiol. 53:2033–2044.

Tong, I.-T., H. H. Liao, and D. C. Cameron. 1991. 1,3-propanediol production by Escherichia coli expressing genes from the Klebsiella pneumoniae dha regulon. Appl. Env. Microbiol. 57:3541–3546.

Tong, I. T., and D. C. Cameron. 1992. Enhancement of 1,3-Propanediol Production by Cofermentation in Escherichia coli expression Klebsiella pneumoniae dha regulon genes. Appl. Biochem. Biotechnol. 34/35:149–159.

Toth, J., A. A. Ismaiel, and J.-S. Chen. 1999. Purification of a coenzyme A-acylating aldehyde dehydrogenase and cloning of the structural gene from Clostridium beijerinckii NRRL B593. Appl. Environ. Microbiol. 65:4973–4980.

Traeger, M., G. N. Qazi, N. Gulan, R. Buse, and V. Onken. 1992. Comparison of direct glucose oxidation by Gluconobacter oxydans ssp. suboxydans and Aspergillus niger in a pilot scale airlift reactor. J. Ferment. Bioeng. 74:274–281.

Tran-Din, K., and G. Gottschalk. 1985. Formation of D(−)-1,2-propanediol and D(−)-lactate from glucose by Clostridium sphenoides under phosphate limitation. Arch. Microbiol. 142:87–92.

Truffaut, N., J. Hubert, and G. Reysset. 1989. Construction of shuttle vectors useful for transforming Clostridium acetobutylicum. FEMS Microbiol. Lett. 58:15–20.

Trunger, V., and W. Boos. 1994. Mapping and cloning of gldA, the structural gene of the Escherichia coli glycerol dehydratase. J. Bacteriol. 176:1796–1800.

Tsai, S. P., and S.-H. Moon. 1998. An integrated bioconversion process for the production of L-lactic acid from starchy potato feed stocks. Appl. Biochem. Biotechnol. 70–72:417–428.

Tsao, G. T., N. J. Cao, J. Du, and C. S. Gong. 1999. Production of mutifunctional organic acids from renewable resources. Adv. Biochem. Eng. Biotechnol. 65:243–280.

Tullo, A. 1999. BDO market embraces new technologies. Chemical Market Reporter April 26.

Tummala, S. B., N. E. Welker, and E. T. Papoutsakis. 1999. Development and characterization of a gene expression reporter system for Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 65:3793–3799.

Turk, R. 1993. Metal free and low metal salt substitutes containing lysine. US Patent. 5229161.

Turner, K. W., and A. M. Roberton. 1979. Xylose, arabinose, and rhamnose fermentation by Bacteroides ruminicola. Appl. Environ. Microbiol. 38:7–12.

Uchida, K. 1974. Lipids of alcoholophilic lactobacilli. II: Occurrence of polar lipids with unusually long acyl chains in Lactobacillus heterochiochii. Biochim. Biophys. Acta 369:146–155.

Uchida, K. 1975a. Alteration of the unsaturated to saturated ratio of fatty acids in bacterial lipids by alcohols. Agric. Biol. Chem. 39:1515–1516.

Uchida, K. 1975b. Effects of cultural conditions on the cellular fatty acid composition of Lactobacillus heterohiochii, an alcoholophilic bacterium. Agric. Biol. Chem. 39:837–842.

Uhlig, H., K. Kahrbaum, and A. Steudel. 1986. Acetobacter methanolicus sp. nov., an acetophilic facultatively methylotrophic bacterium. Int. J. Syst. Bacteriol. 36:317–322.

Ui, S., H. Masuda, and H. Muraki. 1983. Laboratory-scale production of 2,3-butanediol isomers (D(−),L(+), and Meso) by bacterial fermentations. J. Ferment. Technol. 61:253–259.

Ui, S., T. Masuda, H. Masuda, and H. Muraki. 1986. Mechanism for the formation of 2,3-butanediol stereoisomers in Bacillus polymyxa. J. Ferment. Technol. 64:481–486.

Ui, S., M. Odagiri, A. Mimura, H. Kanai, T. Kobayashi, and T. Kudo. 1996. Preparation of a chiral acetoinic compound using transgenic Escherichia coli expressing the 2,3-butandiol dehydrogenase gene. J. Ferment. Bioeng. 81:386–389.

Ui, S., Y. Okajima, A. Mimura, H. Kanai, and T. Kudo. 1997. Molecular generation of an Escherichia coli strain producing only the meso-isomer of 2,3-butanediol. J. Ferment. Bioeng. 84:185–189.

Ui, S., T. Hosaka, K. Watanabe, and A. Mimura. 1998. Discovery of a new mechanism of 2,3-butanediol stereoisomer formation in Bacillus cereus YUF-4. J. Ferment. Bioeng. 85:79–83.

Valentin, H. E., T. A. Mitsky, D. A. Mahadeo, M. Tran, and K. J. Gruys. 2000a. Application of a propionyl coenzyme A synthetase for poly(3-hydroxypropionate-co-3-hydroxybutyrate) accumulation in recombinant Escherichia coli. Appl. Environ. Microbiol. 66:5253–5258.

Valentin, H. E., S. Reiser, and K. J. Gruys. 2000b. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) formation from γ-aminobutyrate and glutamate. Biotechnol. Bioeng. 67:291–299.

Vandak, D., M. Telgarsky, and E. Sturkik. 1995a. Influence of growth factor components on butyrate production form sucrose by Clostridum butyricum. Folia Microbiol. 40:32–42.

Vandak, D., T. M. Zigova, and E. Sturdik. 1995b. Effect of growth supplements and whey pretreatment on butyric acid production by Clostridium butyricum. World J. Microbiol. Biotechnol. 11:363.

Vandak, D., J. Zigova, E. Sturdik, and S. Schlosser. 1997. Evaluation of solvent and pH for extractive fermentation of butyric acid. Process Biochem. 32:245–251.

Van der Werf, M. J., M. V. Guettler, M. K. Jain, and J. G. Zeikus. 1997. Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by Actinobacillus sp. 130Z. Arch. Microbiol. 167:332–342.

van der Westhuizen, A., D. T. Jones, and D. R. Woods. 1982. Autolytic activity and butanol tolerance of Clostridium acetobutylicum. Appl. Environ. Microbiol. 44:1277–1281.

Van Niel, C. B. 1928. The Propionic Acid Bacteria (thesis). Labortorium voor Microbiologie der Techische Hoogeschool Delft, N.V. Uitgeversaak, J. W. Boissevain & Co. Haarlem, The Netherlands.

Varadarajan, S., and D. J. Miller. 1999. Catalytic upgrading of fermentation-derived organic acids. Biotechnol. Prog. 15:845–854.

Vasconcelos, I., L. Girbal, and P. Soucaille. 1994. Regulation of carbon and electron flow in Clostridium acetobutylicum grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J. Bacteriol. 176:1443–1450.

Vega, J. L., S. Prieto, B. B. Elmore, E. C. Clausen, and J. L. Gaddy. 1989. The biological production of ethanol from synthesis gas. Appl. Biochem. Biotechnol. 20/21:781–797.

Verhasselt, P., F. Poncelet, K. Vits, A. van Gool, and J. Vanderleyden. 1989. Cloning and expression of a

Clostridium acetobutylicum α-amylase gene in Escherichia coli. FEMS Microbiol. Lett. 59:135–140.

Verhave, T. H. 1930. Verfahren zur bacteriellen oxydation organisher Verbindugen zwecks Herstellungen von Oxydationsprodukten wie Dioxyacton usw. German Patent 563, 758. Chem. Abstr. 27:1085.

Vick Roy, T. B. 1985. Lactic acid. *In:* M. Moo Young (Ed.) Comprehensive Biotechnology. Pergamon Press. Oxford, New York, NY. 761–776.

Voget, C. E., C. F. Mignone, and R. J. Ertola. 1985. Butanol production from apple pomace. Biotechnol. Lett. 7:43–46.

Vollbrecht, and El Nawawy. 1980. Restricted oxygen supply and excretion of metabolites. Euro. J. Appl. Microbiol. Biotechnol. 9:1–8.

Voloch, M., M. R. Ladisch, V. W. Rodwell, and G. T. Tsao. 1983. Reduction of acetoin to 2,3-butanediol in Klebsiella pneumoniae: A new model. Biotechnol. Bioengin. 25:173–183.

Von Freudenreich, E., and O. Jensen. 1906. Über die im Emmentaler Käse stattfindende Propionsauregärung. Zentralblatt für Bacteriologie, Parasiten Kunde Infectious Krankheiten und Hygiene Abt. II, 17:529–546.

Wagner, T. O., D. S. Gray, B. Y. Zarah, and A. A. Kozinski. 1980. Practicality of alcohols as motor fuel. *In:* Alcohols as Motor Fuels. Society of Automotive Engineers. Warrendale, PA. 249–265.

Walker, S. A., and T. R. Klaenhammer. 2000. An explosive antisense RNA strategy for inhibition of a lactococcal bacteriophage. Appl. Environ. Microbiol. 66:310–319.

Walter, K. A., G. N. Bennett, and E. T. Papoutsakis. 1992. Molecular characterization of two Clostridium acetobutylicum ATCC 824 butanol dehydrogenase isozyme genes. J. Bacteriol. 174:7149–7158.

Walter, K. A., R. V. Nair, J. W. Cary, G. N. Bennett, and E. T. Papoutsakis. 1993. Sequence and arrangement of two genes of the butyrate-synthesis pathway of Clostridium acetobutylicum ATCC 824. Gene 134:107–111.

Walton, M. T., and J. L. Martin. 1979. Production of butanol-acetone by fermentation. *In:* H. J. Peppler and D. Perlman (Eds.) Microbial Technology, 2nd ed. Academic Press. New York, NY. 1:187–209.

Wang, T.-T., and B. H. Lee. 1997a. Plasmids in Lactobacillus. Crit. Rev. Biotechnol. 17:227–272.

Wang, F. L., and S. Y. Lee. 1997b. Poly(3-hydroxybutyrate) production with high productivity and high polymer content by a fed-batch culture of Alcaligenes latus under nitrogen limitation. Appl. Environ. Microbiol. 63:3703–3706.

Wang, F., and S. Y. Lee. 1998a. High cell density culture of metabolically engineered Escherichia coli for the production of poly(3-hydroxybutyrate) in a defined medium. Biotechnol. Bioeng. 58:325–328.

Wang, X., C. S. Gong, and G. T. Tsao. 1998b. Bioconversion of fumaric acid to succinic acid by recombinant E. coli. Appl. Biochem. Biotechnol. 70–72:919–928.

Waterson, R. M., F. J. Castellino, G. M. Hass, and R. L. Hill. 1972. Purification and characterization of crotonase from Clostridium acetobutylicum. J. Biol. Chem. 247:5266–5271.

Wayman, M., M. E. Martin, and G. Graf. 1962. Propionic Acid Fermentation. US Patent 3,067,107. Chem. Abstr. 58:7337.

Weber, G. H., and W. A. Broich. 1986. Shelf life extension of cultured dairy foods. Cult. Dairy Prod. J. 21(4):19–23.

Webster, J. R., S. J. Reid, D. T. Jones, and D. R. Woods. 1981. Purification and characterization of an autolysin from Clostridium acetobutylicum. Appl. Environ. Microbiol. 41:371–374.

Weimer, P. J. 1984a. Control of product fromation during glucose fermentation by Bacillus macerans. J. Gen. Microbiol. 130:103–111.

Weimer, P. J. 1984b. Fermentation of 6-deoxyhexoses by Bacillus macerans. Appl. Environ. Microbiol. 47:263–267.

Weiss, N., U. Schillinger, and O. Kandler. 1983. Lactobacillus trichodes and Lactobacillus heterochiochii, subjective synonym of Lactobacillus fruitivorans. Syst. Appl. Microbiol. 4:507–511.

Weizmann, C. 1915. Improvements in bacterial fermentation of carbohydrates and in bacterial cultures for the same. British Patent. 4845.

Welch, R. W., F. B. Rudolph, and E. T. Papoutsakis. 1989. Purification and characterization of the NADH-dependent butanol dehydrogenase from Clostridium acetobutylicum (ATCC 824). Arch. Biochem. Biophys. 273:309–318.

Welch, R. W. 1991. Purification and studies of two butanol (ethanol) dehydrogenases and the effects of rifampicin and chloramphenicol on other enzymes important in the production of butyrate and butanol in Clostridium acetobutylicum ATCC 824 [PhD thesis]. Rice University. Houston, TX.

Welling, H. 1998. Fibers industry get ready. Apparel Industry Magazine 59:65–66.

Weusthuis, R. A., G. N. M. Huijberts, and G. Eggink. 1997. *In:* G. Eggink, A. Steinbuchel, Y. Poirer, and B. Witholt (Eds.) Proceedings of the 1996 International Symposium on bacterial Polyhydroxyalkanoates. NRC Research Press. 102–109.

Weyer, E. R., and L. F. Rettger. 1927. A comparative study of six different strains of the organism commonly concerned in large-scale production of butyl alcohol and acetone by the biological process. J. Bacteriol. 14:399–424.

White, D. 1995. The Physiology and Biochemistry of Prokaryotes. Oxford University Press. New York, NY.

Whitfield, C. D., and S. G. Mayhew. 1974. Purification and properties of electron-transferring flavoprotein from Peptostreptococcus elsdenii. J. Biol. Chem. 249:2801–2810.

Wiegel, J. 1980. Formation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 36:1434–1446.

Wiegel, J., M. Braun, and G. Gottschalk. 1981. Clostridium thermoautotrophicum species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. Curr. Microbiol. 5:255–260.

Wiegel, J., and L. G. Ljungdahl. 1986. The importance of thermophilic bacteria in biotechnology. Crit. Rev. Biotechnol. 3:39–108.

Wiegel, J. 1992. The obligately anaerobic thermophilic bacteria. *In:* J. K. Kristjansson (Ed.) Thermophilic Bacteria. CRC Press. Boca Raton, FL. 105–184.

Wiegel, J. 1994. Acetate and the potential of homoacetogenic bacteria for industrial applications. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 484–504.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1988. Thiolase from Clostridium acetobutylicum ATCC 824

and its role in the synthesis of acids and solvents. Appl. Environ. Microbiol. 54:2717–2722.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1989a. Coenzyme A transferase from Clostridium acetobutylicum ATCC 824 and its role in the uptake of acids. Appl. Environ. Microbiol. 55:323–329.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1989b. Phosphotransbutyrylase from Clostridium acetobutylicum ATCC 824 and its role in acidogenesis. Appl. Environ. Microbiol. 55:317–322.

Wilke, D. 1995. What should and what can biotechnology contribute to chemical bulk production? FEMS Microbiol. Rev. 16:89–100.

Wilke, D. 1999. Chemicals from biotechnology: Molecular plant genetics will challenge the chemical and the fermentation industry. Appl. Microbiol. Biotechnol. 52:135–145.

Wilkinson, S. R., and M. Young. 1994. Targeted integration of genes into the Clostridium acetobutylicum chromosome. Microbiology 140:89–95.

Wilkinson, S. R., and M. Young. 1995. Physical map of the Clostridium beijerinckii (formerly Clostrdium acetobutylicum) NCIMB 8052 chromosome. J. Bacteriol. 177:439–448.

Wilkinson, S. R., D. I. Young, J. G. Morris, and M. Young. 1995. Molecular genetics and the initiation of solventogenesis in Clostridium beijerinckii (formerly Clostridium acetobutylicum) NCIMB 8052. FEMS Microbiol. Rev. 17:275–285.

Williams, D. R., D. I. Young, and M. Young. 1990. Conjugative plasmid transfer from Escherichia coli to Clostridium acetobutylicum. J. Gen. Microbiol. 136:819–826.

Wills, C., P. Kratofil, D. Londo, and T. Martin. 1981. Characterization of the two alcohol dehydrogenases of Zymomonas mobilis. Arch. Biochem. Biophys. 210:775–785.

Winston, S. J. 1981. Ethanol Fuels: Use, Production & Economics. Solar Energy Information Data Bank, 1st ed. US Government Printing Office.

Winzer, K., K. Lorenz, and P. Dü. 1997. Acetate kinase from Clostridium acetobutylicum: A highly specific enzyme that is actively transcribed during acidogenesis and solventogenesis. Microbiology 143:3279–3286.

Winzer, K., K. Lorenz, B. Zickner, and P. Dü. 2000. Differential regulation of two thiolase genes from Clostridium acetobutylicum DSM 792. J. Molec. Microbiol. Biotechnol. 2:531–541.

Wittlich, P., A. Themann, and K.-D. Vorlop. 2001. Conversion of glycerol to 1,3-propanediol by a newly isolated thermophilic strain. Biotechnol. Lett. 23:463–466.

Wong, H. H., and S. Y. Lee. 1998. Poly(3-hydroxybutyrate) production from whey by high density cultivation of recombinant Escherichia coli. Appl. Microbiol. Biotechnol. 50:30–33.

Wood, H. G., and C. H. Werkman. 1938. The utilization of CO_2 by the propionic acid bacteria. Biochem. J. 32:1262–1271.

Wood, H. G., and C. H. Werkman. 1940. The relationship of bacterial utilization of CO_2 to succinic acid formation. Biochem. J. 34:129–138.

Wood, B. E., and L. O. Ingram. 1992. Ethanol production from cellobiose, amorphous cellulose, and crystalline cellulose by recombinant Klebsiella oxytoca containing chromosomally integrated Zymomonas mobilis genes for ethanol production and plasmids expressing thermostable cellulase genes from Clostridium thermocellum. Appl. Environ. Microbiol. 58:2103–2110.

Wood, A. 2001. DuPont, Genencor extend alliance. Chem. Week 163:38.

Woods, D. R. 1995. The genetic engineering of microbial solvent production. Trends Biotechnol. 13:259–264.

Woolley, R. C., A. Pennock, R. J. Ashton, A. Davies, and M. Young. 1989. Transfer of Tn1545 and Tn916 to Clostridium acetobutylicum. Plasmid 22:169–174.

Woolley, R. C., and J. G. Morris. 1990. Stability of solvent production by Clostridium acetobutylicum in continuous culture: Strain differences. J. Appl. Bacteriol. 69:718–728.

Worden. R. M., A. J. Grethlein, J. G. Zeikus, and R. Datta. 1989. Butyrate production from carbonmonoxide by Butyribacterium methylotrophicum. App. Biochem. Biotechnol. 20/21:687–698.

Worden, R. M., G. A. J., M. K. Jain, and R. Datta. 1991. Production of butanol and ethanol from synthesis gas via fermentation. Fuel 70:615–619.

Worden, R. M., M. D. Bredwell, and A. J. Grethlein. 1997. Engineering issues in synthesis-gas fermentations. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:320–335.

Woskow, S. A., and B. A. Glatz. 1991. Propionic acid production by a propionic acid tolerant strain of Propionibacterium acidipropionici in batch and semicontinuous fermentation. Appl. Environ. Microbiol. 57:2821–2828.

Wyman, C. E. 1994. Ethanol from lignocellulosic biomass: Technology, economics, and opportunities. Bioresource Technol. 50:3–16.

Wyman, C. E. (Ed.). 1996. Handbook on Bioethanol: Production and Utilization. Taylor and Francis. Washington DC.

Wyman, C. E. 2001. Twenty years of trials, tribulations and research progress in bioethanol technology. Appl. Biochem. Biotechnol. 91–93:5–21.

Xavier, A. M. R. B., L. M. D. Gonsalvas, and J. L. Moreira. 1995. Operation patterns affecting lactic acid production in ultrafiltration cell recycle bioreactor. Biotechnol. Bioeng. 45:320–327.

Xue, Y., Y. Xu, Y. Liu, Y. Ma, and P. Zhou. 2001. Thermoanaerobacter tengcongensis sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tongcong, China. Int. J. Syst. Evol. Microbiol. 51:1335–1341.

Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi. 1995. Transfer of two Burkholderia and an Alcaligenes species to Ralstonia gen. nov.: Proposal of Ralstonia pickettii (Ralston, Palleroni and Doudoroff 1973) comb. nov., Ralstonia salanacearum (Smith 1896) comb. nov. and Ralstonia eutropha (Davis 1969) comb. nov. Microbiol. Immunol. 39:897–904.

Yamada, K. 1977. Recent advances in industrial fermentation in Japan. Biotech. Bioeng. 19:1563–1621.

Yamamoto, I., T. Saiki, S.-M. Liu, and L. G. Ljungdahl. 1983. Purification and properties of NADP-dependent formate dehydrogenase from Clostridium thermoaceticum, a tungsten-selenium-iron protein. J. Biol. Chem. 258:1826–1832.

Yan, R.-T., C.-X. Zhu, C. Golemboski, and J.-S. Chen. 1988. Expression of solvent-forming enzymes and onset of solvent production in batch cultures of Clostridium beijerinckii ("Clostridium butylicum"). Appl. Environ. Microbiol. 54:642–648.

Yan, R.-T., and J.-S. Chen. 1990. Coenzyme A-acylating adlehyde dehydrogenase from Clostridium beijerinckii NRRL B592. Appl. Environ. Microbiol. 56:2591–2599.

Yan, R.-T. 1991. Enzymology of butanol formation in Clostridium beijerinckii NRRL B592 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Yang, S. T., H. Zhu, Y. Li, and G. Hong. 1994. Continuous propionate production from whey permeate using a novel fibrous bed bioreactor. Biotech. Bioeng. 43:1124–1130.

Yang, S. T., Z. W. Jin, and B. H. Chollar. 1997. Production of low-cost acetate deicers from biomass and industrial wastes. Snow Removal and Ice Control Technology. Transportation Research Board, National Research Council. Washington DC, 60–69.

Yang, S. T., Y. L. Huang, Z. Jin, Y. Huang, H. Zhu, and W. Qin. 1999. Calcium Magnesium Acetate at Power-production Cost: Production of CMA Deicer from Cheese Whey. US DOT. Publication No. FHWA-RD-98-174.

Yasui, Y. 1958. Process for continuous production of vinegar. Japanese Patent 244,905. Publication No. Sho. 33-3798/1958.

Yeh, C.-S. 1955. Butanol-acetone fermentation [in Chinese]. *In:* Petroleum Communications. China Petroleum Corporation. Taiwan, China. December, Issue 54:10–19.

Yomano, L. P., S. W. York, and L. O. Ingram. 1998. Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production. J. Ind. Microbiol. Biotechnol. 20:132–138.

Yoon, K.-H., and M. Y. Pack. 1990. Nucleotide sequence of the Zymomonas mobilis alcohol dehydrogenase II gene. Nucleic Acids Res. 18:187.

Yoon, K.-H., J.-K. Lee, and B. H. Kim. 1991. Construction of a Clostridium acetobutylicum-Escherichia coli shuttle vector. Biotechnol. Lett. 13:1–6.

Yoshino, S., T. Yoshino, S. Hara, S. Ogata, and S. Hayashida. 1990. Construction of shuttle vector plasmid between Clostridium acetobutylicum and Escherichia coli. Agric. Biol. Chem. 54:437–441.

Young, M., N. P. Minton, and W. L. Staudenbauer. 1989. Recent advances in the genetics of the clostridia. FEMS Microbiol. Rev. 63:301–326.

Young, M. 1993. Development and exploitation of conjugative gene transfer in clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 99–118.

Youngleson, J. S., J. D. Santangelo, D. T. Jones, and D. R. Woods. 1988. Cloning and expression of a Clostridium acetobutylicum alcohol dehydrogenase gene in Escherichia coli. Appl. Environ. Microbiol. 54:676–682.

Youngleson, J. S., D. T. Jones, and D. R. Woods. 1989. Homology between hydroxybutyryl and hydroxyacyl coenzyme A dehydrogenase enzymes from Clostridium acetobutylicum fermentation and vertebrate fatty acid β-oxidation pathways. J. Bacteriol. 171:6800–6807.

Youngleson, J. S., F.-P. Lin, S. J. Reid, and D. R. Woods. 1995. Structure and transcription of genes within the β-hbd-adh1 region of Clostridium acetobutylicum P262. FEMS Microbiol. Lett. 125:185–192.

Yu, E. K. C., and J. N. Saddler. 1982a. Enhanced production of 2,3-butanediol by Klebsiella pneumoniae grown on high sugar concentrations in the presence of acetic acid. Appl. Environ. Microbiol. 44:777–784.

Yu, E. K. C., N. Levitin, and J. N. Saddler. 1982b. Production of 2,3-butanediol by Klebsiella pneumoniae grown on acid-hydrolyzed wood hemicellulose. Biotechnol. Lett. 4:741–746.

Yu, E. K. C., and J. N. Saddler. 1983. Fed-batch approach to production of 2,3-butanediol by Klebsiella pneumoniae grown on high substrate concentrations. Appl. Environ. Microbiol. 46:630–635.

Yu, E. K. C., and J. N. Saddler. 1985a. Biomass conversion to butanediol by simultaneous saccharification and fermentation. Trends Biotechnol. 3:100–104.

Yu, E. K. C., L. Deschatelets, G. Louis-Seize, and J. N. Saddler. 1985b. Butanediol production from cellulose and hemicellulose by Klebsiella pneumoniae grown in sequential coculture with Trichoderma harzianum. Appl. Environ. Microbiol. 50:924–929.

Yu, P.-L., and L. E. Pearce. 1986. Conjugal transfer of streptococcal antibiotic resistance plasmids into Clostridium acetobutylicum. Biotechnol. Lett. 8:469–474.

Zachariou, M., and R. K. Scopes. 1986. Glucose-fructose oxidoreductase, a new enzyme isolated from Zymomonas mobilis that is responsible for sorbitol production. J. Bacteriol. 167:863–869.

Zakpaa, H. D., A. Ishizaki, and K. Shimizu. 1997. Computer-mediated addition of fresh medium in continuous culture of Zymomonas mobilis by monitoring weight changes. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:143–154.

Zanin, G. M., C. C. Santana, E. P. S. Bon, R. C. L. Giordano, F. F. de Moraes, S. R. Andrietta, C. C. de Carvalho Neto, I. C. Macedo, D. L. Fo, L. P. Ramos, and J. D. Fontana. 2000. Brazilian bioethanol program. Appl. Biochem. Biotechnol. 84–86:1147–1161.

Zappe, H., D. T. Jones, and D. R. Woods. 1986. Cloning and expression of Clostridium acetobutylicum endoglucanase, cellobiase and amino acid biosynthesis genes in Escherichia coli. J. Gen. Microbiol. 132:1367–1372.

Zappe, H., D. T. Jones, and D. R. Woods. 1987. Cloning and expression of a xylanase gene from Clostridium acetobutylicum P262 in Escherichia coli. Appl. Microbiol. Biotechnol. 27:57–63.

Zappe, H., W. A. Jones, D. T. Jones, and D. R. Woods. 1988. Structure of an endo-β-1,4-glucanase gene from Clostridium acetobutylicum P262 showing homology with endoglucanase genes from Bacillus spp. Appl. Environ. Microbiol. 54:1289–1292.

Zeikus, J. G. 1980. Chemical and fuel production by anaerobic bacteria. Ann. Rev. Microbiol. 34:423–464.

Zeikus, J. G., M. K. Jain, and P. Elankoven. 1999. Biotechnology of succinic acid production and markets for derived industrial products. Appl. Microbiol. Biotechnol. 51:545–552.

Zeng, A.-P., H. Biebl, and W.-D. Deckwer. 1990. Effect of pH and acetic acid on growth and 2,3-butanediol production of Enterobacter aerogenes in continuous culture. Appl. Microbiol. Biotechnol. 33:485–489.

Zeng, A.-P., and W.-D. Deckwer. 1991. A model for multi-product-inhibited growth of Enterobacter aerogenes in 2,3-butanediol fermentation. Appl. Microbiol. Biotechnol. 35:1–3.

Zeng, A.-P., H. Biebl, H. Schlieker, and W.-D. Deckwer. 1993. Pathway analysis of glycerol fermentation by Klebsiella pneumoniae: Regulation of reducing equivalent balance and product formation. Enz. Microbol. Technol. 15:770–779.

Zeng, A.-P., T.-G. Byun, C. Posten, and W.-D. Deckwer. 1994a. Use of respiratory quotient as a control parame-

ter for optimum oxygen supply and scale-up of 2,3-butanediol production under microaerobic conditions. Biotechnol. Bioengin. 44:1107–1114.

Zeng, A.-P., A. Ross, H. Biebl, C. Tag, B. Gunzel, and W.-D. Deckwer. 1994b. Multiple product inhibition and growth modeling of Clostridium butyricum and Klebsiella pneumoniae in glycerol fermentation. Biotechnol. Bioengin. 44:902–911.

Zeng, A.-P. 1996. Pathway and kinetic analysis of 1,3-propanediol production from glycerol fermentation by Clostridium butyricum. Bioproc. Engin. 14:169–175.

Zeng, A.-P., H. Biebl, and W. D. Deckwer. 1997. Microbial conversion of glycerol to 1,3-propanediol: Recent progress. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. Oxford Press. Oxford, UK. ACS Symposium Series 666:264–279.

Zhang, D. X., and M. Cheryan. 1994a. Starch to lactic acid in a continuous membrane reactor. Process Biochem. 29(2):145–150.

Zhang, H., V. Obias, K. Gonyer, and D. Dennis. 1994b. Production of polyhydroxyalkanoates in sucrose-utilizing recombinant Escherichia coli and Klebsiella strains. Appl. Environ. Microbiol. 60:1198–1205.

Zhang, M., C. Eddy, K. Deanda, M. Finkelstein, and S. Picataggio. 1995. Metabolic engineering of a pentose metabolism pathway in ethanologenic Zymomonas mobilis. Science 267:240–243.

Zhou, X., and R. W. Traxler. 1992. Enhanced butanol production and reduced autolysin activity after chloramphenicol treatment of Clostridium acetobutylicum ATCC 824. Appl. Microbiol. Biotechnol. 37:293–297.

Ziffer, J. A., S. Gaffney, S. Rothenberg, and T. J. Cairney. 1971. Aldonic acid and aldonate compositions and production thereof. British Patent 1,249,347. Chem. Abstr. 79:113.

Zigova, J., E. Sturdik, D. Vandak, and S. Schlosser. 1999. Butyric acid production by Clostridium butyricum with integrated extraction and pertraction. Process Biochem. 34:835–843.

Zigova, J., and E. Sturdik. 2000. Advances in biotechnological production of butyric acid. J. Indust. Microb. Biotechnol. 24:153–160.

Organic Acid and Solvent Production
Part II: Propionic and Butyric Acids and Ethanol

PALMER ROGERS, JIANN-SHIN CHEN AND MARY JO ZIDWICK

Section 6: Propionic Acid and Butyric Acid

Introduction

Both propionic acid and butyric acid together with their acid salts are incorporated into a large number of commercial products (Boyaval and Corre, 1995; Zigova and Sturdik, 2000). These include food additives and flavors, preservatives, cellulose-based plastics, drug formulations, and fragrances. In the future, production of these short-chain organic acids by low-cost efficient fermentation processes also may make them attractive as feedstocks for conversion into various industrial chemicals. Numerous reaction pathways are already available for conversion of lactic acid and succinic acid into useful chemicals that are potentially competitive with petroleum-based routes (Varadarajan and Miller, 1999). As economic conditions favor the growth of a bio-based chemical industry in the twenty-first century, fermentation-derived organic acids will play an increasingly important role as chemical feedstocks.

At present, both propionic and butyric acids are manufactured by a number of chemical processes using petrochemical routes (Sauer, 1991). For example, butyric acid is usually prepared by oxidation of butyraldehyde, which is produced from propylene by oxosynthesis (Billig and Bryant, 1991). Chemical synthesis of these organic acids is favored because chemical processes are still less expensive to perform. In spite of this cost advantage, butyric and propionic acids obtained from natural sources are often required. Consumers prefer that food additives and pharmaceutical products contain ingredients of natural origin. Thus research and development of bacterial fermentation processes yielding propionic or butyric acid has emerged during the last century. Indeed, a small number of pilot plants were designed and tested for the production of these organic acids from inexpensive biomass materials (Playne, 1985). The history of these two fermentations is treated separately below.

The history of the bacterial propionic acid fermentation began from the investigations by Adolph Strecker (Strecker, 1854) in which he observed for the first time the formation of propionic acid from sugar. He showed that a calcium carbonate-sugar mixture, to which cheese and sour milk were added, first formed a thick, calcium-lactate slurry. Later a second fermentation occurred in the mixture that converted the lactic acid to mostly propionic acid, some acetic acid, and a gas (later identified as carbon dioxide [CO_2]).

Pasteur (Pasteur, 1861b; Pasteur, 1879) demonstrated that the phenomena of fermentation, including the butyric and propionic acid fermentations, were due to the activities of living microbes. The earliest investigations of the morphology and physiology of the propionic acid bacteria were reported by Albert Fitz (Fitz, 1878). From experiments using a variety of carbon sources, Fitz predicted a balanced equation for propionic acid production from organic acid and sugars:

$$3\ \text{Lactic acid} \leftrightarrow 2\ \text{propionic acid} + 1\ \text{acetic acid} + 1CO_2 + 1H_2O \qquad (12)$$

Studies by von Freudenreich and Jensen (1906) and Van Niel (1928) defined and differentiated eleven species of the *Propionibacterium* that have a role in propionic acid production during cheese making. Van Niel's thesis on the propionic acid bacteria contains extensive experimental data on growth and fermentations by these bacteria (Van Niel, 1928). He demonstrated a 90–100% yield (m/m) of propionic acid during growth of a number of strains of *Propionibacterium* on glycerol. The significance of this finding is discussed (see "Scientific Background" and "Areas of Research and Development" in this Chapter). Van Niel predicted that the *Propionibacterium* fermentation might form the basis for future industrial production of propionic acid.

Research on the factors affecting the growth and fermentation by the propionic acid bacteria was published in the 1920s and 1930s and is nicely reviewed by Prescott and Dunn (1949).

The early history of process development for the production of propionic acid began about the same time and has extended to the present. A detailed review of development of this process up to 1981 has been presented by Playne (1985). According to Playne (1985), Sherman and Shaw (1923) first promoted developing this fermentation into an industrial process. During the period 1920–1953, 17 patents were granted on propionic acid production by fermentation. Following this early work, three pilot-plant projects were reported which utilized sulfite waste liquors from wood pulp processing. The Wayman process (Wayman et al., 1962) was a continuous process with *Propionibacterium arabinosum* immobilized on calcium carbonate beads (>3 mm). The liquor was recycled, the pH was adjusted to above 5.7, and the operating temperature was 35–38°C. The acids were separated by steam distillation or by solvent extraction. The designed plant would use 4,000 liters/min of sulfite waste and produce 50 tons of acetic and propionic acid per day. Martin et al. (1961) designed a smaller plant based on the same process in which bacterial mass was pre-grown and then immobilized in columns. With nongrowing cells, a much lower amount of yeast extract was required to maintain production and the pH tolerated was lower than pH 5.7, resulting in a higher concentration of acids during production. Utilizing the same Wayman process, Nishikawa et al. (1970) employed immobilized *Propionibacterium freudenreichii* instead of *P. arabinosum* to investigate coproduction of vitamin B_{12} along with propionic and acetic acid after the addition of cobalt ion. Difficulties due to inhibition of growth of this strain with the sulfite liquor were reported.

The history of the butyric acid fermentation began with Pasteur's discovery that a living infusion both grew and produced butyric acid in the absence of air (Pasteur, 1861a). Further, he found that the activities of these rod-shaped bodies were inhibited by exposure to air, suggesting that his bacterium was most likely a butyric clostridium. A number of scientists studied the butyric-acid fermenting bacteria during the 1880s. Beijerinck proposed that the anaerobic spore-forming microbes that were butyric fermenting could be divided into two groups: those producing mostly butyric acid or those producing mostly butyl alcohol (Morris, 1993). The commercial exploitation of the acetone-butanol (AB) fermentation, which began during the First World War (1914–1918), had a great impact on the study of the anaerobic sporeforming bacteria and the history of their utilization for manufacture of chemical products (Morris, 1993). The AB fermentation dependent on strains of *Clostridium acetobutylicum* dominated the market for acetone during the war and then for butanol after the war up to the early 1940s. Even with massive fermentation plants and development of downstream processing, the lower costs of chemical synthesis from petroleum became more economically competitive during the 1950s and indeed up to the present time. There was a major attempt to produce calcium butyrate from a number of waste carbon sources including sawdust by a fermentation process developed by Lefranc and coworkers (Lefranc, 1923). Employing the "Lefranc Process," the calcium butyrate was converted downstream to dipropyl ketone by a pyrolytic process (Société Lefranc et Cie., 1925). The proposal was to use the ketones as a fuel or a fuel additive. Depasse (1945) reported a detailed description of a commercial process that would produce 5,000 liters/day of ketones from beet molasses through butyrate fermentation. There is no evidence that a factory was ever built to manufacture ketones by this process (Playne, 1985). Advances in strain development and in fermentation or downstream process technology for butyric acid production have been extremely slow. In a recent short review, Zigova and Sturdik (2000) outline some of the developments since 1985 referred to below (see "Areas of Research and Development" in this Chapter).

Scientific Background

Propionic Acid Process Table 24 lists the most prominent bacteria in the development of processes for propionic acid production. The propionibacteria *Propionibacterium acidopropionicum*, *P. freudenreichii* subsp. *freudenreichii*, and *P. freudenreichii* subs. *P. shermanii* are the organisms used in the most recent investigations for improvement of the propionic acid fermentation.

Even though these important dairy bacteria play successful roles in Swiss-type cheese production, they suffer three major drawbacks as biocatalysts effective for commercial production of propionic acid from biomass sources. Strains of the genus *Propionibacterium* are characterized by 1) very low growth rates and fermentation rates, so that a batch process would require 7–12 days to complete, 2) low tolerance to acid and to moderate concentrations of propionic or acetic acids resulting in low yields, and 3) simultaneous production of acetic acid and other products complicating downstream purification. In an attempt to allay the problem of acid sensitivity, three relatively acid-tolerant species of propionibacteria (*P. thoenii*, *P. jensenii* and *P. cyclohexanicum*) have been investigated (Table 24). Finally, the obligate anaerobic bacterium, *Clostridium propionicum*, is listed since it converts lactate to propionic acid, acetic acid and CO_2, as do the

Table 24. Bacteria used in developing processes for propionic acid and butyric acid production.

Organism	Properties
1. Propionic acid bacteria	
Propionibacterium acidipropionici	High yield of propionic acid
ATCC 25562, ATCC4875, P9, P68, P200910	Production strains
	Genetic development
P. freudenreichii subsp. *freudenreichii*	Used in Swiss-type cheese
P. freudenreichii subsp. *shermanii*	production
P. shermanii	New production strains
CDB10014, IF012426, TL162, P93, ATCC 6207^T	Genetic development
P. thoenii	Acid-tolerant strain
P20, P38, P54	Genetic studies
P. jensenii	Acid-tolerant strain
P114, P117	Genetic studies
P. cyclohexanicum	Acid-tolerant, cyclohexyl fatty
TA-12^T, IAM 14535^T	acid in membranes
	Acid tolerant
	High lactic acid produced
Clostridum propionicum	Obligate anaerobe
ATCC 25522	Acrylate pathway to propionate
2. Butyric acid bacteria	
Clostridium butyricum	Uses many C-sources
S21	
C. tyrobutyricum	Selective
CIP I-776, CNRZ 596	Uses few sugars: glucose and fructose
	Produces 90% butyrate
C. beijerinckii	Uses many C-sources
ATCC 25732	Will produce solvents: isopropanol, butanol and ethanol
C. acetobutylicum	Uses many C-sources
ATC 4259	Will produce solvents: acetone, butanol and ethanol
Butyribacterium methylotrophicum	Uses CO as a C-source

Abbreviations: CDB, Collection of the Centro de Desenvolvimento Biothechnologico, Brazil; ATCC, The American Type Culture Collection; IFO, The Institute for Fermentation, Osaka; HUT, The Hiroshima University Type Culture Collection; P200910, P20, etc., strains from Iowa State University Collection; IAM, Collection of the Institute of Applied Microbiology (now Inst. of Molecular and Cellular Biosciences), Tokyo University; CIP, Culture Collection of the Institut Pasteur; CNRZ, Institut National de al Recherche Agronomique; and S21, Strain isolated at Slovak Technical Institute, Bratislava.

propionibacteria, according to the Fitz equation (12); see "Introduction" in this Chapter. In contrast to the propionibacteria, *C. propionicum* reduces lactate directly to propionic acid probably through an acrylate intermediate (Sinskey et al., 1981) rather than via succinate, as shown for the propionibacteria (Fig. 22). Even though acrylic acid is an important chemical feedstock presently produced by chemical synthesis, it is unlikely that the *C. propionicum* fermentation will develop as a bioprocess for production of this chemical (Rogers and Gottschalk, 1993).

Organic acid inhibition of the propionic acid fermentation and cell growth is the most serious problem facing the use of both the propionibacteria and *C. propionicum* for any bioconversion process. Rather belatedly, research focused in this area is emerging. Batch fermentations of lactose by *P. acidipropionici* were studied at a broad pH range (4.5–7.1; Hsu and Yang, 1991). As the growth rate fell from 0.23 h^{-1} at pH 6.0–7.1 to 0.08 h^{-1} at pH 4.5, the yield of propionic acid increased from 33% (w/w) to about 63% (w/w), while the yield of acetic acid was unchanged at about 9–12%. The authors also found that after growth ceased, propionic acid production continued for many hours apparently uncoupled from growth. These basic findings have led to development of improved fermentation processes; see "Areas of Research and Development." A propionic acid-tolerant strain of *P. acidipropionici* (strain P200910) was isolated that produced significantly more propionic acid than the parent strain, P9 (Woskow and Glatz, 1991). Strain P200910 contained an increase in the proportion of straight-chain fatty acids in cellular lipids. During batch and semicontinuous fermentations of whey permeate, strain P200910 showed uncoupling of propionic acid production from growth, produced a higher ratio of propionic acid to acetic acid, utilized lactose more efficiently, and produced more propionic acid per gram of cell mass than did the parent, P9, in all fermentations. Further research is needed to determine the biochemical and genetic nature of the changes responsible for the observed superiority

of acid-tolerant strains. Rehberger and Glatz (1998) tested 17 strains of propionibacteria for organic acid production after fermentation of glucose, maltose or fructose. In general, strains of *P. acidipropionici* produced more propionic acid and reached a lower final pH than strains of other species. Strains of *P. acidipropionici*, *P. jensenii* and *P. thoenii* were tested for ability to grow and/or survive at low pH with lactic, propionic (found to be the most inhibitory) or hydrochloric acid. The strains of *P. jensenii* and *P. thoenii* started growth and survived at a lower pH than did the *P. acidipropionici* strains. Apparently the ability to produce large amounts of propionic acid does not coincide with the ability to initiate growth or to survive at a low pH. A newly described acid tolerant propionibacterium, *P. cyclohexanicum*, strain TA-12, grows at pH range 3.2–7.5, whereas the closely related *P. freudenreichii* grows at pH 4.5–8.5 (Kusano et al., 1997). Strain TA-12 is also resistant to heating at 90°C for 10 min. although it is not a sporeformer. The major cellular fatty acid was *w*-cyclohexylundecanoic acid, which was 52.7% of the total fatty acids. It may be that the presence of this unusual lipid contributes to the acid and heat resistance of this organism. Unfortunately, following fermentation of glucose, TA-12 yielded a 5 : 4 : 2 ratio of lactic acid, propionic acid and acetic acid (Kusano et al., 1997). These results are important for design of future experiments to determine the biological basis for acid tolerance and its relationship to acid production; see "Areas of Research and Development" in this Chapter.

The relationship of the propionibacteria to oxygen with respect to both growth rate and fermentation products has been examined. *Propionibacterium shermanii* CDB 10014 was reported to grow well at high oxygen (O_2) transfer rates of 24 mmoles O_2 liter^{-1} h^{-1} (Quesada-Chanto et al., 1998b). Cell growth rate was reduced about one third during growth on glucose in the presence of O_2, compared with anaerobic growth; whereas growth yield in the presence of O_2 increased about 50% over growth yield in anaerobic conditions. Thus, growth of propionibacteria cannot be considered O_2 sensitive. However, in contrast, growth with O_2 completely inhibits propionic acid production and enhances both acetic acid and lactic acid production by *P. shermanii* and *P. acidipropionici* (Quesada-Chanto et al., 1994b; Quesada-Chanto et al., 1998b).

Both cell growth and propionic acid production by propionibacteria are dependent upon the vitamin-nitrogen sources as well as their concentrations in the fermentation broth. Comparative experiments using various sources of yeast extract and corn-steep liquor (CSL) showed slightly better growth and optimal production of propionic acid by either *P. shermanii* or *P. acidipropionici* using the less expensive CSL (Quesada-Chanto et al., 1998a).

The propionibacterium strains of the species listed in Table 24 are rather omnivorous with respect to carbon sources for growth and acid formation. Most strains grow on lactic acid, glycerol and erythritol, all strains grow on a panel of sugars, and *P. thoenii* and *P. acidipropionici* grow on starch (Cummins and Johnson, 1986; Kusano et al., 1997). The general fermentation pathway used by the majority of propionic acid-producing bacteria, including the propionibacteria, is the succinate-propionate pathway combined with the central pathway of carbon metabolism for conversion of glucose (sugars), lactic acid, and glycerol.

The pathway for propionic acid synthesis involves the following reactions, as first proposed by Swick and Wood (1960). Pyruvate is converted to oxaloacetate by a transcarboxylation of CO_2 from (S)-methymalonyl-CoA by a biotin-enzyme as a CO_2-carrier. Oxaloacetate is reduced and converted to fumarate by the enzymes malate dehydrogenase and fumarase. The reduction of fumarate to succinate by a membrane-bound fumarate reductase is coupled in anaerobically grown propionibacteria to an electrochemical proton gradient sufficient to synthesize one ATP. The high growth yields of propionibacteria during fermentation are consistent with ATP formation in this reaction (Gottschalk, 1986). Then succinyl-CoA is formed by transfer (catalyzed by a CoA transferase) of coenzyme A from propionyl-CoA. Rearrangement of succinyl-CoA to (R)-methylmalonyl-CoA is a coenzyme B_{12}-dependent reaction catalyzed by methylmalonyl-CoA mutase (Barker, 1972). Finally, the (R)-methylmalonyl-CoA is converted to (S)-methylmalonyl-CoA by a specific racemase. The S-enantiomer is the specific substrate for the transcarboxylase yielding propionyl-CoA. The CoA transferase recycles the coenzyme A back to another succinic acid, resulting in the release of propionic acid into the broth. Thus, with the interlocking of a "one-carbon" cycle and a CoA cycle, pyruvate is reduced to propionate with a minimum expenditure of energy.

Considering the pathways for the three carbon sources displayed in Fig. 22, under ideal conditions, the propionibacteria will produce the ratio of the three major products as well as smaller amounts of minor products such as succinic acid, lactic acid, etc., according to balance restrictions. For example, Fitz showed the usual product balance from the fermentation of lactate: 3 lactic acid = 2 propionic acid + 1 acetic acid + $1CO_2$ + $1H_2O$. Similarly, when glucose is fermented the same ratio of products is usually obtained:

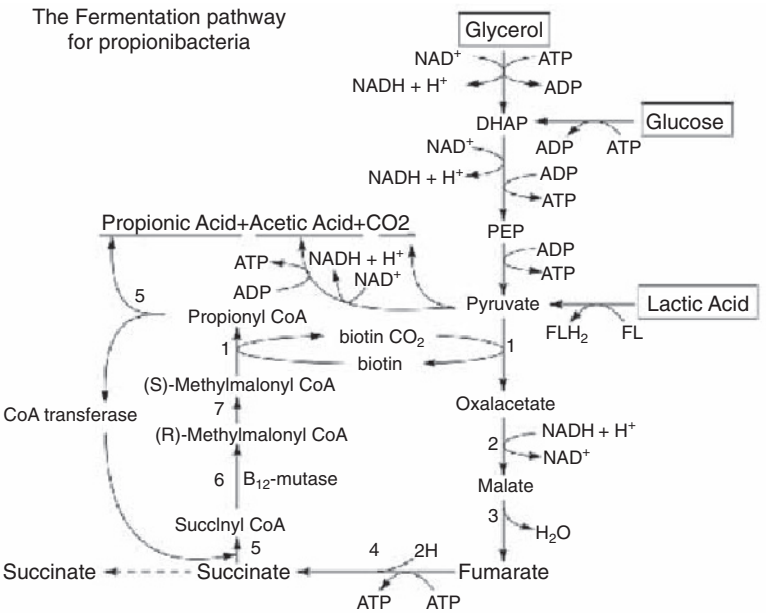

Fig. 22. Fermentation of glucose, glycerol, and lactic acid by propionibacteria utilizing the succinate-propionate pathway. 1, (S)-methylmalonyl-CoA-pyruvate transcarboxylase; 2, malate dehydrogenase; 3, fumarase; 4, fumarate reductase; 5, CoA transferase; 6, (R)-methylmalonyl-CoA mutase; and 7, methylmalonyl-CoA racemase.

$$1.5\ \text{glucose} \leftrightarrow 2\ \text{propionic acid} + 1\ \text{acetic acid} + 1CO_2 + 1H_2O \quad (13)$$

With respect to products produced, the same ratio is expected, and according to the pathways shown in Fig. 22, the major difference is that only 3 ATPs are produced during fermentation of 3 lactic acid molecules, while 6 ATPs are expected when 1.5 glucose molecules are fermented. The maximum theoretical weight yields for sugar from the balanced equation (13) are 54% (w/w) as propionic acid and 22.2% as acetic acid. The yield for the lactic acid fermentation (equation 12) is the same as for sugars. In a recent review, Piveteau (1999) lists a comparison of the relative amounts of propionate and acetate produced from sugars or lactate by 12 strains of propionibacteria grown under 26 different conditions. The molar ratios, P/A, varied between 0.9/1.0 and 3.8/1.0, clustering around 2.0/1.0, as predicted from equations (12) and (13). These ratios are in contrast to that predicted and realized from the fermentation of glycerol to propionic acid. The pathway in Fig. 22 predicts:

$$1\ \text{Glycerol} \leftrightarrow 1\ \text{propionic acid} + 1H_2O \quad (14)$$

In this case, the balance does not demand any formation of CO_2 or acetic acid. Further, 6 ATPs are predicted when 3 glycerol molecules are fermented. It is interesting that Van Niel reported in his thesis (Van Niel, 1928) that seven strains of propionic acid bacteria produced 90–100% propionic acid when grown with glycerol as major carbon source. Research efforts are focusing on production of propionic acid from glycerol; see "Areas of Research and Development" in this Chapter.

Butyric Acid Process Several bacterial species are known to produce significant amounts of butyric acid. They are members of the genera *Clostridium*, *Butyribacterium*, *Butyrivbrio*, *Eubacterium* and *Fusobacterium*. However, it is strains of the genus *Clostridium* (Hippe et al., 1991) that have been used for development of butyric acid or butanol production (Table 24). The productivity of these strains is high and they are relatively stable. In contrast to the *Propionibacterium*, they are strict anaerobes and form heat-resistant endospores. During the 1980s and 1990s, *C. butyricum* was the favored organism for the development of fermentation technologies and fermentations combined with simultaneous product recovery (Vandak et al., 1995a; Vandak et al., 1995b; Vandak et al., 1997). *Clostridium butyricum* can grow well on a large number of sugars including complex mixtures, such as molasses, potato starch, and cheese-whey permeate, producing only butyric acid, acetic acid, CO_2 and H_2 in most fermentations. *Clostridium tyrobutyricum* has been studied, since fermentation of glucose yields mostly butyric acid, CO_2 and H_2 with very little acetic acid or other products. Unfortunately, natural strains of *C. tyrobutyricum* grow and ferment on only a few sugars such as glucose and fructose (Table 24). Both *C. acetobutylicum* and *C. beijerinckii* are important butanol-producing strains, which also synthesize primarily butyric acid under specific growth conditions (Alam et al., 1988; Evans and Wang,

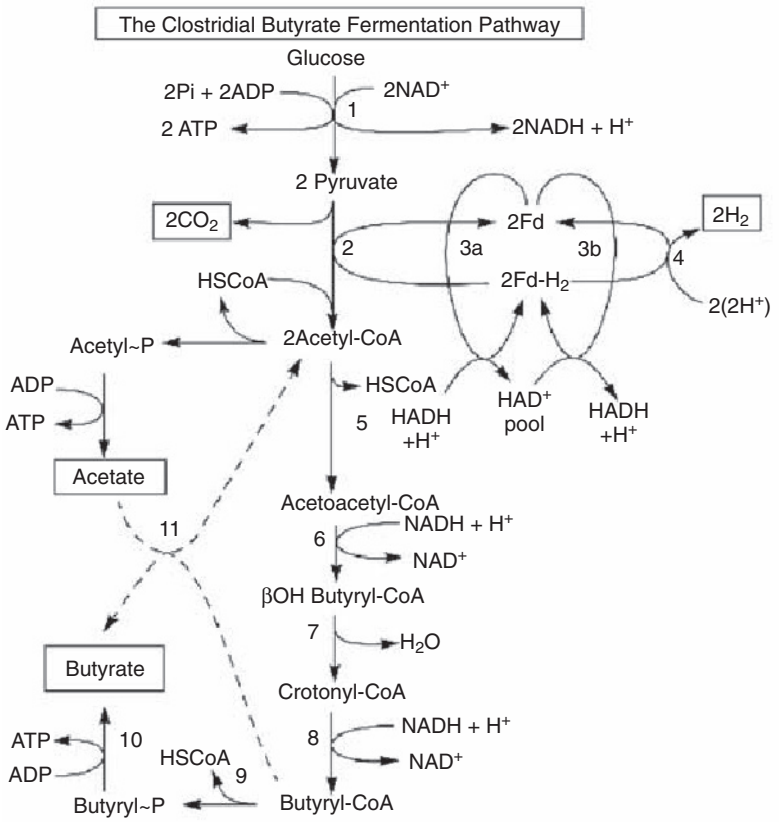

Fig. 23. The pathway of the clostridia for the formation of butyrate and acetate from glucose. 1, Embden-Myerhof-Parnas pathway and hexose phosphotransferase; 2, pyruvate-ferredoxin oxidoreductase; 3a, NAD(P)H-ferredoxin reductase; 3b, ferredoxin-$NAD(P)^+$ reductase; 4, hydrogenase; 5, acetyl CoA-acetyltransferase; 6, β-hydroxylbutyryl CoA dehydrogenase; 7, crotonase; 8, butyryl CoA dehydrogenase; 9, phosphotransbutyrylase; 10, butyrate kinase; and 11, proposed enzyme butyryl CoA-acetate transferase (for recycling of acetate).

1990). A great deal of research effort has been centered on these bacteria, aimed at development of the butanol-acetone process; see "Butanol, Acetone and Isopropanol" in this Volume and Rogers (1999). Because of the extensive knowledge of the regulatory system and the genetics of these organisms, they are excellent candidates for strains to be used in future development of the butyric acid process. Finally, *Butyribacterium methylotrophicum* is listed since it can grow on carbon monoxide (CO) as a sole carbon and energy source and produces primarily butyric acid under specific culture conditions (Worden et al., 1989; Shen et al., 1996). Since CO is produced by gasification of waste biomass, it is potentially a cheap feedstock for butyric acid and acetic acid production.

The pathway for the butyric acid fermentation in the clostridia (and in fact all of the anaerobic bacteria that produce butyric acid) is illustrated in Fig. 23.

It is clearly a branched pathway, yielding both acetic acid and butyric acid in the fermentation. As in the case of the propionic acid fermentation, this is a serious problem for downstream recovery of pure butyric acid. A second problem is that one-third of a carbon source, such as glucose, is removed as CO_2, leaving only two-thirds of the carbon for organic acid production. This loss is a consequence of the conversion of a major portion of the pyruvate produced from sugars to acetyl-CoA, CO_2 and reduced ferredoxin (FdH_2) by the enzyme pyruvate:ferredoxin oxidoreductase, which is ubiquitous in all butyrate-producing bacteria (Fig. 23). Reduced ferredoxin FdH_2 is reoxidized to Fd by hydrogenase which passes the two electrons to hydrogen ions yielding the H_2 gas typically produced by these organisms. The role of the butyrate branch leading from acetyl-CoA is the reoxidation of NADH to NAD^+ so that the pathway from glucose to pyruvate can continue uninterrupted. There is also an additional ATP formed from butyryl-phosphate, so that a net yield of 3ATPs for each butyrate is formed from glucose. In the alternate branch, formation of two acetic acid molecules instead of one butyric acid would not provide for reoxidation of NADH, but a net yield of 4 ATPs results from conversion of glucose to two acetic acids. Since a major characteristic of this fermentation is the simultaneous production of both butyric and acetic acids, the identification of specific control points in the bacterium that will allow selective butyric acid formation is crucial for the eventual development of a commercial process. *Clostridium*

butyricum and *C. tyrobutyricum* are purely acidogenic species and fermentation by them follows this approximate ratio of products:

$$1\ \text{glucose} \leftrightarrow 0.8\ \text{butyrate} + 0.4\ \text{acetate} + 2CO_2 + 2.4H_2 \quad (15)$$

Thauer et al. (1977) have interpreted the production ratio of acetate to butyrate in terms of supplying the energy requirements in the cell. Some work has demonstrated that at high growth rates, acetate production increases, whereas at low rates of growth, butyric acid increases and acetate production either decreases or almost stops (Andel et al., 1985; Michel-Savin et al., 1990a). These observations are consistent with the view that more acetate production results in more ATP synthesis, which would be necessary for rapid growth. Also, for every acetate molecule produced, the excess electrons are removed by the production of extra H_2 gas via hydrogenase, as shown in equation (15) above. The electron traffic is apparently regulated in these cells by allosteric modification of the NADH:ferredoxin oxidoreductase and reduced ferredoxin: NAD^+oxidoreductase (reactions 3a and 3b, Fig. 23). They are sensitive to the CoA/acetyl-CoA ratio and the NAD^+/NADH ratio, respectively (Petitdemange et al., 1976). Measurements of the intracellular concentrations of NADH and acetyl-CoA in *C. butyricum* at various growth rates in a glucose-limited chemostat confirm this view of the control of the butyrate-to-acetate ratio (Abbad-Andaloussi et al., 1996). In all cases, the amount of acetic acid production remains lower than that of butyric acid. This is probably due to the unfavorable potential difference between the Fd/FdH_2 couple and the NAD^+/NADH couple slowing transfer of electrons from NADH to Fd, which is essential when acetate is produced (reaction 3a, Fig. 23).

It was observed that fed-batch supply of glucose increased the productivity for butyrate by *C. tyrobutylicum*. The ratio of butyrate to acetate (selectivity) was influenced by growth rate. At high growth rates, both acetate and butyrate were produced, whereas in glucose-limited fed-batch cultures, acetate (which accumulated at high growth rates) was recycled and converted to butyrate (Michel-Savin et al., 1990a). This recycling process (probably involving a CoA transferase; Fig. 23 [dashed lines]) provides no direct energetic advantage for re-utilization of acetate. The purpose of recycling in the organism may be to detoxify the medium by reducing total hydrogen ion concentration, which occurs when one butyrate substitutes for two acetates. In nonlimited glucose fed-batch cultures, *C. tyrobutyricum* produced a butyrate concentration of 42.5 g/liter with a selectivity of 0.90 and a yield of 36.6 g/100 gm of glucose. (Michel-Savin et al., 1990b). This unusually high selectivity may be related to recycling of acetate by these bacteria.

Commercial Applications

Major Uses of Propionic and Butyric Acids

These organic acids serve important roles in a great variety of products and industrial processes. Propionic acid and its sodium, potassium and calcium salts are excellent antifungicides. They are used extensively in the food industry to suppress mold growth in breads, meats, fruits, and on the surfaces of cheeses and also as preservatives for grains, silage and tobacco during storage and transport. Dipping and spraying food containers, caps, and wrappers with solutions containing propionate salts are used for preservation. Solutions of propionic acid mixed with acetic and lactic acids have been shown to be effective in growth inhibition of *Listeria monocytogenes* in foods. The Food and Drug Administration (FDA) considers the sodium, potassium and calcium propionate salts as safe additives (GRAS), since they are found normally in a number of foods and are metabolized easily by all mammals. Propionic acid is used in the production of cellulose-based plastics (such as cellulose acetate propionate), which are used in textiles, filters, reverse osmosis membranes, sheeting, film products, lacquers, and molding plastics. Propionic acid esters and other derivatives are used as anti-arthritic and antibiotic drug preparations, as perfumes and flavors (e.g., citronellyl propionate and geranyl propionate), as plasticizers (e.g., phenyl propionate and glycerol tripropionate), and as specialized solvents.

A major use of butyric acid is in the manufacture of cellulose acetate butyrate plastics. They are used as textile fibers and in situations where resistance to heat and sunlight is essential. Calcium butyrate has been used in some leather tanning processes. Butyric acid esters are added as flavors in some soft drinks and chewing gums. Various derivatives of butyric acid are used as vasoconstrictor drugs, in anesthetics, and as antioxidants (Playne, 1985).

Market Size and Prices

The main producers of propionic acid in the United States are Eastman, Hoechst Celanese, and Union Carbide (Sauer, 1991). Eastman and Hoechst Celanese also manufacture butyric acid. The estimates of propionic acid production are 120×10^6 lbs/per yr in the United States and 80×10^6 lbs/per yr in Western Europe (Boyaval and Corre, 1995). The price for propionic acid is currently \$1.01–1.08 per kgm (Anonymous, 2001a). The production of butyric acid in the United States was

about 48×10^6 lbs/yr in 1991, and no estimate of world production is available (Sauer, 1991). The price for butyric acid is not available, but the price for synthetic butyraldehyde is \$1.19–1.28/per kgm (Anonymous, 2001b). Normally synthetic butyric acid is produced by air oxidation of butyraldehyde (Billig and Bryant, 1991). Thus, the price may be close to the price quoted for butyric acid, \$1.50/per kgm, in May 1982 (Playne, 1985).

Processes for Production of Propionic and Butyric Acids Chemical processes dominate the production of short chain organic acids. The primary route of synthesis employs the "Oxo" process (Billig and Bryant, 1991). Propionic acid is made by oxo synthesis of propionaldehyde from ethylene, CO and H_2 with a rhodium catalyst. Liquid-phase oxidation of the aldehyde yields propionic acid. Butyric acid is made by air oxidation of butyraldehyde, which is synthesized by the oxo process from propylene, CO and H_2. The triphenylphosphine-modified rhodium oxo process, termed the "LP Oxo" process, is the industry standard for the hydroformylation of ethylene and propylene (Billig and Bryant, 1991). Also pure propionic acid can be obtained from propionitrile or by oxidation of propane gas.

Three changing economic forces have combined to revive a search for alternative production routes for propionic and butyric acids. The decreasing supply of world crude oil reserves will eventually increase the cost of substrates for chemical synthesis. The increasing amounts of food industry by-products and unused agricultural biomass can serve as substrates for microbial conversion. The increasing consumer demand for organic natural products in food additives, pharmaceutical products, and preservatives has produced a favorable business climate for the emergence of the microbial fermentation route for production of these organic acids. For example, organic acids produced by fermentation are favored over chemically synthesized acids when the fermentation product can be labeled a "natural preservative" for foods or feeds. Several products produced by *Propionibacterium* fermentation of milk or whey after concentration and drying have been marketed as preservatives. These include: Upgrade, made by Microlife Techniques; CAPARVE, produced by PTX Food Corp.; and Microgard, made by Wesman Foods, Inc. (Lyon and Glatz, 1995). The United States Food and Drug Administration approved the use of Microgard in cottage cheese to prolong its shelf life and an estimated 30% of the cottage cheese made in the United States contains this preservative (Weber and Broich, 1986).

Significant improvements in the yield, productivity, and selectivity of the fermentations producing propionic acid or butyric acid would be required if these methods were to become competitive with chemical synthesis methods used for industrial applications of these acids as commodity chemicals.

Research and Development

Improvement of Bacterial Strains Modern genetic methods are being applied to the strains of propionibacteria that are listed in Table 24. Lyon and Glatz (1995) have reviewed the plasmid biology, the application of DNA transfer methods, and mutagenesis and selection techniques applied to these organisms. The cryptic plasmid pRG01 is present in a number of strains of *P. acidipropionici*, *P. freudenreichii*, *P. jensenii* and *P. thoenii*. Kiatpapan et al. (2000) sequenced this plasmid and constructed a shuttle vector, pPK705, using parts of pRG01, the *Escherichia coli* plasmid pUC18, and the hygromycin B-resistant gene as a drug marker. The pPK705 has been transferred into *P. freudenreichii* strains by electroporation at an efficiency of 8×10^6 CFU/microgram of DNA. This and other similar vectors will facilitate genetic analysis of strains of propionibacteria and allow transfer of genes for producing new industrial strains.

The solvent-forming clostridia have been the focus of a great deal of research effort over the past twenty years because of the potential to apply their ability to convert biomass into the important commodity chemicals butanol, acetone, and isopropanol. The metabolic pathways for solvent and acid production as well as the basic mechanisms of regulation of these pathways have been determined for *C. acetobutylicum* and related clostridia (see reviews by Rogers and Gottschalk, 1993, and Rogers, 1999). Genetic systems for gene manipulation of the clostridia have been developed including ones involving mutagenesis (Jones, 1993), conjugative gene transfer (Young, 1993), clostridial cloning vectors (Minton et al., 1993), and transformation and electroporative transformation (Reysset and Sebald, 1993). With these techniques, the cloning, structure and expression of acid and solvent pathway genes of *Clostridium acetobutylicum* have been examined in some detail (Papoutsakis and Bennett, 1993; Mermelstein et al., 1994; Dürre et al., 1995). The obvious application of these basic studies is to develop clostridial strains that will be useful in processes for commercial solvent production (Papoutsakis and Bennett, 1999). However, since these solventogenic organisms also produce butyric acid, strains can be developed for production of this acid as well (Alam et al., 1988; Evans and Wang, 1990). Fur-

thermore, the mechanisms of regulation of the fermentation pathways in the solvent-forming clostridia have been fairly well defined (Girbal et al., 1995; Rogers, 1999). Using these studies as a model, regulation of carbon and electron flow in *C. butyricum* is being examined under conditions for optimal production of butyric acid or of 1,3-propanediol (Saint-Amans et al., 2001).

The complete genome sequence of the bacterium *C. acetobutylicum* ATCC 824 was determined including a 192-kb megaplasmid that contains the majority of the genes necessary for solvent production (Nolling et al., 2001). With this information, it is now possible to effectively develop industrial strains that can be utilized for either butanol or butyric acid production from a variety of biomass sources.

A major barrier blocking commercialization of bacterial fermentation for production of propionic or butyric acid is that the available strains of propionibacteria and clostridia have low tolerance to organic acids and sensitivity to acid stress in general. The acid tolerance response (ATR) of *P. freudenreichii* was studied (Jan et al., 2000). Survival at pH 2 was conferred by preexposure of the bacteria in media at pH 4–5, so that adaptation was established within 3–10 min. Later, it was shown that new "stress" proteins are synthesized rapidly during adaptation. These proteins were identified as chaparonins GroEL and GroES. Also, specific DNA synthesis and repair enzymes were upregulated (Jan et al., 2001). However, in what way is sensitivity to accumulated organic acids related to production capacity? Rehberger and Glatz (1998) showed that *P. jensenii* and *P. thoenii* survive at a lower pH than *P. acidipropionici*, yet *P. acidipropionici* produces the highest yield (g/liter) of propionic and acetic acids during fermentation. The newly described propionibacterium, *P. cyclohexanicum* (Kusano et al., 1997), grows and ferments at a low pH optimum of pH 5.5–6.5, while the pH optimum of *P. freudenreichii* and other dairy propionibacteria is 6.5–7.0. *Propionibacterium cyclohexanicum*, strain TA-12, *w*-cyclohexyl undecanoic acid is the major cellular fatty acid (52.7% of the total fatty acids). Thus there appears to be a number of factors governing the tolerance of propionibacteria to organic acids as well as their sensitivity to acids. The low tolerance of the clostridia to organic acids and to solvents has been a major factor preventing their use in commercial production of acetic acid and of solvents such as butanol (see "Acetic Acid" in Organic Acid and Solvent Production, Part I in this Volume and "Butanol, Acetone and Isopropanol" in this Volume). Although numerous mutants and so-called "butanol tolerant strains" of *C. acetobutylicum* and *C. beijerinckii* have been selected, this research has had only a limited impact on our understanding of tolerance or on producing industrially useful strains (Rogers, 1999). On the other hand, important research has emerged on the basic elements of the general stress response and of the σ factors and the phosphorelay pathway essential for the initiation of endosporulation in *Clostridium* (Bahl et al., 1995; Sauer et al., 1995; Wilkinson et al., 1995). It was found that the transcription factor SpOA in *C. beijerinckii* not only controls entry into the sporulation cycle but also upregulates expression of genes for the solvent pathways and downregulates expression of the genes for organic acid production (Brown et al., 1994). As organic acid concentration increases and the pH drops, *C. acetobutylicum* rapidly accumulates a set of stress proteins, the chaperonins, required for proper folding of newly synthesized proteins in the bacterium; in addition, a series of ATP-dependent proteases (Lon, ClpP, and ClpX) is induced (Bahl et al., 1995). This research may be applied to the future development of organic acid-tolerant strains of the clostridia useful for commercial fermentations.

New Process Technologies: Propionic Acid Production Comparative fermentations with a variety of substrates confirmed the earlier finding reported by Van Niel (1928) that numerous species of the propionibacteria produce propionic acid selectively with very little acetic acid when fermenting glycerol (Boyaval et al., 1994; Barbirato et al., 1997; Himmi et al., 2000). Propionic acid/acetic acid molar ratios for *P. acidipropionici* and *P. freudenreichii* subs *P. shermanii* were 5.7 and 3.8, respectively, with glycerol, and 1.7 and 1.3, respectively, with glucose (Himmi et al., 2000). Barbirato et al. (1997) obtained an even better propionic acid to acetic acid ratio of 37 with *P. acidipropionici*. Since selective acid production simplifies downstream processing for recovery of propionic acid, the choice of glycerol for this process is suggested. The high proportion of acetic acid produced with glucose or lactic acid as substrate explains the lower yields of propionic acid compared to that reached when fermenting glycerol, as recorded in Table 25. Inexpensive industrial grades of glycerol are readily available as by-products of processing of natural fats and oils during production of fatty acids.

The two major weaknesses of the fermentation process for propionic acid production are the low final product concentration and the extremely low productivity due to the sensitivity of the bacterial cells to organic acids and their slow fermentation and growth rates. Two developments in fermentation technology that improve the process are the high cell-density reactor and improved ultrafiltration membranes

Table 25. Experimental fermentation processes for production of propionic acid.

Organism	Process	Substrate	Time of operation (h)	Product concentration (g liter^{-1})	Product productivity (g liter^{-1} h^{-1})	Yield for major product (g/g)	References
P. acidipropionici ATCC 25562	Batch	Glucose	60–80	11.9	0.17	0.59	Barbirato et al., 1997
		Glycerol	60–80	13.6	0.18	0.68	
		Lactic acid	60–80	12.3	0.17	0.61	
P. acidipropionici ATTC 4965	CSTR, high cell density, with cell recycle UF module (5 m^3 pilot plant)	Whey (lactose)	950	35	0.65	0.54–0.62	Colonban et al., 1993
P. acidipropionici DSM 8250	CSTR, high cell density, with cell recycle UF module (1.5 liters)	Sucrose	>336	3.0–16.2	3.0–8.0	0.4	Quesdada-Chanto et al., 1994
		Blackstrap molasses (sugarcane)	>336	17.7	4.4	0.5	
P. thoenii NCDO1082	CSTR, with high cell density, cell recycle and UF module (1.5 liter)	Glycerol	300	10	1.0	>0.5	Boyaval et al., 1994
P. acidopropionici P200910	Immobilized cells: Ca alginate beads, 10^{10} cells per g of beads (fed batch)	Glucose	250	57	0.23	0.57	Paik and Glatz, 1994
		Com steep liquor	250	45.6	0.18		
P. thoenii P20	Immobilized cells: Ca alginate beads, 2×10^{11} cells per g of beads (repeated fed batch)	Glucose (75 g liter^{-1})	12	34	2.8	0.45	Rickert et al., 1998
P. acidipropionici ATCC4875	Immobilized cells: fibrous bed bioreactor (continuous RT 52 h)	Lactose (42 g liter^{-1}) in whey perm.	4 mo	20	0.38	0.48	Yang et al., 1994
P. acidipropionici and *S. lactis* OSU 588	Immobilized cells: fibrous bed bioreactor (continuous RT 68 h)	Lactose (45 g liter^{-1}) in whey perm.	4 mo	22	0.32	0.49	Yang et al., 1994
P. acidipropionici 4875	Hollow fiber men. Extractive ferm. (amine extractant)	Lactose (50 g liter^{-1})	120	75	0.98	0.66	Jin and Yang, 1998
P. thoenii P20	Hollow fiber mem. Exractive ferm. (alamine extractant) Immobilized cells: Ca alginate beads, repeated fed batch	Glucose (75 g liter^{-1})	200	71	0.4–0.6	0.5–0.6	Gu et al., 1999

Abbreviations: CSTR, continuously stirred tank reactor; UF, ultrafiltration; RT, room temperature; perm., permeate; ferm., fermentation; mem., membrane; and alamine, trilaurylamine.

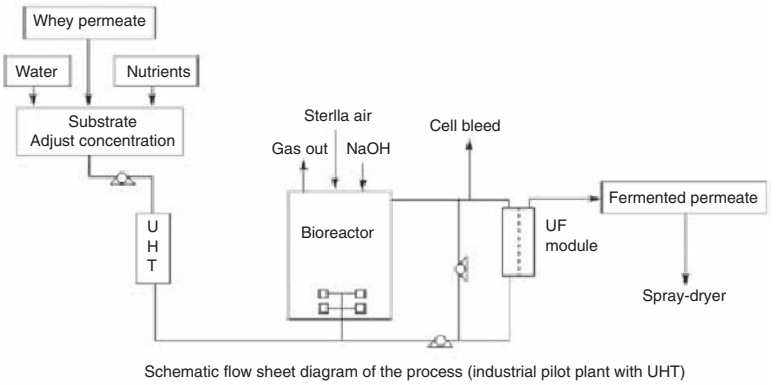

Fig. 24. Diagram of an industrial scale pilot plant for production of propionic acid from whey permeate by sequential fermentation, ultrafiltration, and cell recycling. Redrawn with permission from Colomban et al. (1993).

for recovery of the product. The Laboratory of Dairy Technology Research of the Institut National de la Recherche (INRA) in Rennes, France, is investigating the application of these techniques for improved propionic acid production (Boyaval and Corre, 1995). Colomban et al. (1993) demonstrated the production of propionic acid from whey permeate by *P. acidipropionici* at high cell density combined with sequential cell recycling and ultrafiltration. Figure 24 is a diagram of the process employing a 5-m^3 industrial pilot plant bioreactor.

Initially the fermentation was run batchwise for about 100 h until the lactose concentration reached less than 5 g·liter^{-1} in the bioreactor. The culture was then pumped through an ultrafiltration (UF) module to produce a permeate fraction (fermented permeate) that contained propionic and acetic acids plus unfermented lactose (Fig. 24). The permeate was eventually concentrated to 36% (w/w) propionic acid and 11% acetic acid. The retentate from the UF module containing the cell biomass and about 25% of the culture volume was recycled back into the bioreactor together with a stream of fresh medium sufficient to replace the culture volume. Figure 25 presents the results of a typical run in the 5-m^3 pilot plant, showing the sequential cycles of usually 70 to 40 h.

In a typical pilot plant run, the biomass was maintained at about 40 g dry wt liter^{-1} by partial cell bleeding (B) at the times shown during the 15 cycles for a total time of 950 h (Fig. 25a). At this biomass density, the lactose is exhausted regularly after 50–70 h, leading to a new cycle of ultrafiltration and feeding (Fig. 25b). During this high cell density fermentation, the propionic acid and acetic acid concentrations reached were normally 35 and 9 g·liter^{-1}, respectively (Fig. 25c), which are 3 times greater than those observed in batch cultures (Table 25). The productivity of propionic acid was normalized at about 0.65 g·liter^{-1} h^{-1}, which is 3.8-fold better than in batch cultures (Table 25). Experimental fermentations by *P. acidipropionici* using sucrose or blackstrap molasses (cane sugar) as substrate were studied employing the same high cell density and continuous recycle process as described above (Table 25). In a 1.5-liter reactor, at a cell density of 125 g dry wt liter^{-1}, sucrose was converted to propionic acid at the high productivity of 3.0–8.0 g·liter^{-1}h^{-1} and at a concentration of 30–16.2 g·liter^{-1}, dependent on the dilution rate (Quesada-Chanto, 1994a). At a cell density of 75 g dry wt liter^{-1}, blackstrap molasses was fermented to propionic acid at a productivity of 4.4 g·liter^{-1}h^{-1}, and at a concentration of 17.7 g·liter^{-1}. Stable production was maintained at a constant product concentration "over many weeks" (Quesada-Chanto, 1994a). The most encouraging report was that of the experimental fermentation of glycerol to propionic acid by *P. thoenii* NCDO 1082 by the continuous high cell density cell recycle process (Boyaval et al., 1994). During stable operation with a cell density of only 20 g dry wt liter^{-1}, a propionic acid concentration of 10 g·liter^{-1} and a productivity of 1.0 g·liter^{-1}h^{-1} were maintained, while the acetic acid concentration was only 0.1 g·liter^{-1}. During the continuous ultrafiltration, the only nutrient added to the reactor was glycerol. At 140 h, a short 10-h regeneration period with yeast extract was inserted. The application of the high cell density reactor with ultrafiltration to the fermentation of glycerol as a substrate appears very promising for future development of a commercial propionic acid production process (Boyaval and Corre, 1995).

Experimental fermentation processes employing cell immobilization have been applied to propionic acid production by the propionibacteria. The objectives are to create a stable high-density cell module that will produce a high concentration of propionic acid in a relatively short time. *Propionibacterium acidipropionici* was immobilized in Ca alginate beads at about 10^{10} cells/g of

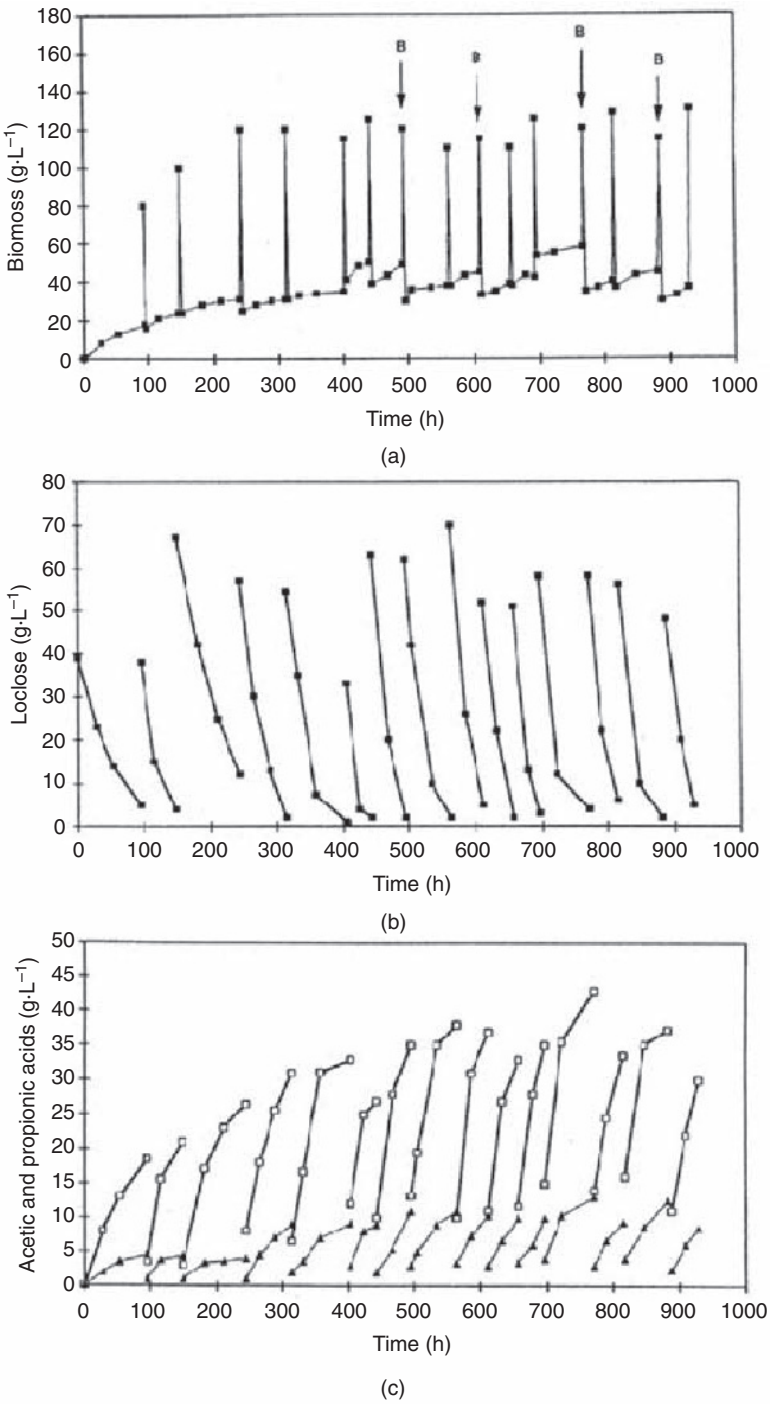

Fig. 25. (a) Biomass evolution during a typical run in a 5-m^3 bioreactor. Each peak results from the concentration of the cells by ultrafiltration. At the times indicated by the arrows, a cell bleed, B, was done. (b) Lactose concentration during the same run. (c) Propionic acid ("box") and acetic acid ("triangle") concentrations in the same run. From Colomban et al. (1993), with permission.

beads. Fed-batch fermentations with 80 g of beads in 290 ml medium were incubated 250 h with either glucose or corn steep liquor as substrate (Paik and Glatz, 1994). Unusually high concentrations of propionic acid were obtained: 45.6 g·liter^{-1} with corn steep liquor and 57 g·liter^{-1} with glucose (Table 25). Using Ca alginate immobilized *P. thoenii* P20 at 2×10^{11} cells per g of beads, Rickert et al. (1998) demonstrated complete conversion of high concentrations of glucose (65–115 g·liter^{-1}) to propionic acid during a short 12-h fermentation time. For example, 75 g·liter^{-1} glucose yielded 34 g·liter^{-1} of propionic acid (Table 25). A second type of immobilization technique, which appears promising, is the fibrous bed bioreactor (Lewis and Yang, 1992), which consists of a piece of cotton towel (15 × 45 cm) rolled up with a stainless steel mesh and placed in a 5

×15 cm glass column. *Propionibacterum acidipropionici* is added to the reactor with 290 ml of growth medium and incubated for 2–3 days until about 40–60 g dry wt liter^{-1} of immobilized cells is reached. Cells form a biofilm on the fiber surfaces and are entrapped in the fibrous matrix. Reactor performance was studied during continuous operation at a variety of retention times to determine the best conditions for converting the lactate or lactose in whey permeate to propionic and acetic acids (Lewis and Yang, 1992; Yang et al., 1994). These reactors are capable of complete conversion of high concentrations of whey permeate-lactose to 20–22 g·liter^{-1} propionic acid at a productivity of 0.38 g·liter^{-1} h^{-1} for 4–6 months of stable operation (Table 25). Co-immobilization of *P. acidipropionici* and *S. lactis* did not improve the productivity of the fermentation (Yang et al., 1994; see Table 25). The nature of this immobilization system allows constant cell renewal, and consequently, the bioreactor could operate continuously for 6 months without clogging, degeneration or contamination.

Extractive fermentation is a relatively new technology that combines the fermentation process with product separation from beer. This process has been applied to commercial production of both lactic acid and ethanol, and experimental results suggest application to the propionic acid process will overcome some of the barriers indicated above. Solichien et al. (1995) examined a series of microporous membranes and polymer films for performance in membrane-based extraction of propionic and acetic acids with a range of organic solvents and acid-complexing carriers. The hollow-fiber membrane extractors, Celgard X20-400 or X-30, were found to be most satisfactory for laboratory-scale fermentations. Each extractor consisted of 200–800 hydrophobic, microporous, polypropylene hollow fibers jacketed in an extractor shell such that the extraction solvent could flow through the hollow fibers while the fermentation broth with the cells circulated on the shell side of the extractor. Solvent leakage from the membrane pores was prevented by applying back pressure to the shell side of the extractor. The hydrophobic structure of the fibers prevented the broth from entering the solvent phase. This set-up allowed the extractive fermentation to operate continuously. The solvent selected for extractive fermentation of propionic acid was 40% Alamine 304-1 (trilaurylamine) in oleyl alcohol because it was nontoxic, provided good partitioning of propionic acid, and allowed free recovery of the acid by distillation (Gu et al., 1998; Gu et al., 1999). Jin and Yang (1998) used a similar extraction solvent: 4–15% Adogen 283 (a secondary amine, ditridecylamine; Sherex Chemical Co., Dublin, OH) in oleyl alcohol. Jin and Yang (1998) demonstrated a fed-batch extractive fermentation of lactose by *P. acidipropionici* for propionic acid production. Lactose was added up to 50 g·liter^{-1} every 20 h, while the culture was cycled through the extractor module for 120 h. The extracted propionic acid reached 75 g·liter^{-1}, at a productivity of 0.98 g·liter^{-1}h^{-1} and a yield of 0.66 g/g of lactose (Table 25). This was the first report of a successful demonstration of extractive fermentation of propionic acid. Further, the fed batch extractive fermentation produced a propionic to acetic acid ratio (P/A ratio) of 9.8, probably owing to the selectivity of the extraction step for propionic acid combined with the low pH (5.3) of the fermentation (Jin and Yang, 1998). Production of propionic acid by fermentation of glucose by immobilized cells of *P. thoenii* P20 was combined with concurrent liquid extraction employing a hollow-fiber membrane extractor (Gu et al., 1999). Cells immobilized in Ca alginate beads were suspended in 300 ml broth with glucose maintained at 75 g·liter^{-1} in a fed-batch operation. The culture was continuously cycled through the shell side of the extractor with concurrent circulation of the organic extraction solvent through the tube side for more than 200 h. A total of 71 g·liter^{-1} of propionic acid and 16 g·liter^{-1} of acetic acid was produced with a propionic acid productivity varying between 0.4 and 0.6 g·liter^{-1}h^{-1} during the fermentation (Table 25). Gu et al. (1999) also noted a partial selectivity of the extractive fermentation for propionic acid with a P/A ratio of 4.4.

New Process Technologies: Butyric Acid Production The development of processes for the production of butyric acid by fermentation has centered on two species of *Clostridium*, *C. butyricum* and *C. tyrobutyricum*. Experiments employing *C. tyrobutyricum* seem the most promising primarily because strains of this bacterium are highly selective for butyric acid over acetic acid, both of which are produced simultaneously during fermentation of carbohydrates. For example, in batch culture *C. butyricum* produces a butyric acid to acetic acid weight ratio (B/A) of 2.0–2.5, whereas the B/A for *C. tyrobutyricum* is 4.0–6.0. Thus, compared to *C. butyricum*, *C. tyrobutyricum* produces higher yields of butyrate per g of carbohydrate and higher product concentrations (see Table 26).

The most successful process reported for fermentation of glucose by *C. tyrobutyricum* was a continuous fermentation with cell recycle through a microfiltration membrane module for about 20 days (Michel-Savin, 1990c). With glucose fed to maintain 65 g·liter^{-1} and at a dilution rate D = 0.32 h^{-1}, an excellent butyric acid concentration of 29.7 g·liter^{-1} and a remarkable productivity of 9.5 g·liter^{-1}h^{-1} were obtained and

Table 26. Experimental fermentation processes for production of butyric acid.

Organism	Process	Substrate	Time of operation (h)	Product concentration ($g \cdot liter^{-1}$)	Product productivity ($g \cdot liter^{-1}\ h^{-1}$)	Yield for major product (g/g)	References
Clostridium tyrobutyricum CNRZ 596	Batch	Glucose	70	44	0.59	0.38	Michel-Savin et al., 1990b, c
	Continuous culture D = 0.1 h^{-1}			16.8	1.68	0.35	
	Continuous culture D = 0.2 h^{-1}			9.7	1.94	0.37	
	Continuous with cell recycle, UF		>400	29.7	9.5	0.45	
C. tyrobutyricum CIP I-776	Batch	Wheat flour hydrolysate (glucose)	140	45	0.32	0.34	Fayolle et al., 1990
	Fed batch, constant feeding		50	55.3	1.17	0.44	
	Fed batch, feeding controlled by gas production rate		50	62.8	1.25	0.45	
	Fed batch	Com steep liquor	120	72	0.6	0.48	
C. butyricum S21	Batch	Glucose	30	7.3	0.24	0.24	Zigova et al., 1999
	Extractive batch	Sucrose	45	10.0	0.23	0.30	
	Pertractive fed-batch	Sucrose	95	20.0	0.21	0.19	

Abbreviations: CIP, Culture Collection of the Institut Pasteur; CNRZ, Institute National de la Recherche Agronomique; S21, Strain isolated at Slovak Technical Institute, Bratislava; D, dilution rate; and UF, ultrafiltration.

remained constant for more than 15 days (see Table 26). Importantly, the selectivity for butyric acid production improved to a B/A ratio of 16, or 0.94 g of butyric acid per g of total acid produced. Fayolle et al. (1990) demonstrated fed-batch fermentation of wheat flour hydrolysate (glucose) or corn steep liquor by *C. tyrobutyricum* compared to batch fermentation (see Table 26). Again productivity was greatly improved over batch fermentation and selectivity for butyric acid reported was a B/A ratio of 10–30. The reason for the high selectivity for butyric acid observed for the *C. tyrobutyricum* fermentation may be related to the reutilization of acetate discovered by Michel-Savin et al. (1990a); see "Scientific Background" in this Chapter. In a very preliminary investigation, butyric acid production by *C. butyricum* was demonstrated with simultaneous extraction or pertraction of butyric acid using as an organic phase, Hostarex A327 (*n*-octyl[*n*-decyl]amine; 20% w/w in oleylalcohol; Zigova et al., 1999). They reported an increase in butyric acid concentration compared to the control batch fermentation with either integrated extraction or pertraction during the fermentation (see Table 26).

Final recovery and purification of organic acids from the fermentation broth is the most energy-intensive and costly stage in any bulk manufacturing process. Experiments using the modern solvents for simultaneous extraction during the fermentations of propionic or butyric acid resulted in rather low concentrations of organic acids (50–70 g·liter^{-1}), as shown in Tables 25 and 26. Thus further separation steps would be necessary. Playne (1985) reviewed nine different approaches to the problem of separation of organic acids available at that time, with no real solution. Boyaval et al. (1993) demonstrated the application of electrodialysis with bipolar membranes and electro-electrodialysis for concentration of propionic acid up to 130 g·liter^{-1} from fermentation broth with 40 g·liter^{-1}. Boyaval and Corre (1995) propose that this technique considerably improves the recovery and purification step, but it had not been evaluated for an industrial process at that time.

Propionic and Butyric Acid Production by Fermentation

Today, chemical synthesis of propionic and butyric acids dominates the production of these and most other organic acids used in industry. However, since these organic acids are added to products for human consumption by both the food and pharmaceutical industries, consumer demand for "natural sources" of these acids is substantial. Thus fermentation can play a role in supplying these markets. The prospect of future challenge of the dominant chemical methods by fermentation technology will depend upon decreased availability and increasing cost of ethylene, propylene, or other chemical precursors.

Developments of the propionic acid fermentation catalyzed by propionibacteria are outlined above (see "Areas of Research and Development" in this Chapter). First, selectivity for propionic acid was improved to better than 95% of total organic acids by using glycerol as a carbon source. Low-cost industrial-grade glycerol is available and is used in production of 1,3-propanediol by *C. butyricum* (Papanikolaou, 2000). Secondly, the research laboratories at INRA, France, combined new production technologies to demonstrate an economical production of propionic acid from cheese whey with propionibacteria (Colomban et al., 1993). Downstream processing with electrodialysis membrane modules facilitated recovery and purification from the fermentation medium (Boyaval et al., 1993). A second approach is the simultaneous extractive fermentation, using a hollow fiber membrane module and an amine extractant to keep the organic acid concentration in the growth medium low and to recover the propionic acid in a separate solution (Jin and Yang, 1998; Gu et al., 1999). Although this experimental work appears promising, these new technologies have yet to reach a commercial production stage.

Work on fermentation processes yielding butyric acid is not as advanced as that for propionic acid. *Clostridium tyrobutyricum* was found superior to *C. butyricum* in that *C. tyrobutyricum* is more selective, yielding 90% of total organic acids as butyric acid. Some preliminary studies gave high productivities and product concentrations (Fayolle et al., 1990; Michel-Savin et al., 1990c). Unfortunately, this clostridium will grow and ferment only glucose and fructose as carbon sources. However, there is every reason to be optimistic about the future of this fermentation for two reasons. First, a great deal of genetic and physiologic work on the closely related butanol-producing strains such as *C. acetobutylicum* has been done (see "Areas of Research and Development" in this Chapter). Thus, the panel of carbon sources available to *C. tyrobutyricum* can be changed easily. Secondly, fermentation processes for the production of 1,3-propanediol have been greatly developed (see "1,3- and 1,2-propanediols" in Butanol, Acetone, and Isopropanol in this Volume). The methods developed for production of 1,3-propanediol, employing strains of *C. butyricum*, will certainly be applicable to developing processes for butyric acid production (Papanikolaou et al., 2000).

Section 7: Ethanol

Introduction

Among alcohols, ethanol is the best known because it is the intoxicating ingredient of alcoholic beverages, which mankind has consumed since ancient times. The use of fermentation to convert starchy or sugary material into ethanol-containing liquid is thus among the earliest practices of applied microbiology. It has been suggested that alcoholic fermentation played a role in making water potable where other methods for the treatment of water were not available. The commercial value of the alcoholic beverages alone would rank ethanolic fermentation (for the production of wine and spirits) as one of the most important applications of microbial activities.

Despite the long practice of ethanolic fermentation by mankind, the active agent for this process was not known until the mid-nineteenth century. In the 1830s, after the successful synthesis of urea from inorganic materials, chemists debated intensely the subject of whether ethanol is a product of biological action. That yeast is the agent responsible for ethanol production in sugary liquid was eventually established by Louis Pasteur (1822–1895). Since the discovery of anaerobic bacteria by Pasteur, the number of known mesophilic ethanol-producing bacteria has been rising continuously. During the past 20 years, many saccharolytic thermophilic anaerobic bacteria have been isolated, and some of them can produce ethanol from hexoses and pentoses with a high yield (see Ethanol-producing Microorganisms in this Chapter).

Among ethanol-producing bacteria, *Zymomonas mobilis* is unusual in having an ethanol productivity higher than that of the yeast *Saccharomyces cerevisiae* and an ethanol tolerance comparable to that of the yeast (Buchholz et al., 1987). However, the substrate range of *Z. mobilis* is as limited as the yeast's. The high ethanol productivity of *Z. mobilis* is partly attributable to the high levels of pyruvate decarboxylase and alcohol dehydrogenase in *Z. mobilis*. This property has been utilized in the engineering (see Metabolic Engineering in this Chapter) of *Z. mobilis*, *Escherichia coli*, *Klebsiella oxytoca*, and other bacteria to create strains that can produce high yields of ethanol from all sugars that are monomers of hemicellulose and cellulose.

Ethanol has many uses. Besides being the key ingredient of alcoholic beverages, ethanol is traditionally used as an industrial chemical and as a component of pharmaceuticals and other healthcare and consumer products. As a chemical, ethanol can be produced either through chemical synthesis or by fermentation. Owing to changes in the availability and cost of the raw materials and the needs of the market, the major route for ethanol production has changed back and forth between fermentation and chemical synthesis since the 1930s. In the United States, synthetic ethanol began to dominate the market in the 1950s, and the trend continued until the early 1980s when fermentation again became the major route (see Commercial Production in this Chapter). At present, the United States production of fermentation ethanol at over 1.6 billion gallons a year has greatly exceeded previous levels achieved by the synthetic route. In Brazil, over 3.6 billion gallons of fermentation ethanol were produced during the 1997–1998 harvest (Zanin et al., 2000). This dramatic increase in ethanol production is because of the use of ethanol as an automobile fuel, which results from the jump in oil prices in the early 1970s and from the uncertainty of an unobstructed supply of crude oil. Ethanol has been used as a fuel for various purposes, and its use as an automobile fuel also has a long history (Jackson and Moyer, 1991). However, the extensive use of ethanol (as an automobile fuel) did not start until the implementation of the Brazilian National Alcohol Program (the Pro-Alcohol Program) in 1975 (Jackson and Moyer, 1991; Zanin et al., 2000).

Fermentation ethanol has traditionally been produced by using yeasts with sugars from corn, sugar beet, and sugarcane juice as the raw material. During the past 20 years, great advances have been made in the development of a bacterial process for the production of ethanol from lignocellulosic biomass (Sheehan, 2000). A commercial plant is under construction to use an engineered strain of *E. coli* to produce 20 million gallons of ethanol per year from sugarcane residues and rice hull (see Commercial Production). Further improvement of bacteria for the commercial production of ethanol from lignocellulosic materials should increase the proportion of ethanol produced by bacteria.

Fermentation ethanol that is produced from the lignocellulosic biomass has been identified as bioethanol, to distinguish it from ethanol produced from feed grains. Lignocellulosic materials represent the bulk of plant biomass and are a desirable source of energy because they are less expensive to produce than grains and are not used for food or feed. When taking into consideration the use of fuel through all stages of production, producing ethanol from lignocellulosic biomass requires much less fossil fuel than producing ethanol from feed grains. Up to 7 gallons of oil may be needed to produce 8 gallons of ethanol from feed grains, whereas only about one gallon of oil is needed to produce 7 gallons of ethanol from cellulosic biomass (Lugar and Woolsey, 1999). Comprehensive reviews on the

technology, economics, and policy issues of bioethanol production by bacterial fermentation are available (Himmel et al., 1997; Sheehan, 2000; Wyman, 2001).

Scientific Background

PHYSICOCHEMICAL PROPERTIES Ethanol or ethyl alcohol (CH_3CH_2OH, mol. wt. 46.07) is a colorless, flammable liquid. Anhydrous ethanol has a boiling point of 78.5°C and is hygroscopic. It has a specific gravity of 0.7893 at 20°C (relative to water at 4°C), or a weight of 6.578 lb (20°C) per gallon. Ethanol and water form an azeotrope, which has an ethanol concentration of 95.57% (by weight), 94.9% (by volume), or 92.3% (by weight of ethanol at 15.56°C). The azeotropic mixture has a boiling point of 78.15°C. Therefore, distillation of a fermentation broth yields the "95%" ethanol, which must be further processed to yield the anhydrous ethanol. The 95% ethanol can be used directly as a fuel for automobiles equipped with a specially designed engine (Jackson and Moyer, 1991; Zanin et al., 2000). However, anhydrous ethanol must be used when it is blended with gasoline.

After 1975, a major use for ethanol was as a fuel or fuel additive for automobiles (see Commercial Applications in this Chapter). Ethanol has a number of desirable properties when used as a fuel in an internal combustion engine (Winston, 1981; Jackson and Moyer, 1991). These desirable properties include: 1) High heat of combustion relative to volume. Ethanol has a heating value of 11,500–12,800 British thermal units (Btu)/lb, which is about two-thirds that of gasoline (18,900–20,260 Btu/lb). 2) High rate of flame propagation. The rate of flame propagation of ethanol is roughly comparable to that of gasoline. A high rate of propagation is important because the fuel in the combustion chamber must burn completely during the combustion stroke or it is blown out in the exhaust stroke without doing useful work. 3) High octane rating. The octane rating measures the relative tendency of fuels to pre-ignite, which is an undesirable property, when a mixture of fuel and air is compressed on the second stroke in an automobile (Otto cycle) engine. *Iso*-octane was chosen as a standard and assigned an octane rating of 100. Fuels that burn less readily than *iso*-octane are assigned octane ratings higher than 100. Ethanol has a Research Octane Number (RON) of 106 and a Motor Octane Number (MON) of 92. In comparison, "straight run" gasolines have RON and MON ranging as low as 80s and 60s, respectively. Gasoline sold as an automobile fuel in the United States generally has an octane rating between 87 and 93, which is based on (RON + MON)/2. Ethanol is therefore an effective octane-rating enhancer for gasoline and is a substitute for MTBE (methyl *tert*-butyl ether), which replaces the lead-based octane-rating enhancer in gasoline; however, use of MTBE in the United States is now being phased out because of its threat to the environment (Houge, 2000).

Ethanol has an oxygen content of 35% (on a weight basis) and is hence also described as an oxygenate when used as a fuel additive. When blended with gasoline, ethanol can improve the combustion of fuel in the automobile engine to reduce the amount of carbon monoxide in the exhaust gas (Sheehan, 2000). Therefore, besides its usefulness as an octane-rating enhancer, ethanol has been blended with gasoline to be used in regions where carbon monoxide emission from automobiles is a problem, especially in cold climate.

ETHANOL-PRODUCING MICROORGANISMS Ethanol, a product of anaerobic metabolism, is produced by a wide range of eukaryotic and prokaryotic microorganisms when growth or redox metabolism takes place under oxygen-free or low-oxygen conditions (Wiegel, 1980). Because alcohol dehydrogenases (see Toxicity of Ethanol to Microbial Cells) catalyze a readily reversible reaction, it is natural for short-term ethanol production to occur when the intracellular condition favors the formation of ethanol. Thus, even obligate aerobic bacteria like *Pseudomonas* and *Alcaligenes* can produce ethanol if they are incubated under anaerobic conditions (Vollbrecht and El Nawawy, 1980). In general, however, ethanol is a product of obligate and facultative anaerobes, including *Bacillus subtilis* (Nakano et al., 1997), which may produce ethanol when grown under anaerobic conditions in the absence of external electron acceptors.

Among ethanol-producing microorganisms, the yeast *Saccharomyces cerevisiae* is the best known, and it is still the most widely used organism for the production of industrial ethanol from glucose (from corn) and sucrose (from sugarcane or sugar beet). *Saccharomyces cerevisiae* can metabolize glucose, fructose, galactose, maltose and sucrose, but not starch or other polysaccharides (Helbert, 1982). The lager beer yeast *Saccharomyces uvarum* is sometimes used because it is more flocculent and sediments faster than *S. cerevisiae* (Reed, 1982). In addition to *S. cerevisiae* and *S. uvarum*, other ethanol-producing yeasts are used to ferment raw materials containing sugars not efficiently used by *S. cerevisiae* (Reed, 1982). *Candida utilis* is used for the fermentation of the sulfite liquor (waste from the paper industry) because it also ferments pentoses. The fermentation of whey (waste from

cheese-making) requires the use of a "dairy" yeast, such as *Kluyveromyces fragilis* and *Kluyveromyces lactis*, because the dairy yeasts can ferment lactose at a rate comparable to that of glucose utilization by *S. cereviase*. Besides yeasts, several other fungi within the genera of *Aspergillus*, *Fusarium* and *Penicillium* can produce more than a mole of ethanol per mole of glucose consumed, but these fungi do not grow or metabolize sugars as fast as yeasts or bacteria do (Wiegel, 1980).

Many bacteria have the ability to produce ethanol, but relatively few taxa contain bacteria that produce ethanol as a major end product. Ethanol-producing species can be found among aerobic, anaerobic and facultative anaerobic bacteria within the family Enterobacteriaceae and the genera *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Lactobacillus* and many others (Wiegel, 1980). Examples of mesophilic (Table 27) and thermophilic (Table 28) bacteria that can produce one or more moles of ethanol per mole of glucose metabolized have been compiled by Wiegel (Wiegel, 1980; Wiegel, 1992).

Mesophilic Ethanol-Producing Bacteria Several mesophilic species are potentially useful for the production of industrial or fuel ethanol. Among them, the facultative anaerobes *Escherichia coli*

Table 27. Mesophilic bacterial species producing ethanol as a major end product.

Organism	Moles of ethanol produced per mole of glucose metabolized
Clostridium sporogenes	Up to 4.15[a]
Clostridium indolis (pathogenic)	1.96[a]
Clostridium sphenoides	1.8[a] (1.8)[b]
Clostridium sordelii (pathogenic)	1.7
Zymomonas mobilis (syn. *Z. anaerobica*)	1.9
Zymomonas mobilis subsp. *pomaceae*	1.7
Spirochaeta aurantia	1.5 (0.8)
Spirochaeta stenostrepia	0.84 (1.46)
Spirochaeta litoralis	1.1 (1.4)
Erwinia amylovora	1.2
Leuconostoc mesenteroides	1.1
Streptococcus lactis	1.0
Sarcina ventriculi (syn. *Zymosarcina*)	1.0

[a]In the presence of high amounts of yeast extract.
[b]Values in parentheses were obtained with resting cells.
Adapted from Wiegel (1980).

Table 28. Thermophilic bacterial species producing ethanol as a major end product.

Organism	T_{max}	Moles of ethanol produced per mole of glucose metabolized
Thermoanaerobacter tengcongensis[a]	80	0.7
Thermoanaerobacter ethanolicus	78	1.8; 1.4 (xylose)
Thermoanaerobacter thermohydrosulfuricus (formerly *Clostridium thermohydrosulfuricum*)[b]	78	12–1.5
Bacillus stearothermophilus	78	1.0 (anaerobic, above 55°C)
Thermoanaerobacter brockii subsp. *brockii* (formerly *Thermoanaerobium brockii*)[b,c]	78	0.95
Thermoanaerobacter brockii subsp. *finnii* (formerly *Thermoanaerobacter finnii*)[c,d]	75	1.67; 1.26 (xylose)
Thermoanaerobacter mathranii[e]	75	1.1 (xylose)
Clostridium sp. (cellulolytic)	75	0.8
Thermoanaerobacterium thermosaccharolyticum (formerly *Clostridium thermosaccharolyticum*)[f]	68	1.1
Clostridium thermocellum	68	1.0

[a]Xue et al. (2001).
[b]Lee et al. (1993).
[c]Cayol et al. (1995).
[d]Wiegel (1992).
[e]Larsen et al. (1997).
[f]Collins et al. (1994) and Cann et al. (2001).
Adapted from Wiegel (1980), with the addition of new data.

and *Zymomonas mobilis* have been extensively investigated. The metabolic capabilities of *E. coli* (Ingram et al., 1998b; Ingram et al., 1999) and *Z. mobilis* (Zhang et al., 1995) have been expanded by genetic manipulations to enable these species to produce ethanol efficiently from sugars derived from hemicellulosic materials (see Metabolic Engineering in this Chapter). Besides the sugar-fermenting species, the CO-utilizing *Butyribacterium methylotrophicum* (Worden et al., 1991), *Clostridium ljungdhalii* (Tanner et al., 1993), and *Clostridium autoethanogenum* (Abrini et al., 1994) can produce ethanol from synthesis gas (CO plus H_2), which may be derived by gasification of biomass (Klasson et al., 1992; Philips et al., 1994; Clausen and Gaddy, 1996) or coal (Barik et al., 1988). Members of the genus *Acetobacterium*, including *Acetobacterium woodii*, *Acetobacterium carbinolicum* and *Acetobacterium wieringae*, which do not normally produce ethanol from glucose and CO_2, shift their metabolism to produce ethanol when the phosphate concentration of the growth medium is below a certain level (Buschhorn et al., 1989). The amount of ethanol produced by *A. woodii* is moderate (below 15 mM), but its production of ethanol in the stationary phase when there is an excess of glucose or fructose in the medium and when the phosphate concentration is low (below 8.4 mM) suggests an interesting regulatory mechanism. In addition, growth of *A. woodii* can tolerate an ethanol concentration up to 1 M (4.6%, w/v), which is unusual among bacteria (Buschhorn et al., 1989). Properties of extensively investigated mesophilic ethanol-producing bacteria are described below.

Zymomonas mobilis is a remarkable ethanol producer (Swings and De Ley, 1977; Rogers et al., 1982). It is one of the natural fermentative agents in the production of palm and cactus wines and the Mexican drink "pulque" and has been isolated from various fermenting, sugar-rich fluids (Rogers et al., 1982). About 20 different names have been used for this organism since its original naming as "*Termobacterium mobile*;" the names and the history have been reviewed (Swings and De Ley, 1977). *Zymomonas mobilis* is a facultative anaerobic rod (some strains are obligately anaerobic, whereas others can tolerate some O_2). It can produce almost 2 moles of ethanol and 2 moles of CO_2 from each mole of glucose or fructose metabolized. Some strains can also ferment sucrose, but no other carbon sources are used. *Zymomonas mobilis* is the only species in the genus (Swings and De Ley, 1984). It is excluded from the Enterobacteriaceae on the basis of its polar flagellation, its inability to reduce nitrates, its growth at pH 4, and its growth in the presence of 5% ethanol. Genetically, phenotypically, and ecologically, *Zymomonas* is related to the acetic acid bacteria, especially *Gluconobacter*: they both occur in acidic, sugary and alcoholic niches such as tropical plant juices and beer (Buchholz et al., 1987).

Zymomonas mobilis can produce ethanol to a concentration of 10–12%, which is comparable to the performance of the yeasts *S. cerevisiae* (Buchholz et al., 1987) and *S. uvarum* (Kosaric, 1996). Compared with the yeasts, the bacterium *Z. mobilis* has higher rates (two-fold or greater) of sugar uptake and ethanol production. It converts sugar to ethanol with a similar yield (above 90%) and tolerates high sugar concentrations (above 40%). *Zymomonas mobilis* ferments sugars exclusively via the Entner-Doudoroff pathway. The key intermediate of the Entner-Doudoroff pathway, 2-keto-3-deoxy-6-phosphogluconate (KDPG), is cleaved by KDPG aldolase to pyruvate and glyceraldehyde-3-phosphate, and the latter is further metabolized to pyruvate. Thus, two moles of pyruvate are produced per mole of glucose, but only one mole of ATP is obtained per mole of glucose consumed. *Zymomonas mobilis* uses pyruvate decarboxylase (PDC) to convert pyruvate directly to acetaldehyde and CO_2, whereas most other ethanol-producing organisms convert pyruvate to acetyl-CoA and CO_2 first and require an acetaldehyde dehydrogenase to convert acetyl-CoA to acetaldehyde, the substrate for alcohol dehydrogenase (ADH). The high ethanol productivity of *Z. mobilis* is correlated with the organism's high cellular level of PDC and ADH.

Under anaerobic growth conditions, *Escherichia*, *Klebsiella* and other enteric bacteria produce ethanol as a minor end product (Gottschalk, 1986). The native ethanol productivity of these bacteria is not useful for industrial applications. However, the enteric bacteria can ferment hexose as well as pentose sugars derived from hemicellulosic materials (Ingram and Doran, 1995a). To take advantage of their broad substrate range, the enteric bacteria have been recipients of the *Z. mobilis* PDC and ADH genes, and recombinant strains of *E. coli* will be used in the commercial production of fuel ethanol (see Metabolic Engineering in this Chapter).

In addition to the organisms that convert carbohydrates into ethanol, there are mesophilic anaerobic bacteria that can convert CO into ethanol. A strain of *Butyribacterium methylotrophicum* that was adapted to grow on 100% CO (with a doubling time of 12 h) produced small amounts of butanol and ethanol (Grethlein et al., 1990; Worden et al., 1991). Ethanol production in a continuous culture doubled (from 0.026 to 0.056 g/liter) when the pH decreased from 6.8 to 6.0 (Grethlein et al., 1990). *Clostridium autoethanogenum* is another anaerobe that can grow on 100% CO and pro-

duce ethanol besides acetate and CO_2 (Abrini et al., 1994). *Clostridium autoethanogenum* was isolated from rabbit feces, which is known to contain active CO-utilizing bacteria. Besides 100% CO, this organism can also grow on CO_2 plus H_2, pyruvate, arabinose, xylose, fructose, rhamnose and L-glutamate. From chicken yard waste, another CO-utilizing anaerobe, *Clostridium ljungdahlii*, was isolated (Barik et al., 1988; Vega et al., 1989; Tanner et al., 1993). *Clostridium ljungdahlii* can grow on CO, CO_2 plus H_2, ethanol, pyruvate, arabinose, xylose, fructose or glucose. The amount of ethanol produced by *C. ljungdahlii* was low initially (Klasson et al., 1992), but the productivity increased in response to changes in growth conditions (Philips et al., 1994). By maintaining the pH at 4–4.5 and using a nutrient-limited medium, the ethanol concentration was raised to 20 g/liter, whereas the acetate concentration was lowered to 2–3 g/liter (Clausen and Gaddy, 1996). Ethanol production from CO and H_2 (synthesis gas) has a low volumetric productivity, which is attributed to low cell densities, production of unwanted by-products, and slow transfer of the gases into the liquid phase (Worden et al., 1997). These engineering and biological problems will need to be resolved before synthesis gas, which may be generated through gasification of coal or cellulosic materials, can be biologically converted into ethanol on a commercial scale.

Thermophilic Ethanol-Producing Bacteria

Thermophilic bacteria have been proposed for the production of useful chemicals (Wiegel and Ljungdahl, 1986; Slapack et al., 1987; Lynd, 1989; Wiegel, 1992; Lowe et al., 1993). Wiegel (1980) listed seven advantages of using thermophilic anaerobic bacteria for ethanol fermentation. They include higher ethanol yields due to the utilization of a wide range of substrates as well as less biomass production (relative to aerobes), fast fermentation, little or no possibility for microbial contamination, no need for aeration or cooling, and a growth condition conducive to continuous distillation for the removal of ethanol and other volatile products. Commercial utilization of the thermophiles for ethanol production will be more attractive when genetic tools for engineering these organisms are further developed.

Ethanol-producing thermophiles are members of the genera *Clostridium*, *Thermoanaerobacterium* and *Thermoanaerobacter*. The latter two genera include several species that were at first identified as members of the genus *Clostridium* (Wiegel, 1992; Lee et al., 1993). The first thermophilic anaerobe that is still validly described (being included in the Approved Lists of Bacterial Names) is the cellulose-degrading, ethanol-producing *Clostridium thermocellum*; however, it took nearly 20 years after its first description before a pure culture was obtained (Wiegel, 1992). In early 2001, there are over 20 named species in the two genera *Thermoanaerobacterium* and *Thermoanaerobacter*. A taxonomic key was proposed for the preliminary identification of saccharolytic, ethanol-producing, thermophilic anaerobic bacteria (Lee et al., 1993). However, the description for the genus *Thermoanaerobacterium* has been emended concerning the location of sulfur droplets (Liu et al., 1996) or the lack of reduction of thiosulfate to sulfur (Cann et al., 2001). The lack of defining phenotypic characteristics for some species makes their assignment to a proper genus difficult. Therefore, the following taxonomic key may be revised when useful criteria for further differentiation emerge.

A. Does not reduce thiosulfate, ferments cellulose
   1. *Clostridium thermocellum* (Wiegel, 1992)
B. Does reduce thiosulfate, does not ferment cellulose
   1. May reduce thiosulfate to elemental sulfur
      Thermoanaerobacterium aotearoense (Liu et al., 1996)
      Thermoanaerobacterium polysaccharolyticum (Cann et al., 2001)
      Thermoanaerobacterium saccharolyticum (Lee et al., 1993)
      Thermoanaerobacterium thermosaccharolyticum (Cann et al., 2001)
      Thermoanaerobacterium thermosulfurigenes (Lee et al., 1993)
      Thermoanaerobacterium xylanolyticum (Lee et al., 1993)
      Thermoanaerobacterium zeae (Cann et al., 2001)
   2. Reduces thiosulfate to hydrogen sulfide (H_2S)
      Thermoanaerobacter brockii (Lee et al., 1993)
      Thermoanaerobacter ethanolicus (Lee et al., 1993)
      Thermoanaerobacter mathranii (Larsen et al., 1997)
      Thermoanaerobacter thermohydrosulfuricus (Lee et al., 1993)
      Thermoanaerobacter tengcongensis (Xue et al., 2001)
      Thermoanaerobacter wiegelii (Cook et al., 1996)

Among the ethanol-producing thermophiles, *C. thermocellum* was extensively studied for its ability to degrade cellulose and produce ethanol (Wiegel, 1980; Wiegel, 1992). Strains of *C. thermocellum* are readily isolated from nearly all

decaying organic material. They produce 0.3 to nearly 1 mole of ethanol per mole of glucose-equivalent contained in the cellulose utilized. It was possible to develop strains with an increased tolerance to ethanol (up to 8%; based on cellulose degradation) and an improved ratio of ethanol to acetate produced (from 1 : 1 to 10 : 1); however, growth of the mutants was still inhibited to 50% when 5% of ethanol was present in the growth medium (reviewed by Wiegel, 1980). Isolation of strains that tolerate 4–5% ethanol was reported (Rani and Seenayya, 1999). *Clostridium thermocellum* forms stable mixed cultures with saccharolytic organisms in a beneficial, syntrophic association. Cocultures of *C. thermocellum* and the saccharolytic ethanol-producing *Thermoanaerobacter ethanolicus* produced a significantly higher amount of ethanol from cellulose than the pure cultures produced (Wiegel, 1980), suggesting that the cellulolytic activity of *C. thermocellum* is in practice more important than its ethanol-producing activity for the production of ethanol from cellulose. Since the early 1980s, the cellulolytic enzymes of *C. thermocellum* have been subjects of active research, and the cellulosome, an extracellular supramolecular machine that can efficiently degrade crystalline cellulosic substrates, was identified through this research (Shoham et al., 1999).

The genera *Thermoanaerobacter* and *Thermoanaerobacterium* contain saccharolytic species with a high ethanol productivity. The thermophilic anaerobes that have T_{max} above 72°C and can produce more than 1.5 moles of ethanol per mole of glucose consumed are *Thermoanaerobacter ethanolicus* (including the former *Clostridium thermohydrosulfuricum* strain 39E), *Thermoanaerobacter brockii* subspecies *finii* (formerly *Thermoanaerobacter finii*), and *Thermoanaerobacter thermohydrosulfuricus*. Some of these thermophiles were isolated from hot springs, where the glucose concentration is not high. Therefore, the observed high ethanol productivity of the newly isolated strains, when provided with high glucose concentrations in the laboratory, may represent a lack of refined regulatory mechanisms for these organisms to respond to rich growth conditions. After repeated subculturing in a rich medium, these organisms may adapt to a metabolism producing more acetate and less ethanol (Wiegel, 1992).

Thermoanaerobacter ethanolicus has a broad pH optimum between 5.5 and 8.5 (growth occurs between pH 4.5 and 9.5), and it ferments glucose to ethanol (up to 1.8 moles ethanol per mole of glucose used) as efficiently as yeast and *Zymomonas* (Wiegel, 1992). In addition, *T. ethanolicus* has a broad substrate range: glucose, xylose, oligomers of xylose, xylan, mannose, ribose, arabinose, lactose, cellobiose, maltose, starch and pyruvate are used. These properties make *T. ethanolicus* an attractive candidate for the conversion of lignocellulosic material or whey into ethanol.

ENZYMOLOGY OF ETHANOL FORMATION Pyruvate is the central metabolic intermediate during sugar metabolism. The saccharolytic ethanol-producing bacteria can be divided into two groups based on how pyruvate is converted into the direct precursor for ethanol formation. One group, which encompasses the majority of fermentative bacteria, converts pyruvate into acetyl-CoA and CO_2, and acetyl-CoA is then reduced to acetaldehyde by aldehyde dehydrogenase. Like yeasts, the other group, including *Z. mobilis*, *Sarcina ventriculi*, *Erwinia amylovora*, and the acetic acid bacteria, converts pyruvate directly into acetaldehyde and CO_2 by pyruvate decarboxylase (Bringer-Meyer et al., 1986). Acetaldehyde is reduced to ethanol by alcohol dehydrogenase. The alcohol-producing organisms have multiple alcohol dehydrogenases (ADHs), and in many cases, the physiological importance of each ADH isozyme remains to be determined.

Pyruvate Decarboxylase The thiamine pyrophosphate- and Mg^{2+}-dependent pyruvate decarboxylase (EC 4.1.1.1) catalyzes the nonoxidative decarboxylation of pyruvate to form acetaldehyde and CO_2. Pyruvate decarboxylase (PDC) is widely present in plants and fungi, but its occurrence among the bacteria is limited. Constituting 4–6% of the total soluble protein of *Z. mobilis*, PDC is one of the most abundant proteins in this organism (Bringer-Meyer et al., 1986). Also, PDC has been purified from *Z. mobilis* (Hoppner and Doelle, 1983; Bringer-Meyer et al., 1986), and its structural gene has been cloned from two strains of *Z. mobilis* (Brau and Sahm, 1986; Conway et al., 1987; Neale et al., 1987; Reynen and Sahm, 1988). The *Z. mobilis* PDC is a tetrameric protein consisting of apparently identical subunits with a molecular weight of 56,500. The *pdc* gene of *Z. mobilis* ATCC 29191 encodes a polypeptide of 567 amino acids (Neale et al., 1987; Reynen and Sahm, 1988), whereas the *pdc* gene of *Z. mobilis* ATCC 31821 encodes a polypeptide of 559 amino acids (Conway et al., 1987). The difference involves two internal amino acids and seven at the C-terminus. The K_M of PDC for pyruvate was first reported at 4.4 mM (Hoppner and Doelle, 1983). But when the PDC activity was measured in the presence of Mg^{2+} (20 mM) and thiamine pyrophosphate (1.5 mM), a K_M of 0.4 mM was found (Bringer-Meyer et al., 1986). The lower K_M value of *Z. mobilis* PDC for pyruvate should allow the enzyme to compete favorably against the other pyruvate-utilizing

enzymes (e.g., pyruvate formate lyase [K_M = 2.05 mM] and D-lactate dehydrogenase [K_M = 4.4 mM]) in *E. coli* (Brau and Sahm, 1986). This difference in K_M values for pyruvate could explain the increase in ethanol production from 6.5 mM to 41.5 mM and the lack of acid production when a recombinant strain of *E. coli* harboring the *Z. mobilis pdc* gene was compared with the parent strain (Brau and Sahm, 1986). The apparent benefit of expressing *Z. mobilis* PDC in *E. coli* for ethanol production is confirmed by the successful engineering of *E. coli* for fuel ethanol production (see Metabolic Engineering in this Chapter). Antiserum raised against *Z. mobilis* PDC did not detect any hybridizing protein in cell extracts of *Sarcina ventriculi*, *Erwinia amylovora*, *Gluconobacter oxydans* or *Saccharomyces cerevisiae*, although the PDCs of *Z. mobilis* and *S. cerevisiae* have a similar molecular size and subunit structure (Bringer-Meyer et al., 1986).

In anaerobic bacteria, oxidation of pyruvate is generally catalyzed by a ferredoxin- or flavodoxin-linked pyruvate dehydrogenase (Chen, 1987; Chen, 1993) to form acetyl-CoA, CO_2, and reduced ferredoxin or flavodoxin. In CO-utilizing organisms, acetyl-CoA is synthesized by the nickel-containing CO dehydrogenase/acetyl-CoA synthase (Ragsdale and Riordan, 1996). Acetyl-CoA is reduced by acyl-CoA-dependent aldehyde dehydrogenases (ALDHs) to acetaldehyde.

CoA-Acylating Aldehyde Dehydrogenase The reduction of acetyl-CoA to acetaldehyde is catalyzed by CoA-acylating aldehyde dehydrogenase (EC 1.2.1.10), which catalyzes the reaction Acyl-CoA + NAD(P)H + H^+ ⇔ aldehyde + CoASH + $NAD(P)^+$. Although more than 150 full-length aldehyde dehydrogenase (ALDH) sequences are now compiled and aligned at the website (http://www.psc.edu/biomed/pages/researchCol_HBN_ALDH.html), relatively few ALDHs, especially CoA-acylating ALDHs, have been isolated and characterized from ethanol-producing organisms. Most organisms have several distinct ALDH genes, and 13 have been recognized in the *E. coli* genome (Perozich et al., 1999). Nevertheless, only the *adhE* gene has been shown to be responsible for the CoA-linked ALDH activity and is required for ethanol production in *E. coli* (Clark, 1989; Holland-Staley et al., 2000). The ADH-E protein of *E. coli* has been purified as a polymer, and it has a third function, the pyruvate-formate lyase (PFL) deactivase activity, besides the acetyl-CoA reducing activity and ADH activity (Kessler et al., 1991; Kessler et al., 1992). Based on its molecular size and its ADH and CoA-acylating ALDH activity, the ALDH purified from *E. coli* by Fromm and coworkers (Rudolph et al., 1968; Shone and Fromm, 1981) appears to be the *adhE* product. Results of steady-state measurements (in the direction of CoA-acylation) suggest that the enzyme uses a bi-uni-uni-uni ping-pong mechanism in which NAD^+ binds to the free enzyme followed by acetaldehyde; the product NADH is then released before CoASH can bind, and acetyl-CoA is the final product released (Shone and Fromm, 1981).

In the butanol- and ethanol-producing clostridia, two types of CoA-acylating ALDHs have been found (Toth et al., 1999). *Clostridium acetobutylicum* has an *adhE*- or *aad*-encoded enzyme very similar to the ADH-E of *E. coli* (Fischer et al., 1993; Nair et al., 1994), whereas *Clostridium* species NRRL B643 (Palosaari and Rogers, 1988) and *C. beijerinckii* (Yan and Chen, 1990; Toth et al., 1999) have a dimeric enzyme with a subunit of 55–56 kDa. The gene (*ald*) encoding the *C. beijerinckii* ALDH has been cloned and sequenced (Toth et al., 1999). Results of Southern analysis suggest that each species of the butanol- and ethanol-producing clostridia uses either an aldehyde-alcohol dehydrogenase or an aldehyde dehydrogenase, but not both, for the reduction of acyl-CoA to the respective aldehyde. The ALDH of *C. beijerinckii* is most similar to the *eutE*-encoded ALDH of *Salmonella typhimurium* and *E. coli*, which use the ALDH during growth on ethanolamine.

Alcohol Dehydrogenase Alcohol dehydrogenase (ADH; EC 1.1.1.1 for NAD^+-linked ADH; EC1.1.1.2 for $NADP^+$-linked ADH; EC 1.1.1.71 for $NAD(P)^+$-linked ADH) catalyzes the readily reversible reaction: aldehyde or ketone + NAD(P)H + H^+ ⇔ primary or secondary alcohol + $NAD(P)^+$. ADHs may be differentiated by their coenzyme and substrate specificities, metal requirement, and the number and size of their subunits (Reid and Fewson, 1994).

Multiple ADHs are often present in an organism (Chen, 1995). Two ADHs have been found in *Z. mobilis* (Wills et al., 1981; Kinoshita et al., 1985; Neale et al., 1986). Both ADHs use NADH to reduce acetaldehyde to ethanol and were reported to be dimeric or tetrameric. ADH-I is a zinc enzyme with a subunit molecular weight of 40,000 (Kinoshita et al., 1985; Neale et al., 1986), whereas ADH-II is activated by iron (Scopes, 1983; Neale et al., 1986) and has a subunit molecular weight of 37,000 (Neale et al., 1986) or 38,000 (Kinoshita et al., 1985). ADH-II accounts for 90% of ADH activity in cell extracts (Neale et al., 1986). It has been proposed that ADH-I forms a complex with glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (Aldrich et al., 1992). Mutants lacking ADH-II but not ADH-I have been obtained

(O'Mullan et al., 1995). These mutants were isolated by using allyl alcohol as the selecting agent. ADH-I mutants may not be detectable by this procedure because of the high activity of ADH-II, which would reduce allyl alcohol to toxic acrolein more rapidly than would ADH-I, and perhaps because of the presence of ADH-I in an enzyme complex. The level of ADH-I and ADH-II can be modulated by iron and zinc (Mackenzie et al., 1989). The structural gene for ADH-I, designated *adhA*, has been cloned and sequenced (Keshav et al., 1990), and it encodes a polypeptide of 337 amino acids with a molecular weight of 36,096. The structural gene for ADH-II, designated *adhB*, encodes a polypeptide of 383 amino acids with a molecular weight of 40,141 (Conway et al., 1987; Yoon and Pack, 1990). The *adhB* gene of *Z. mobilis* has been used in the engineering of *E. coli* for ethanol production from lignocellulosic material (see Metabolic Engineering in this Chapter).

ADHs have been purified from the ethanol-producing thermophiles *Thermoanaerobacter brockii* subsp. *brockii* (Lamed and Zeikus, 1981) and *Thermoanaerobacter ethanolicus* (Bryant et al., 1988; Burdette and Zeikus, 1994). Although these thermophiles do not produce secondary alcohols, an NADP(H)-dependent ADH purified from these organisms is active in reducing both aldehydes and ketones to the respective primary or secondary alcohols. In addition, the secondary ADH from *T. ethanolicus* 39E can produce ethanol from acetyl-CoA, indicating the presence of acetyl-CoA reductive thioesterase (CoA-acylating ALDH) activity in the enzyme (Burdette and Zeikus, 1994). The structural gene for the primary-secondary ADH has been cloned from *T. ethanolicus* 39E (Burdette et al., 1996) and *T. brockii* subsp. *brockii* (Peretz et al., 1997). X-ray crystallographic data (Korkhin et al., 1998) show that the primary-secondary ADH of *T. brockii* subsp. *brockii* is structurally related to the primary-secondary ADH of *Clostridium beijerinckii* NRRL B593, which produces isopropanol in addition to *n*-butanol and ethanol (Korkhin et al., 1998).

Fermentative growth of *E. coli* depends on the *adhE*-encoded protein, which is a polypeptide of 891 amino acids, with a molecular weight of 96,008, and has CoA-acylating ALDH activity in addition to ADH activity (Clark, 1989; Goodlove et al., 1989). Thus, ADH-E is responsible for the two reductive reactions in converting acetyl-CoA to ethanol and the regeneration of NAD^+. The *adhE* gene is expressed only under anaerobic growth conditions, and the ADH-E protein is inactive in air. Interestingly, mutations in the promoter region and in the coding region of *adhE* allowed production of active ADH-E under aerobic growth conditions, and the amino acid Glu568 alone determines the oxygen sensitivity of ADH-E (Holland-Staley et al., 2000). In addition to the aldehyde-alcohol dehydrogenase, *E. coli* has a 1,2-propanediol oxidoreductase (encoded by the *fucO* gene), which has 41.7% identity at the amino acid level with the iron-containing ADH-II of *Z. mobilis* (Conway and Ingram, 1989).

Toxicity of Ethanol to Microbial Cells

Short-chain alcohols (C_1 to C_5) elicit a number of cellular responses that are potentially cytotoxic, and this cytotoxicity is manifested in all cell types, although the threshhold level of alcohol cytotoxicity may vary from organism to organism (Baker and Kramer, 1999). Effects of alcohols on microorganisms have been reviewed (Ingram and Buttke, 1984; Ingram, 1986; Buchholz et al., 1987; Mishra and Kaur, 1991). Reviews are also available on ethanol tolerance in bacteria (Ingram, 1990), *E. coli* (Ingram and Dombek, 1989), *Z. mobilis* (Ingram and Dombek, 1989; Rogers et al., 1989) and clostridia (Linden and Kuhn, 1989). Cytotoxicity is the basis for the practical use of alcohols as a disinfectant or as a preservative, but it is also a major factor limiting the final ethanol concentration during commercial fermentation.

The cytotoxicity of short-chain alcohols ranges from reversible effects to cell death. Besides the toxicity to microbes, human exposure to ethanol and other short-chain alcohols in the environment is widespread because these alcohols are extensively used as industrial solvents and are increasingly used as a component of automobile fuels. In mammalian cells, aberrations in phospholipid and fatty acid metabolism, changes in the cellular redox state, disruptions of the energy state, and increased production of reactive oxygen metabolites have been implicated in cellular damage resulting from acute or chronic exposure to short-chain alcohols. Such cellular damage can result in disruptions of the intracellular signaling cascades and decreases in phosphorylation potential and lipid peroxidation, which are the suggested mechanisms by which alcohols can affect the rate of cell proliferation (Baker and Kramer, 1999). Nonoxidative metabolism of short-chain alcohols, including synthesis of alcohol phospholipids and fatty acid alcohol esters, is an additional mechanism by which alcohols can affect membrane structure and compromise cell function. The reasons for specific cell types to exhibit different sensitivities to individual short-chain alcohols have been attributed to unique membrane compositions (Buchholz et al., 1987; Ingram, 1990) and to unique metabolites produced in the affected cell and to perturbations of functions unique to the cell type (Baker and Kramer, 1999).

Concentrations of ethanol above 15% result in immediate inactivation of most vegetative organisms, with spores being considerably more resistant, whereas low concentrations of ethanol also render bacteria more sensitive to inactivation by ionizing radiation and by lipophilic acids (Ingram and Buttke, 1984). The toxicity of ethanol to yeasts and bacteria has a major impact on the cost of industrial ethanol fermentation. As ethanol accumulates in the growth medium, the rate of conversion of sugars into ethanol declines progressively and the fermentation terminates when an ample supply of substrates is still available in the medium. The limit of the achievable ethanol concentration in a reactor and the decreasing rate of ethanol accumulation during fermentation increase the cost of the process because the production facility cannot be used more efficiently. It is thus important to understand the mechanism of ethanol toxicity so that bacterial strains more tolerant to ethanol may be developed for industrial uses. However, when studying ethanol toxicity or tolerance, it is useful to consider that rather than the increasing ethanol concentration, other growth-stage related changes in the medium composition might inhibit growth. It has been reported that salt accumulation resulting from the addition of base for pH control, not ethanol, limits the growth of an ethanol-producing organism (Lynd et al., 2001).

The cell membrane has been identified as the prime target for the toxic effect of ethanol, but other cellular components can also be deleteriously affected by ethanol to contribute to the cytotoxicity of ethanol (Ingram, 1986; Ingram and Dombek, 1989; Mishra and Kaur, 1991). A primary action of ethanol in biological systems is to increase membrane leakage. The rates of leakage of hydrogen, magnesium, and potassium ions increased with added ethanol. Nucleotides are lost from cells at a surprising rate in the absence of ethanol, and this rate of loss is accelerated by the addition of ethanol (Ingram, 1990). Loss of magnesium ions and nucleotides can cause a decrease in glycolytic flux and hence lower fermentation activity. At higher ethanol concentrations, the integrity of the membrane is further disrupted, and large molecules like proteins are also lost and cell death will follow.

From a consideration of the possible structural and functional changes in the membrane as a result of a change in the fatty acyl composition of the membrane lipids, it is possible to infer the mechanism by which ethanol inhibits growth and survival through its actions on the plasma membrane (Ingram, 1986; Ingram, 1990). Ethanol exerts its effects on the membrane by affecting hydrophobic associations. Thus, the potency of alcohols as inhibitors of cellular functions is directly related to hydrophobicity, rather than involving specific steric interactions or receptors. Ethanol is less polar than water but more polar than the hydrophobic core of the membrane. High concentrations of ethanol in the culture fluid and cytoplasm alter the colligative properties of the culture fluid and cytoplasm. Ethanol increases the dielectric strength and the strength of ionic or coulombic interactions in the aqueous milieu, hence causing a decrease in the ability of the aqueous environment to accommodate charged species and a shift of the p*Ka* of all ionizable groups toward the conjugate neutral form. Such changes may alter the conformation of and interactions between macromolecules, the pH optima for enzymic reactions, or the pH of the environment itself (Ingram, 1990).

When situated within the hydrophobic core of the membrane, ethanol decreases the dielectric strength of the membrane, the primary barrier for the cell, which in turn increases the ability of the membrane core to accommodate charged or polar molecules. The passage of molecules across the membrane is restricted primarily by the energy barrier for their transfer from an aqueous to a hydrophobic environment. Ethanol decreases this energy barrier both by its effects on the aqueous milieu (decreasing polarity) and its effects on the hydrophobic core of the membrane (increasing polarity), thus increasing the permeability of the membrane to polar and charged molecules (Ingram, 1990).

Research conducted with *E. coli* and *S. cerevisiae* shows that in response to elevated levels of ethanol in the surrounding medium, the composition of the fatty acids in the cell membrane changes with an increase in the proportion of unsaturated long-chain fatty acids, especially the *cis*-18:1 (Δ11) fatty acid (vaccenic acid) for *E. coli* and the *cis*-18:1 (Δ) fatty acid (oleic acid) for *S. cerevisiae* (Ingram, 1986). These findings suggest that the changed fatty acid compositions of the membrane lipids and hence the properties of the cell membrane may afford the cells a greater tolerance toward ethanol. The fatty acid composition of the membrane lipids of naturally ethanol-tolerant organisms provides supporting evidence for this relationship.

Zymomonas mobilis, *Lactobacillus homohiochii* and *Lactobacillus heterohiochii* are among the most ethanol-tolerant organisms so far identified. (*Lactobacillus heterohiochii* is now considered a subjective synonym of *Lactobacillus fruitivorans* because of high DNA/DNA homology between the strains; Weiss et al., 1983.) *Zymomonas mobilis* is unusual among the ethanol-producing bacteria in that it can produce ethanol to a concentration of 12% (w/v), which is comparable to that of the yeast *S. cerevisiae*. In comparison, growth of *A. woodii* can tolerate an

ethanol concentration of 4.6% (w/v), which is considered high for bacteria (Buschhorn et al., 1989). The membrane of *Z. mobilis* maintains a stable and exceptionally high amount (70% of the fatty acids) of vaccenic acid and unusual hopanoids, which are evolutionary precursors of sterols (Buchholz et al., 1987). Ethanol-dependent changes in the membranes of *Z. mobilis* include a decrease in the phospholipid content, an increase in the proportion of cardiolipin and phosphatidylcholine, and an increase in the proportion of hopanoids. *Lactobacillus homohiochii* and *L. heterohiochii* are not ethanol-producing bacteria; they were originally isolated as spoilage organisms from Japanese rice wine (Kitahara et al., 1957; Demain et al., 1961). These two lactobacilli can grow in ethanol concentrations above 16% (w/v), and both organisms exhibit ethanol-inducible changes in fatty acid composition. During growth in the presence of high concentrations of ethanol, the membranes of *L. homohiochii* contain less than 3% of saturated fatty acids, with the balance being primarily the *cis*-18:1 (Δ11) vaccenic acid (Uchida, 1975b). *Lactobacillus heterohiochii* exhibits an ethanol-inducible increase in the proportion of long-chain, *cis*-mono-unsaturated fatty acids including 18:1, 20:1, 22:1, 24:1, 26:1, 28:1, and 30:1 fatty acids (Uchida, 1974; Uchida, 1975a). During growth in the presence of ethanol, over 30% of the fatty acyl chains in the membranes of *L. heterohiochii* are monounsaturated and have a chain length of 20 or more carbons, which is a property not yet observed in any other organism (Ingram, 1986).

The relationship between the fatty acid composition of the membrane lipids and an organism's ethanol tolerance also has been investigated using mutants of *E. coli*. The membrane fatty acyl composition of *E. coli* is easily manipulated using mutants defective in lipid biosynthesis and fatty acid supplements (Ingram and Buttke, 1984). Mutant strains of *E. coli*, which are unable to synthesize vaccenic acid (an 18:1 fatty acid), contain membranes composed of nearly equal proportions of palmityl and palmitoleyl residues. These mutants are hypersensitive to growth inhibition and death caused by exposure to ethanol in comparison to the wildtype cells. Exogenously added fatty acids are readily incorporated into *E. coli* cells, and addition of 18:1 fatty acids to the growth medium resulted in an increase in the growth rate of the mutant in the presence of ethanol and a restoration of the tolerance level to that of the wildtype strain. No other fatty acids were found beneficial. Similar enhancement in ethanol tolerance was observed in ethanol-producing yeasts when the fermentation medium was supplemented with unsaturated fatty acids or sterols (Ingram and Buttke, 1984; Mishra and Kaur, 1991). These studies have led to the conclusion that an elevated level of ethanol tolerance involves an increase in the fatty acyl chain length and in the proportion of *cis*-mono-unsaturated fatty acids in the membrane lipids, and with *E. coli* at least, the increase in chain-length appears to be more significant than the increase in *cis*-unsaturation. The adaptative changes in the fatty acyl composition of the membrane of the ethanol-tolerant cells indicate that the plasma membrane is a flexible system that may be manipulated by genetic engineering to increase the ethanol tolerance of the cell.

Metabolic Engineering Efforts to use genetic tools to improve bacteria for ethanol production have focused on the enteric bacteria (Ingram et al., 1998b; Ingram et al., 1999) and *Z. mobilis* (Himmel et al., 1997). The rationale for this endeavor resides in the fact that for subtropical or temperate regions the abundant and renewable lignocellulosic biomass is the only choice of carbon substrates for an expanded fermentation industry because the starchy grains will be increasingly needed as food and feed and hence cannot meet the growing need of the ethanol industry. The yeast *S. cerevisiae* is not chosen for several reasons: 1) it cannot efficiently use the pentose sugars that are the major component of hemicellulose, 2) it is less amenable to genetic manipulation than the prokaryotes, and 3) it has different requirements for O_2 during growth and during ethanol production, which practically limit the duration of the production period. On the other hand, enteric bacteria can ferment these pentose sugars, and *Z. mobilis* has superb ethanol tolerance and high specific rates of sugar uptake and ethanol production, both under anaerobic conditions. Therefore, efforts of metabolic engineering have been directed to either expanding the substrate range of *Z. mobilis* or increasing the ethanol productivity of enteric bacteria.

Z. mobilis Results of earlier genetic studies with *Z. mobilis* have been reviewed (Rogers et al., 1982), and mutants with better ethanol tolerance and better viability in liquid media were selected. The use of recombinant DNA techniques allowed the construction of a chimeric plasmid carrying a xylose assimilation operon (*xylA* and *xylB* genes under the promoter for glyceraldehyde-3-phosphate) and a pentose phosphate pathway operon (*tal* and *tktA* genes under the promoter for enolase) consisting of the *E. coli* genes or gene homolog encoding xylose isomerase (*xylA*), xylulokinase (*xylB*), transaldolase (*tal*) and transketolase (*tkt*) under the control of strong promoters from *Z. mobilis*

(Zhang et al., 1995). The recombinant *Z. mobilis* harboring the plasmid expresses the respective enzymes, can grow on xylose as the sole carbon source, and produces 1.43 moles ethanol per mole of xylose consumed (86% theoretical yield). In the presence of a mixture of glucose and xylose, the recombinant strain fermented both sugars to ethanol at 95% of theoretical yield within 30 hours. Glucose is used at a faster rate than xylose by the recombinant strain, although there is not an apparent diauxic effect. A recombinant strain of *Z. mobilis* capable of fermenting arabinose to ethanol has also been constructed (Himmel et al., 1997). These accomplishments provide a foundation for the further broadening of the substrate range of *Z. mobilis*.

Enteric Bacteria The metabolic engineering of enteric bacteria for the production of ethanol from hexose and pentose sugars, the major components of hemicellulose syrup, has been reviewed (Ingram and Doran, 1995a; Ingram et al., 1998b; Ingram et al., 1999). Initial efforts involved the introduction of recombinant plasmids carrying either the *pdc* gene or the *pdc* and *adhB* genes of *Z. mobilis* into enteric bacteria to obtain high ethanol production from pentose and hexose sugars. When the *pdc* gene alone was introduced into wildtype *Klebsiella planticola* (Tolan and Finn, 1987) or *K. planticola* mutant deficient in pyruvate formate lyase (Feldmann et al., 1989) or *E. coli* (Brau and Sahm, 1986), increased ethanol production from xylose or glucose was observed. However, the recombinant strains exhibited plasmid instability, decreased ethanol tolerance, decreased growth rate under fermentative conditions, decreased cell yield, incomplete conversion of substrate, and accumulation of acidic fermentation products. When recombinant plasmids carrying both *pdc* and *adhB* genes of *Z. mobilis* were introduced into *E. coli* (Ingram et al., 1987; Ingram and Conway, 1988; Neale et al., 1988) or *Klebsiella oxytoca* (Ohta et al., 1991b), the central metabolism was effectively redirected into ethanol production under both aerobic and anaerobic conditions, resulting in diminished acid production and dramatically increased final cell density. The ADH-II of *Z. mobilis* has a K_M of 12 μM for NADH, which is much lower than those of the competing dehydrogenases of *E. coli* (Ingram and Conway, 1988). A high level of ADH-II activity in the recombinant *E. coli* results in efficient removal of acetaldehyde to increase ethanol production, to facilitate the continued action of pyruvate decarboxylase, and to prevent the production of acids. The results indicate that the acids produced by enteric bacteria are more damaging than ethanol is to the cell (Ingram and Conway, 1988).

For an efficient transfer of both the *pdc* and the *adhB* genes of *Z. mobilis* into different recipient cells, an artificial operon, dubbed the "PET operon" for the production of ethanol, has been constructed as a portable ethanol production cassette (Ingram et al., 1987). The PET operon consists of the *Z. mobilis pdc* and *adhB* genes under the control of the *lac* promoter, and it was initially introduced into recipient enteric bacteria on a pUC18 plasmid. Use of a putative *Z. mobilis* promoter in place of the *E. coli lac* promoter for the PET operon gave optimal expression of PDC and ADH-II activities and much higher ethanol production in recombinant *E. coli* strains harboring the plasmid (Ingram and Conway, 1988).

To alleviate the problem of plasmid instability, *Z. mobilis pdc* and *adhB* genes were successfully inserted into the chromosome of *E. coli* at the *pfl* site under the control of the *pfl* promoter (Ohta et al., 1991a). The *pfl* site (encoding pyruvate formate lyase or PFL) was chosen because PFL represents a competing branch point (causing diversion of pyruvate to acid production) and because the *pfl* gene is expressed at very high levels during anaerobic growth. The latter is important for high-level expression of PDC and ADH-II from single-copy genes in *E. coli*. Additional genetic modifications, which include a selection for resistance to a high level of chloramphenicol, inactivation of succinate production and block of homologous recombination, were required to increase the level of expression, to decrease acid production, and to ensure genetic stability. The resulting *E. coli* strain was designated *E. coli* KO11 (Ohta et al., 1991a). Ethanol concentrations of 54.4 and 41.6 g/liter were obtained from 10% glucose and 8% xylose, respectively. Although the high yield was partly attributed to the utilization of fermentable complex nutrients in the medium, that *E. coli* can accumulate ethanol above 5% is significant. *Escherichia coli* KO11 has since been used widely to demonstrate the fermentation of hemicellulose hydrolysates from various lignocellulosic materials (Ingram et al., 1999). *Zymomonas mobilis pdc* and *adhB* genes have also been integrated into the *pfl* gene on the chromosome of *K. oxytoca* to enable the recombinant strain P2 to produce ethanol from cellulose and hemicellulose (Wood and Ingram, 1992). In 1991, the use of recombinant *E. coli* KO11 harboring the *Z. mobilis pdc* and *adhB* genes for ethanol production was awarded the United States patent no. 5,000,000 (Ingram et al., 1991).

When autoclaved crystalline cellulose is the substrate, commercial fungal (*Trichoderma*) cellulase is added to the growth medium to allow simultaneous saccharification and fermentation or SSF (Doran and Ingram, 1993; see "Polyhy-

droxyalkanoic Acids" in Organic Acid and Solvent Production, Part I in this Volume). When sugarcane bagasse is the substrate, a pretreatment is essential to render the cellulose accessible to enzymic digestion. Either ammonia freeze explosion (AFEX) or hydrolysis of hemicellulose with dilute sulfuric acid is an effective pretreatment to increase cellulose digestibility (Doran et al., 1994). At present, low sugar concentrations in hemicellulose hydrolysate rather than alcohol tolerance of the bacteria limit the final concentration of ethanol achieved during fermentation.

During the pretreatment of lignocellulose with acid, substances toxic to enteric bacteria are produced. At present, additional treatments of the acid hydrolysate are necessary, which include the separation of liquid (hemicellulose syrup) and solid (cellulose and lignin) followed by washing and detoxification steps. The hemicellulose syrup containing the monosaccharides of hemicellulose is fermented to ethanol by *E. coli* KO11. The fibrous solid containing cellulose and lignin is converted to ethanol by SSF using recombinant *K. oxytoca* and commercial fungal cellulase, with the lignin-rich residue after SSF burned to provide energy (Ingram et al., 1999).

Other Bacteria The *Z. mobilis pdc* gene was introduced into the soft-rot bacterium *Erwinia chrysanthemi* to produce ethanol from xylose and arabinose (Tolan and Finn, 1987). Plasmids carrying the PET operon have been introduced into the soft-rot bacteria *Erwinia carotovora* and *E. chrysanthemi* (Beall and Ingram, 1993). These plant pathogenic bacteria have the native ability to secrete a battery of hydrolases and lyases to aid in the solubilization of lignocellulose and to macerate and penetrate plant tissues. Strains harboring the PET operon produced ethanol efficiently from glucose, cellobiose, and xylose. The PET operon has also been introduced into *Bacillus subtilis* and *Bacillus polymyxa* (de F. S. Barbosa and Ingram, 1994). The *Z. mobilis* genes encoding PDC and ADH are expressed in these recombinant strains; however, the level of expression is not high enough for ethanol production. The presence of multiple proteinases in these Gram-positive bacteria may limit high-level expression. Although the glycohydrolases of Gram-positive organisms may prove advantageous for the production of ethanol from cellulosic material, further improvement in the expression level of the introduced ethanol-pathway enzymes will be needed to make the recombinant strains useful.

Commercial Applications

Commercial Production Ethanol has traditionally been produced by chemical synthesis (mostly via catalytic hydration of ethylene) or by fermentation using yeasts. The synthetic procedures for ethanol production can be found in Lowenheim and Moran (1975) or Logsdon (1994), and the ethanolic fermentation using yeasts can be found in Reed (1982). The bacterial fermentation to be used by BC International Corp. (McCoy, 1998) at its plant in Jennings, Louisiana, is based on United States patent no. 5,000,000 awarded to Dr. L. O. Ingram and coworkers (Ingram et al., 1991) for a recombinant *E. coli* that can ferment both pentose and hexose sugars to ethanol (see Metabolic Engineering in this Chapter).

The proportion of ethanol produced by the synthetic and the fermentation processes is largely determined by the production costs, the market condition, and the government policies at the time. In 1935, 90% of the United States supply of industrial ethanol came from fermentation plants; less than 10% was of synthetic origin. By 1954, 70% was synthetic, and by 1963, synthetic ethanol accounted for 91% of the production (Lowenheim and Moran, 1975). However, the trend has reversed since the early 1980s. During the past 25 years, the annual production of ethanol by the United States and Brazil, the two largest ethanol producers in the world, increased dramatically (Table 29), whereas the United States production of synthetic ethanol decreased from a peak of 360 million gallons in 1969 (Anonymous, 1979) to 100 million gallons or less in the early 1990s (Table 29). The United States ethanol production is about 1.7 billion gallons per year; roughly 350 million gallons (20%) are used in industry and beverage-making. The unprecedented increase in ethanol production in the United States after 1980 Renewable Fuels Association (http://www.ethanolrfa.org) and Brazil after 1975 (Zanin et al., 2000) is mainly because of the use of ethanol in the automobile fuel to reduce the consumption of gasoline. With properly modified gasoline engines, anhydrous ethanol can be blended with gasoline up to a concentration of 85% ethanol (E85) for automobile use, whereas hydrated ethanol (the azeotropic mixture with water, containing about 95% ethanol) can be used as straight motor fuel in alcohol-fueled automobiles (Jackson and Moyer, 1991; Zanin et al., 2000). Dehydration of ethanol can now be accomplished by using a molecular sieve dehydrator according to Commercial Alcohols, Inc. (http://www.comalc.com), which is the largest alcohol producer and distributor in Canada, or by the traditional procedure of adding benzene to form a ternary azeotrope containing 74% benzene, 18.5% ethanol, and 7.5% water, which has a boiling point of 68°C, much lower than that (78.15°C) of the ethanol-water azeotrope. Fuel ethanol and industrial ethanol can be

Table 29. Production of ethanol in the United States and Brazil (in million gallons).

Year	United States		Brazil
	Synthetic[a]	Fermentation[b]	Fermentation[c]
1975	217.2		132
1976	227.4		
1977	203.6		
1978	192.6		
1979	214.1	~10[d]	
1980	220.6	175	
1981	200.2	215	
1982	155.5	350	
1983	163.7	375	
1984	161.1	430	
1985	98.7	610	
1986	80.4	710	3,434
1987	87.3	830	
1988	85.4	845	
1989	83.5	870	
1990	83.0	900	
1991	80.0	950	
1992	106.1	1,100	
1993	103.1	1,200	
1994	98.5	1,350	
1995	95.2	1,400	
1996		1,100	
1997		1,300	3,471
1998		1,400	3,646
1999		1,470	
2000		1,630	

[a]Converted from data in million lbs, as collected by the International Trade Commission and reported annually in *Chemical & Engineering News* until 1995, which was the last year that such data were collected or published.
[b]From the Renewable Fuels Association (Washington, DC, United States).
[c]Converted from data in million or billion liters as reported in Zanin et al. (2000).
[d]Sheehan (2000).

manufactured by the same fermentation process, but with different finishing steps.

Essentially all of the fermentation ethanol is produced from sugars derived from corn (Table 30) and sugarcane juice (Zanin et al., 2000) using yeasts as the fermentation agent. However, BC International is building a production facility in Louisiana, in the United States, that will use *E. coli* KO11 (see Metabolic Engineering in this Chapter) to convert sugars present in bagasse and rice hull to ethanol (McCoy, 1998; Table 30). The BC International facility has an annual production capacity of 20 million gallons, which is comparable to or larger than the capacity of over 35 United States companies that are producing fuel ethanol from grains or waste materials (http://www.ethanolrfa. org). Positive results at the BC International plant can lead to expanded commercial uses of recombinant bacteria for the conversion of lignocellulosic material to ethanol.

Non-fuel Uses Alcoholic beverages are perhaps the most important use for ethanol, other than its industrial uses and its use as a fuel. The use of pure ethanol in alcoholic beverages is likely limited, because besides neutral spirits such as Vodka, ethanol is but one of the many crucial ingredients of each distinctive beverage. Useful information on the processes for the manufacturing of alcoholic beverages can be found in the following references: wine (Benda, 1982), beer (Helbert, 1982) and distilled spirits (Brandt, 1982).

Ethanol is one of the most versatile oxygen-containing organic chemicals. It has important uses as a solvent and in the manufacturing of other organic chemicals, pharmaceuticals, perfumes, toiletries, detergents, flavors, disinfectants and other products (Logsdon, 1994). The consumption of ethanol in these non-fuel products can be expected to grow, especially because ethanol is easily degraded in the environment and can be produced from renewable raw materials.

Fuel Ethanol The dramatic increase in the production and use of ethanol as an automobile fuel started in 1975 when the Brazilian National Alcohol Program or Pro-Alcohol Program was implemented (Jackson and Moyer, 1991; Zanin et al., 2000). Between 1975 and 1986, annual Brazilian ethanol production (from sugarcane juice) increased from 0.5 billion liters (132 million gallons) to 13 billion liters (3,434 million gallons). However, ethanol production decreased in the early 1980s before it stabilized after 1986 because of policy changes which reduced incentives for sugarcane production, the manufacturing of alcohol-driven automobiles, and the guaranteed price for ethanol. The production level was maintained at 14.16 billion liters (3,741 million gallons) for the 1996–1997 harvest and 13.8 billion liters (3,646 million gallons) for the 1997–1998 harvest. The performance of the Pro-Alcohol Program and the evolution of alcohol-fueled automobiles in Brazil were reviewed (Zanin et al., 2000).

Several reviews examined the merit, the technology, and the economics of producing fuel ethanol from lignocellulosic biomass, also known as "bioethanol" or "cellulosic ethanol" (Lynd et al., 1991; Ballerini et al., 1994; Wyman, 1994; Wyman, 1996; Himmel et al., 1997; Lugar and Woolsey, 1999; Sheehan, 2000). One review summarized key events of the last 20 years of this effort (Wyman, 2001). It was stressed that the production and use of bioethanol cause very low net greenhouse gas emissions, as shown by full fuel-cycle analysis. Fuel properties of ethanol,

Table 30. Feedstock and production capacity (million gallons per year)[a] of selected United States companies producing fuel ethanol.

Company	Location	Feedstock	Capacity
Archer Daniels Midland	Decatur, Illinois	Corn	797
	Peoria, Illinois	Corn	
	Cedar Rapids. Iowa	Corn	
	Clinton, Iowa	Corn	
	Walhalla, North Dakota	Corn, barley	
Minnesota Corn Processor	Columbus, Nebraska	Corn	110
	Marshall, Minnesota	Corn	
Cargill, Inc.	Blair, Nebraska	Corn	100
	Eddyville, Iowa	Corn	
Williams Bio-Energy	Pekin, Illinois	Corn	100
New Energy Corp.	South Bend, Indiana	Corn	85
High Plains Corp.	York, Nebraska	Corn, milo	70
	Colwich, Kansas		
	Porteles, New Mexico		
Chief Ethanol	Hastings, Nebraska	Corn	62
AGP	Hastings, Nebraska	Corn	52
BC International	Jennings, Louisiana	Bagasse, rice hull	20[b]
Central Minnesota	Little Falls, Minnesota	Corn	18
Pro-Corn	Preston, Minnesota	Corn	18
Agri-Energy, LLC	Luverne, Minnesota	Corn	17
Exol, Inc.	Albert Lea, Minnesota	Corn	17
Al-Corn Clean Fuel	Claremont, Minnesota	Corn	17
Georgia-Pacific Corp.	Bellingham, Washington	Paper waste	7
J. R. Simplot	Caldwell, Idaho	Potato waste	6
	Burley, Idaho		
Golden Cheese Co. of California	Corona, California	Cheese whey	5
Kraft, Inc.	Melrose, Minnesota	Cheese whey	2.6

[a]Total capacity including those under construction, 2,184 million gallons per year.
[b]Under construction (http://www.ethanolrfa.org; June, 2001).
From data of the Renewable Fuels Association (http://www.ethanolrfa.org).

other additives of gasoline, and unleaded regular gasoline can be found in Jackson and Moyer (1991) and Wyman (1994). More information about the fuel properties of ethanol can be found in a book produced by the Solar Energy Information Data Bank and published by the United States Government Printing Office (Winston, 1981).

Research and Development of Bacterial Ethanol Production

The technology is now available to produce fuel ethanol from lignocellulosic material. The challenge is to assemble the various process options into a commercial venture and to begin the task of incremental improvements (Ingram et al., 1999). The task of incremental improvements is a continuing one so that an initially successful process can remain competitive. For example, *E. coli* KO11 (Ohta et al., 1991a) is remarkable in having the capacity to ferment the constituent pentoses and hexoses of hemicellulose into ethanol with a high yield. Nevertheless, its ability to ferment simultaneously all the sugars in a mixture may be limited due to catabolite repression. The high ethanol productivity of *E. coli* KO11 may also be an unstable trait under certain conditions, and the ethanol tolerance of *E. coli* KO11 is still lower than that of yeasts used for commercial ethanol production. These are some of the shortcomings of *E. coli* KO11 that will require continued attention to improve. Mutants of *E. coli* KO11 have been obtained that are unable to ferment glucose and other sugars transported by the phosphoenolpyruvate-dependent sugar transport system, but these mutants retain the ability to ferment arabinose and xylose not transported by this system. Such mutants can use xylose efficiently in the presence of high concentrations of glucose, and a second fermentation step or a coculture may be employed to utilize the remaining glucose. Additional mutants have been obtained that retain the ability to ferment all sugars and can produce 60 g/liter of ethanol from 120 g/liter of xylose in 60 hours (Lindsay et al., 1995). Using an enrichment method that selects alternatively for ethanol tolerance and for ethanol production, more ethanol-tolerant mutants of *E. coli* KO11 were isolated (Yomano et al., 1998). Conditions have been identified that cause *E. coli* KO11 to lose

its high ethanol productivity. For example, maintenance of *E. coli* KO11 on xylose in chemostat cultures leads to the irreversible loss of high ethanol productivity (Dumsday et al., 1999). Such knowledge is important to the further improvement of this organism.

Research on growth and ethanol production by *Z. mobilis* is also in progress at the United States National Renewable Energy Laboratory or NREL (Himmel et al., 1997) and elsewhere (Zakpaa et al., 1997; Joachimsthal et al., 1998; McLellan et al., 1999; Silveira et al., 2001). The topics of these studies range from the selection of a mutant capable of ethanol production from glucose in the presence of 20 g/liter of sodium acetate (Joachimsthal et al., 1998) to the mechanism that causes an oscillatory behavior in the continuous fermentation of *Z. mobilis* (McLellan et al., 1999). The development of acetate-tolerant ethanol-producers is important because during acid hydrolysis of hemicellulose, acetic acid (which inhibits fermentation) is produced at a high ratio relative to fermentable sugars.

The conversion of cellulose into fermentable glucose requires the use of expensive fungal cellulase, and it remains a limiting factor in the conversion of lignocellulosic material to ethanol. There is intense research on cellulase, with an aim of decreasing the cost of cellulase production (Himmel et al., 1997). There has also been success in adding the ability to produce and secrete high levels of endoglucanase to *K. oxytoca* P2 so that the requirement for fungal cellulase is reduced in the simultaneous saccharification and fermentation (SSF) process for cellulose (Ingram et al., 1999). An alternative approach is to convert lignocellulosic material into CO and H_2 (synthesis gas), which can be converted to ethanol by some anaerobic bacteria (Clausen and Gaddy, 1996). At present, the synthesis-gas fermentation has a low volumetric productivity owing to physical and biological limitations (Worden et al., 1997). Improvements in both bioreactor design and metabolic capacity of the organism will be needed to make the process commercially useful.

The thermophilic anaerobic bacteria in the genera *Thermoanaerobacter* and *Thermoanaerobacterium* may emerge as useful organisms for ethanol production from lignocellulosic material because of their high ethanol productivity and their natural ability to use a wide range of sugars, oligosaccharides and polysaccharide. New ethanol-producing species of *Thermoanaerobacter* and *Thermoanaerobacterium* are reported (Cook et al., 1996; Liu et al., 1996; Larsen et al., 1997; Cann et al., 2001; Xue et al., 2001). These organisms offer a growing pool of potentially useful traits for use in metabolic engineering. Besides metabolic engineering, the performance of these organisms may also be enhanced under special growth conditions. A coculture of *T. ethanolicus* and *C. thermocellum* can convert cellulose into ethanol more efficiently than pure cultures can, without the addition of fungal cellulase (Wiegel, 1980). To make the thermophilic bacteria useful for commercial ethanol production, genetic tools are needed to refine their properties both now and in future incremental improvement. Electrotransformation procedures (Klapatch et al., 1996; Mai et al., 1997) as well as shuttle vectors and homologous recombination for chromosomal integration (Mai and Wiegel, 2000) have been developed for *Thermoanaerobacterium saccharolyticum* or *Thermoanaerobacterium thermosaccharolyticum*. Genes for hydrolytic enzymes have been introduced into *T. saccharolyticum* and expressed (Mai and Wiegel, 2000). These genetic tools should facilitate research with thermophiles such as *T. ethanolicus* and *C. thermocellum*.

Patents and Regulatory Issues

There are patents that cover three critical areas of bacterial ethanol production: the use of genetically engineered bacteria for ethanol production, the commercial production of cellulase for the saccharification of cellulosic materials, and the development of the simultaneous saccharification and fermentation (SSF) process.

The issuance of the United States patent no. 5,000,000 (Ethanol production by *Escherichia coli* strains coexpressing *Zymomonas mobilis pdc* and *adh* genes; Ingram et al., 1991) is a landmark event attesting to the innovative nature of using rationally constructed bacteria (see Metabolic Engineering in this Chapter) for producing ethanol from lignocellulosic material. Further improvement and extension of this approach have led to additional patents, for example, United States patents 5,028,539 (Ethanol production using engineered mutant *Escherichia coli*; Ingram and Clark, 1992), 5,424,202 (Ethanol production by recombinant hosts; Ingram et al., 1995b), 5,482,846 (Ethanol production by Gram-positive microbes; Ingram and Barbosa-Alleyne, 1996), and 5,821,093 (Recombinant cells that highly express chromosomally integrated heterologous genes; Ingram and Ohta, 1998a).

The development of manufacturing processes for stable cellulytic enzymes has resulted in United States patents 3,990,945 (Enzymatic hydrolysis of cellulose; Huff and Yata, 1976), 5,275,944 (Thermostable purified endoglucanase from *Acidothermus cellulyticus*; Himmel et al., 1994), and 5,712,142 (Method for increasing thermostability in cellulase enzymes; Thomas et al., 1997).

The introduction of simultaneous saccharification and fermentation (SSF) has been considered the most important process improvement made

for the enzymatic hydrolysis of biomass for bioethanol production (Sheehan, 2000). United States patent 3,990,944 (Manufacturing alcohol from cellulosic materials using plural ferments; Gauss et al., 1976) describes the SSF process. In the SSF process, the hydrolysis of cellulosic materials to glucose by a separately prepared cellulase and the fermentation of glucose by an alcohol-producing organism occur simultaneously in a reactor under anaerobic conditions. The advantages of the SSF process include the reduction of the number of reactors required and the alleviation of product inhibition of the hydrolytic enzymes.

The construction of the 20-million gallons per year plant by BC International Corporation (see Commercial Production in this Chapter) to utilize these technologies is an endorsement to the commercial value of the bacterial process. Future growth of the fermentation industry for producing fuel ethanol will depend on the price and availability of crude oil, the environmental issues, public policy and legislative trends, and public opinion (Himmel et al., 1997). At present, the costs of producing fuel ethanol by fermentation are still high that without tax credits and other forms of subsidy (Sheehan, 2000), fermentation ethanol will not be able to compete against gasoline, especially during periods when the price of oil drops. As illustrated by what occurred in Brazil in the 1980s, the production of fermentation ethanol is vulnerable to changes in economic and political factors. The "Brazilian miracle" ended when oil prices dropped and stabilized, the government-guaranteed price for ethanol was lowered, the special credit line for the cultivation of sugarcane was cut, and the tax incentive for manufacturing alcohol-driven automobiles was reduced (Zanin et al., 2000). The continuous growth of the demand for hydrated ethanol, which cannot be used in gasoline, further constrained the production of anhydrous ethanol. Thus, the production of fermentation ethanol in Brazil leveled off after 1986. On the other hand, the production of fuel ethanol in the United States is still on the rise (Table 30), and there are laws and regulations in the United States that encourage an increase in use of ethanol in automobile fuels.

The 1990 amendments to the Clean Air Act, requiring the addition of oxygenates to gasoline to reduce the formation of carbon dioxide and ozone in urban areas where the climate conditions favor the production of these pollutants, have created demand for ethanol, which is an oxygenate. The use of methyl *tert*-butyl ether (MTBE) as an octane-rating enhancer in gasoline is being phased out because of its leakage into the groundwater, hence creating another need for ethanol, which has a high octane rating (see Physicochemical Properties in this Chapter). The Biomass R&D Act of 2000 helps to support biomass conversion projects, which can help lower the production costs for ethanol as less expensive enzymes may be developed for converting lignocellulosic material into fermentable substrates. Any legislation that would require all United States gasoline to contain a certain amount of renewable fuels will further expand the market for ethanol.

Prospects

Production of ethanol by microbial fermentation is an ancient art. The tool of molecular biology has made it possible to alter the metabolic pathways of enteric bacteria and to allow their use in commercial production of ethanol from lignocellulosic material. As illustrated by Ingram and Conway (1988), the relative activity levels of pyruvate decarboxylase and alcohol dehydrogenase in genetically engineered *E. coli* have a major influence on ethanol productivity. In *E. coli* KO11 (Ohta et al., 1991a) and *K. oxytoca* P2 (Wood and Ingram, 1992), expression of these two enzymes is under the control of one promoter, the *pfl* promoter. It is conceivable that further improvement in ethanol productivity by these bacteria may be realized when the expression of these two enzymes is further fine-tuned and the competing reactions or limitations further decreased. The thermophilic ethanol producer *T. ethanolicus* (Wiegel, 1992) is an attractive candidate for future commercial use because of its high ethanol productivity and its natural ability to use a wide range of substrates including pentose and hexose sugars, lactose, and oligosaccharides containing xylose. To use lignocellulosic material as the raw material for ethanol production, the polymer must be pretreated to convert it into fermentable substrates—a costly process. Future improvement in this area holds the greatest promise in making bacterial ethanolic fermentation a more successful commercial process.

Ethanol tolerance remains a potential limiting factor for ethanol production by bacteria. However, it has been shown that by suppressing acid production, the recombinant *E. coli* can produce ethanol to a much higher level than what is achievable by the acid-producing parent strain, suggesting that carboxylic acids are more detrimental than ethanol to the cell. Therefore, when ethanol is the predominant end product, it not only facilitates product recovery but also circumvents inhibition caused by other products. At present, the low sugar concentration in the hemicellulose hydrolysate, rather than ethanol toxicity, is limiting the final ethanol concentration obtained with hemicellulosic substrates. Use of bacterial strains that are more tolerant to ethanol will improve the productivity of a process

when ethanol toxicity becomes the limiting factor for the process.

The use of ethanol in automobile fuels is by far the largest use for this chemical. Inclusion of ethanol in diesel fuels will further increase its market size. The future growth of the fuel ethanol market, however, will not be determined solely by the fuel properties of ethanol, the technological capabilities for its production, and economic factors. Public policies and the attitude of the consumer will play an important role in determining whether ethanol will remain the most substantial commercial product of microbial action.

Literature Cited

Abbad-Andaloussi, S., C. Manginot-Durr, J. Amine, E. Petitdemange, and H. Petitdemange. 1995. Isolation and Characterization of Clostridium butyricum DSM 5431 mutants with increased resistance to 1,3-propanediol and altered production of acids. Appl. Environ. Microbiol. 61:4413–4417.

Abbad-Andaloussi, S., C. Durr, G. Raval, and H. Petitdemange. 1996. Carbon and electron flow in Clostridum butyricum grown in chemostat culture on glycerol and glucose. Microbiol. 142:1149–1158.

Abbad-Andaloussi, S., E. Guedon, E. Spiesser, and H. Petitdemange. 1996. Glycerol dehydratase activity: The limiting step for 1,3-propanediol production by Clostridium butyricum DSM 5431. Letts. Appl. Microbiol. 22:311–314.

Abrini, J., H. Naveau, and E.-J. Nyns. 1994. Clostridium autoethanogenum, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch. Microbiol. 16:345–351.

Adamse, A. D. 1980. New isolation of Clostridium aceticum, (Wieringa). Ant v. Leeuwenhoek 46:523–531.

Adler, H. I., and W. Crow. 1987. A technique for predicting the solvent-producing ability of Clostridium acetobutylicum. Appl. Environ. Microbiol. 53:2496–2499.

Afschar, A. S., K. H. Bellgardt, C. E. Vaz Rossell, A. Czok, and K. Schaller. 1991. The production of 2,3-butanediol by fermentation of high test molasses. Appl. Microbiol. Biotechnol. 34:582–585.

Afschar, A. S., C. E. Vas Rossell, R. Jonas, A. Quesada Chanto, and K. Schaller. 1993. Microbial production and downstream processing of 2,3-butanediol. J. Biotechnol. 27:317–329.

Agreda, V. H., and J. R. Zoeller. 1993. Acetic Acid and Its Derivatives. Dekker. New York, NY.

Ahn, W. K., S. J. Park, and S. Y. Lee. 2000. Production of poly(3-hydroxybutyrate) by fed-batch culture of recombinant Esherichia coli with a highly concentrated whey solution. Appl. Environ. Microbiol. 66:3624–3627.

Ahn, W. S., S. J. Park, and S. Y. Lee. 2001. Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant Esherichia coli. Biotechnol. Lett. 23:235–240.

Ahrens, K., K. Menzel, A. P. Zeng, and W. D. Deckwer. 1998. Kinetic, dynamic, and pathway studies of glycerol metabolism by Klebsiella pneumoniae in anaerobic continuous culture. III: Enzymes and fluxes of glycerol dissimilation and 1,3-propanediol formation. Biotechnol. Bioengin. 59:544–552.

Alam, S., D. Stevens, and R. Bajpai. 1988. Production of butyric acid by batch fermentation of cheese whey with Clostridum beijerinckii. J. Indust. Microbiol. 2:359–364.

Alam, S., F. Capit, W. A. Weigand, and J. Hong. 1990. Kinetics of 2,3-butanediol fermentation by Bacillus amyloliquefaciens: Effect of initial substrate concentration and aeration. J. Chem. Tech. Biotechnol. 47:71–84.

Albertsson, A.-C., and R. H. Marchessault. 1994. Governmental policy, regulations and standards. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 313–318.

Aldrich, H. C., L. McDowell, M. de. F. S. Barbosa, L. P. Yomano, R. K. Scopes, and L. O. Ingram. 1992. Immunocytochemical localization of glycolytic and fermentative enzymes in Zymomonas mobilis. J. Bacteriol. 174:4504–4508.

Allcock, E. R., and D. R. Woods. 1981a. Carboxymethyl cellulase and cellobiase production by Clostridium acetobutylicum in an industrial fermentation medium. Appl. Environ. Microbiol. 41:539–541.

Allcock, E. R., S. J. Reid, D. T. Jones, and D. R. Woods. 1981b. Autolytic activity and an autolysis-deficient mutant of Clostridium acetobutylicum. Appl. Environ. Microbiol. 42:929–935.

Allgeier, R. J., and F. M. Hildebrandt. 1960. Developments in vinegar manufacture. Adv. Appl. Microbiol. 8:163–182.

Alpers, J. 1999. Engineering metabolism for commercial gains. Science 283:1625–1626.

Altaras, N. E., and D. C. Cameron. 1999. Metabolic engineering of a 1,2-propanediol pathway in Escherichia coli. Appl. Environ. Microbiol. 65:1180–1185.

Altaras, N. E., and D. C. Cameron. 2000. Enhanced production of (R)-1,2-propanediol by metabolically engineered Escherichia coli. Biotechnol. Prog. 16:940–946.

Altaras, N. E., M. R. Etzel, and D. C. Cameron. 2001. Conversion of sugars to 1,2-propanediol by Thermoanaerobacterium thermosaccharolyticum HG-8. Biotechnol. Prog. 17:52–56.

Althouse, J. W., and L. L. Tavlarides. 1992. Analysis of organic extractant systems for acetic acid removal for calcium magnesium acetate production. Ind. Eng. Chem. Res. 31:1971–1981.

Aminabhavi, T. M., and R. H. Balundgi. 1990. A review of biodegradable plastics. Polym. Plast. Technol. Eng. 29:235–262.

Amrane, A., and Y. Prigent. 1996. A novel concept of bioreactor: Specialized function two-stage continuous reactor, and its application to lactose conversion into lactic acid. J. Biotechnol. 45:195–203.

Anastassiadis, S., A. Aivasidis, and C. Wandrey. 1999. Process for the production of gluconic acid with a strain of Aureobasidium pullulans (deBray) Arnaud. US Patent 5692286.

Van Andel, J. G., G. R. Zoutberg, P. M. Crabbendam, and A. M. Breure. 1985. Glucose fermentation by Clostridium butyricum grown under a self-generated gas atmosphere in chemostat culture. Appl. Microbiol. Biotechnol. 23:21–26.

Anderson, A. J., and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.

Andreesen, J. R., G. Gottschalk, and H. G. Schlegel. 1970. Clostridium formicoaceticum nov. spec. isolation

description, and distinction from C. aceticum and C. thermoaceticum. Arch. Mikrobiol. 72:154–174.

Andrianova, Y. E., T. L. Bakuridze, V. G. Yargunov, I. V. Zhurov, and V. G. Vinter. 1998. Effects of succinate on the growth rates of potato, Rauwolfia, and ginseng in vitro. Prikl. Biokhim. Mikrobiol. 34:435–438.

Annous, B., and H. P. Blaschek. 1991. Isolation and characterization of Clostridium acetobutylicum mutants with enhanced amylolytic activity. Appl. Environ. Microbiol. 57:2544–2548.

Anonymous. 1956. Trends of world solvents production for the past ten years and the management of the Chiai (Taiwan) Solvents Works [in Chinese]. Petroleum Communications. China Petroleum Corporation. Taiwan, China. June, Issue 60:42–57.

Anonymous. 1958. New direction of development for the Chiai (Taiwan) Solvents Works [in Chinese]. Petroleum Communications. China Petroleum Corporation. Taiwan, China. May, Issue 83:6–7.

Anonymous. 1996a. Chemical profile: Propylene glycol. Chem. Mark. Rep. 249:37.

Anonymous. 1996b. Facts and figures for the chemical industry: Production by the US chemical industry. Chem. Eng. News June 24:41.

Anonymous. 1997. Facts and figures for the chemical industry—production: mixed in 1996. Chem. Eng. News June 23:41.

Anonymous. 1999. Shell Chemicals Research Team wins ACS 2000 award for innovation. Chem. Mark. Rep. 256.

Anonymous. 2001a. Chemical prices. Chem. Mark. Rep. 259:8–21.

Anonymous. 2001b. Chemical profile: Isopropanol. Chem. Mark. Rep. November 12:31.

Anonymous. 1979. Facts and figures for the U.S. chemical industry—production of organic chemicals. Chem. Eng. News June 11:37.

Anonymous. 1993. Chemical Marketing Reporter March 1:7.

Anonymous. 1999. All-microbial route yields chiral building blocks. Chem. Eng. News 77(8):57.

Anonymous. 2000. Chemical prices. Chemical Market Reporter 257(23):26–33.

Anonymous. 2001a. Chemical prices. Chem. Market. Rep. 160(17):23–26.

Anonymous. 2001b. Glycerol, propionic acid, butyric acid. Chem. Market. Rep. 259(13):30–33.

Arihara, K., and J. B. Luchansky. 1995. Dairy lactobacilli. *In:* Y. H. Hui and G. G. Khachatouriani (Eds.) Food Biotechnology: Microorganisms. VCH. New York, NY. 609–643.

Arzberger, C. F., W. H. Peterson, and E. B. Fred. 1920. Certain factors that influence acetone production by Bacillus acetoethylicum. J. Biol. Chem. 44:465–479.

Asai, T. 1968. Acetic Acid Bacteria: Classification and Biochemical Activities. University Press of Tokyo and University Park Press. Tokyo, Japan. Baltimore, MD.

Attwood, M. A., J. P. van Dijken, and J. T. Pronk. 1991. Glucose metabolism and gluconic acid production by Acetobacter diazotrophicus. J. Ferm. Bioeng. 72:101–105.

Auvray, F., M. Codderville, P. Ritzenthaler, and L. Dupont. 1997. Plasmid integration in a wide range of bacteria mediated by intgrase of Lactobacillus delbrueckii bacteriophage mv4. J. Bacteriol. 179:1837–1845.

Azeddoug, H., J. Hubert, and G. Reysset. 1992. Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of Clostridium acetobutylicum. J. Gen. Microbiol. 138:1371–1378.

Babb, B. L., H. J. Collett, S. J. Reid, and D. R. Woods. 1993. Transposon mutagenesis of Clostridium acetobutylicum P262: Isolation and characterization of solvent deficient and metronidazole resistant mutants. FEMS Microbiol. Lett. 114:343–348.

Babel, W., U. Iske, M. Jechorek, and D. Miethe. 1987. Method for production of products by fermentation of methanol. German Patent GDR 251,571. Chem. Abstr. 109:36639.

Babel, W., R. Mueller, D. Miethe, and U. Iske. 1988. Continuous microbial synthesis of products of incomplete oxidation, such as gluconic acid. German Patent DD 253,836. Chem. Abstr. 109:108918.

Babel, W., D. Miethe, U. Iske, K. Sattler, H. P. Richter, J. Schmidt, W. Babel, N. Lofhagen, D. Miethe, R. Mueller, U. Iske, M. Jechorek, and R. Dueresch. 1991. Microbial manufacture of gluconic acid. German Patent DD 293, 135. Chem. Abstr. 115:278185.

Bahl, H., W. A. Andersch, K. Braun, and G. Gottschalk. 1982a. Effect of pH and butyrate concentration on the production of acetone and butanol by Clostridium acetobutylicum grown in continuous culture. Eur. J. Appl. Microbiol. Biotechnol. 14:17–20.

Bahl, H., W. Andersch, and G. Gottschalk. 1982b. Continuous production of acetone and butanol by Clostridium acetobutylicum in a two-stage phosphate limited chemostat. Appl. Microbiol. Biotechnol. 15:201–205.

Bahl, H., H. Müller, S. Behrens, H. Joseph, and F. Naberhaus. 1995. Expression of heat shock genes in Clostridium acetobutylicum. FEMS Microbiol. Rev. 17:341–348.

Bahner, B. 1994. Chemical Marketing Reporter March 21st:14.

Baker, R. C., and R. E. Kramer. 1999. Cytotoxicity of short-chain alcohols. Ann. Rev. Pharmocol. Toxicol. 39:127–150.

Balch, W. E., S. Schoberth, R. S. Tanner, and R. S. Wolfe. 1977. Acetobacterium, a new genus of hydrogen-oxidizing carbon dioxide-reducing, anaerobic bacteria. Int. J. Syst. Bacteriol. 27:355–361.

Baldus, J. M., B. D. Green, P. Youngman, and C. P. Morgan Jr. 1994. Phosphorylation of Bacillus subtilis transcription factor Spo0A stimulates transcription from the spoIIG promoter by enhancing binding to weak 0A boxes. J. Bacteriol. 176:296–306.

Ballerini, D., J. P. Desmarquest, J. Pourquie, F. Nativel, and M. Rebeller. 1994. Ethanol production from lignocellulosics: Large scale experimentation and economics. Bioresource Technol. 50:17–23.

Ballongue, J., J. Amine, E. Masion, H. Petitdemange, and R. Gay. 1985. Induction of acetoacetate decarboxylase in Clostridium acetobutylicum. FEMS Microbiol. Lett. 29:273–277.

Barber, J. M., F. T. Robb, J. R. Webster, and D. R. Woods. 1979. Bacteriocin production by Clostridium acetobutylicum in an industrial fermentation process. Appl. Environ. Microbiol. 37:433–437.

Barbirato, F., C. Camarasa-Claret, J. P. Grivet, and A. Bories. 1995. Glycerol fermentation by a new 1,3-propanediol-producing microorganism: Enterobacter agglomerans. Appl. Microbiol. Biotechnol. 43:786–793.

Barbirato, F., J. P. Grivet, P. Soucaille, and A. Bories. 1996a. 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by enterobacterial species. Appl. Environ. Microbiol. 62:1448–1451.

Barbirato, F., P. Soucaille, and A. Bories. 1996b. Physiologic mechanisms involved in accumulation of 3-hydroxypropionaldehyde during fermentation of

glycerol by Enterobacter agglomerans. Appl. Environ. Microbiol. 62:4405–4409.

Barbirato, F., D. Chedaille, and A. Bories. 1997. Propionic acid fermentation from glycerol: Comparison with conventional substrates. Appl. Microbiol. Biotechnol. 47:441–446.

Barbirato, F., S. Astruc, P. Soucaille, C. Camarasa, J. M. Salmon, and A. Bories. 1997a. Anaerobic pathways of glycerol dissimilation by Enterobacter agglomerans CNCM 1210: Limitations and regulations. Microbiology 143:2423–2432.

Barbirato, F., A. Larguier, T. Conte, S. Astruc, and A. Bories. 1997b. Sensitivity to pH, product inhibition, and inhibition by NAD^+ of 1,3-propanediol dehydrogenase purified from Enterobacter agglomerans CNCM 1210. Arch. Microbiol. 168:160–163.

Barbirato, F., E. H. Himmi, T. Conte, and A. Bories. 1998. 1,3-propanediol production by fermentation: An interesting way to valorize glycerin from the ester and ethanol industries. Indust. Crops Prod. 7:281–289.

Barik, S., S. Prieto, S. B. Harrison, E. C. Clausen, and J. L. Gaddy. 1988. Biological production of alcohols from coal through indirect liquefaction: Scientific note. Appl. Biochem. Biotechnol. 18:363–378.

Barker, H. A. 1972. Co-enzyme B_{12}-dependent mutases causing carbon chain rearrangements. *In:* P. D. Boyer (Ed.) The Enzymes. Academic Press. New York, NY. 6:509–537.

Barnard, G. N., and J. K. M. Sanders. 1989. The poly-β-hydroxybutyrate granule in vivo: A new insight based on NMR spectroscopy of whole cells. J. Biol. Chem. 264:3286–3291.

Basu, R., J. S. Bershears, and E. C. Clausen. 1999. . Calcium Magnesium Acetate at Lower Production Costs: Production of CMA Deicer from Biomass. US DOT. Publication No. FHWA-RD-98-055.

Bata, R. M., A. C. Elrod, and T. P. Lewandowski. 1991. Butanol as a blending agent with gasoline for I. C. engines. *In:* R. W. Hurn, W. F. Marshall, and J. R. Allsup (Eds.) Oxygenates in Motor Fuel Formulation. Society of Automotive Engineers. Warrendale, PA. 35–40.

Bates, E. E. M., H. J. Gilbert, G. P. Hazelwood, J. Huckle, J. I. Laurie, and S. P. Mann. 1989. Expression of a Clostridium thermocellum endoglucanase gene in Lactobacillus plantarum. Appl. Environ. Microbiol. 55:2095–2097.

Beall, D. S., and L. O. Ingram. 1993. Genetic engineering of soft-rot bacteria for ethanol production from lignocellulose. J. Ind. Microbiol. 11:151–155.

Beesch, S. C. 1952. Acetone-butanol fermentation of sugars. Ind. Engin. Chem. 44:1677–1682.

Beesch, S. C. 1953. Acetone-butanol fermentation of starches. Appl. Microbiol. 1:85–95.

Benda, I. 1982. Wine and brandy. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 293–402.

Bennett, G. N., and F. B. Rudolph. 1995. The central metabolic pathway from acetyl-CoA to butyryl-CoA in Clostridium acetobutylicum. FEMS Microbiol. Rev. 17:241–249.

Bennett, G. N., and K. Y. San. 2001. Microbial formation, biotechnological production and application of 1,2-propanediol. Appl. Microbiol. Biotechnol. 55:1–9.

Benninga, H. 1990. A History of Lactic Acid Making. Kluwer Academic Publishers. Dordrecht, Germany. Boston, MA.

Beppu, T. 1993. Genetic organization of Acetobacter for acetic acid fermentation. Ant. v. Leeuwenhoek 64:121–135.

Berglund, K. A., P. Elankovan, and D. A. Glassner. 1991. Carboxylic acid purification and crystallization process. US Patent. 5,034,105.

Berglund, K. A., S. Yedur, and D. D. Dunuwila. 1999. Succinic acid production and purification. US Patent. 5,958,744.

Bermejo, L. L., N. E. Welker, and E. T. Papoutsakis. 1998. Expression of Clostridium acetobutylicum ATCC 824 genes in Escherichia coli for acetone production and acetate detoxification. Appl. Environ. Microbiol. 64: 1079–1085.

Bernhauer, K. 1924. Zum Problem der Säurebildung durch A. niger. Biochem. Z. 153:517–521.

Bernhauer, K. 1928. Über die Characterisierung der Stämme von A. niger auf Grund ihres biochemischen Verhaltens. Biochem. Z 197:278–287.

Beronio Jr., P. B., and G. T. Tsao. 1993. Optimization of 2,3-butanediol production by Klebsiella oxytoca through oxygen transfer rate control. Biotechnol. Bioengin. 42:1263–1269.

Bertram, J., and P. Dü. 1989. Conjugal transfer and expression of streptococcal transposons in Clostridium acetobutylicum. Arch. Microbiol. 151:551–557.

Bertram, J., A. Kuhn, and P. Dü. 1990. Tn916-induced mutants of Clostridium acetobutylicum defective in regulation of solvent formation. Arch. Microbiol. 153:373–377.

Bhowmik, T., and J. L. Steele. 1994. Cloning, characterization, and insertional inactivation of Lactobacillus helveticus D(-)-lactate dehydrogenase. Appl. Microbiol. Biotechnol. 41:432–439.

Biebl, H. 1991. Glycerol fermentation of 1,3-propanediol by Clostridium butyricum: Measurement of product inhibition by use of a pH-auxostat. Appl. Microbiol. Biotechnol. 35:701–705.

Biebl, H., S. Marten, H. Hippe, and W. D. Deckwer. 1992. Glycerol conversion to 1,3-propanediol by newly isolated clostridia. Appl. Microbiol. Biotechnol. 36:592–597.

Biebl, H., and S. Marten. 1995. Fermentation of glycerol to 1,3-propanediol: Use of cosubstrates. Appl. Microbiol. Biotechnol. 44:15–19.

Biebl, H., A. P. Zeng, K. Menzel, and W. D. Deckwer. 1998. Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by Klebsiella pneumoniae. Appl. Microbiol. Biotechnol. 50:24–29.

Biebl, H., K. Menzel, A. P. Zeng, and W. D. Deckwer. 1999. Microbial production of 1,3-propanediol. Appl. Microbiol. Biotechnol. 52:289–297.

Bigelis, R., and S. P. Tsai. 1995. Microorganisms for organic acid production. *In:* Y. H. Hui and G. G. Khachatourians (Eds.) Food Biotechnology: Microorganisms. VCH. New York, NY. 239–280.

Billig, E., and D. R. Bryant. 1991. Oxoprocess. Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 17:903–919.

Billig, E. 1992. Butyl alcohols. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 4:691–700.

Birrer, G. A., W. R. Chesbro, and R. M. Zsigray. 1994. Electro-transformation of Clostridium beijerinckii NRRL B-592 with shuttle plasmid pHR106 and recombinant derivatives. Appl. Microbiol. Biotechnol. 41:32–38.

Blaschek, H., B. Annous, J. Formanek, and C.-K. Chen. 2002. Method of producing butanol using a mutant strain of Clostridium beijerinckii. US Patent. 6358717.

Blom, R. H., V. F. Pfeiffer, A. J. Moyer, D. H. Traufler, H. F. Conway, C. K. Crocker, R. E. Farison, and D. V. Hannibal. 1952. Sodium gluconate production-fermentation with A. niger. Ind. Eng. Chem. 44:435–440.

Blomqvist, K., M. Nikkola, P. Lehtovaara, M.-L. Suihko, U. Airaksinen, K. B. Straby, J. K. C. Knowles, and M. E. Penttila. 1993. Characterization of the genes of the 2,3-butanediol operons from Klebsiella terrigena and Enterobacter aerogenes. J. Bacteriol. 175:1392–1404.

Bock, A., and G. Sawers. 1996. "Fermentation" in Escherichia coli and Salmonella. *In:* F. C. Neidhardt (Eds.) Cellular and Molecular Biology. ASM Press. 262–282.

Boenigk, R., S. Bowien, and G. Gottschalk. 1993. Fermentation of glycerol to 1,3-propanediol in continuous cultures of Citrobacter freundii. Appl. Microbiol. Biotechnol. 38:453–457.

Bolt, J. A. 1980. A Survey of alcohol as a motor fuel. Alcohols as Motor Fuels. Society of Automotive Engineers. Warrendale, PA. 21–33.

Boutroux, L. 1880. Sur une fermentation nouvelle du glucose. CR Acad. Sci. 91:236–238.

Bouvet, O. M. M., P. Lenormand, J. P. Carlier, and P. A. D. Grimont. 1994. Phenotypic diversity of anaerobic glyerol dissimilation shown by seven enterobacterial species. Res. Microbiol. 145:129–139.

Bowles, L. K., and W. L. Ellefson. 1985. Effects of butanol on Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:1165–1170.

Bowring, S. N., and J. G. Morris. 1985. Mutagenesis of Clostridium acetobutylicum. J. Appl. Bacteriol. 58:577–584.

Boyaval, P., J. Seta, and C. Gavach. 1993. Concentrated propionic acid production by electrodialysis. Enzyme Microb. Technol. 15:683–686.

Boyaval, P., C. Corre, and M. N. Madec. 1994. Propionic acid production in a membrane bioreactor. Enzyme Microb. Technol. 16:883–886.

Boyaval, P., and C. Corre. 1995. Production of propionic acid. Lait 75:453–461.

Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1996a. Cloning, sequencing and expression of clustered genes encoding β-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from Clostridium acetobutylicum ATCC 824. J. Bacteriol. 178:3015–3024.

Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1996b. Cloning, sequencing, and expression of genes encoding phosphotransacetylase and acetate kinase from Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 62:2758–2766.

Boynton, Z. L., J. J. Koon, E. M. Brennan, J. D. Clouart, D. M. Horowitz, T. U. Gerngross, and G. W. Huisman. 1999. Reduction of cell lysate viscosity during processing of poly(3-hydroxyalkanoates) by chromosomal integration of the Staphylococcal nuclease gene in Pseudomonas putida. Appl. Environ. Microbiol. 65:1524–1529.

Brandt, D. A. 1982. Distilled beverage alcohol. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 468–491.

Brau, B., and H. Sahm. 1986. Cloning and expression of the sturctural gene for pyruvate decarboxylase of Zymomonas mobilis in Escherichia coli. Arch. Microbiol. 144:296–301.

Braun, M., F. Mayer, and G. Gottschalk. 1981. Clostridium aceticum (Wieringa) a microorganism producing acetic acid from molecular hydrogen and carbon dioxide. Arch. Microbiol. 128:288–293.

Bringer-Meyer, S., K.-L. Schmiz, and H. Sahm. 1986. Pyruvate decarboxylase from Zymomonas mobilis: Isolation and partial characterization. Arch. Microbiol. 146:105–110.

Brown, A. T., and L. C. Breeding. 1980. Carbon dioxide metabolism by Actinomyces viscosus: Pathways for succinate and aspartate production. Infect. Immun. 28:82–91.

Brown, D. P., L. Ganova-Raevn, B. D. Green, S. R. Wilkinson, M. Young, and P. Youngman. 1994. Characterization of spoOA homologues in diverse Bacillus and Clostridium species identifies a probable DNA-binding domain. Molec. Microbiol. 14:411–426.

Bryant, F. O., J. Wiegel, and L. G. Ljungdahl. 1988. Purification and properties of primary and secondary alcohol dehydrogenases from Thermoanaerobacter ethanolicus. Appl. Environ. Microbiol. 54:460–465.

Bryant, R. S. 1990. Microbial enhanced oil recovery and compositions therefor. US Patent. 4905761.

Bryn, K., and F. C. Stormer. 1976. Decreased riboflavin formation in mutants of Aerobacter (Enterobacter) aerogenes deficient in the butanediol pathway. Biochim. Biophys. Acta 428:257–259.

Buchholz, S. E., M. M. Dooley, and D. E. Eveleigh. 1987. Zymomonas—an alcoholic enigma. Trends Biotechnol. 5:199–204.

Bucholz, K., and B. Gödelmann. 1978. Macrokinetics and operational stability of immobilized glucose oxidase and catalase. Biotechnol. Bioeng. 20:1201–1220.

Buchta, K. 1983. Lactic acid. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. Verlag Chemie. Weinheim, Germany. 409–417.

Bulthuis, B. A., A. A. Gatenby, S. L. Laynie, A. K. Hsu, and R. D. Lareau. 1998. Method for the production of glycerol by recombinant organisms. Patent Cooperation Treaty (PCT) Application WO 98/21340.

Burdette, D., and J. G. Zeikus. 1994. Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from Thermoanaerobacter ethanolicus 39E and characterization of the secondary-alcohol dehydrogenase (2°dh) as a bifunctional alcohol dehydrogenase-acetyl-CoA reductive thioesterase. Biochem. J. 302:163–170.

Burdette, D. S., C. Vieille, and J. G. Zeikus. 1996. Cloning and expression of the gene encoding the Thermoanaerobacter ethanolicus 39E secondary-alcohol dehydrogenase and biochemical charaterization of the enzyme. Biochem. J. 316:115–122.

Busche, R. M. 1985. Acetic acid manufacture—fermentation alternatives. *In:* P. N. Cherenisinoff and R. P. Ouellette (Eds.) Biotechnology Applications and Research. Technomic. Lancaster, PA. 88–102.

Busche, R. M. 1991. Extractive fermentation of acetic acid: Economic tradeoff between yield of Clostridium and concentration of Acetobacter. Appl. Biochem. Biotechnol. 28/29:605–621.

Buschhorn, H., P. Dü, and G. Gottschalk. 1989. Production and utilization of ethanol by homoacetogen Acetobacterium woodii. Appl. Environ. Microbiol. 55:1835–1840.

Byrom, D. 1992. Production of poly-β-hydroxybutyrate: Poly-β-hydroxyvalerate copolymers. FEMS Microbiol. Rev. 103:247–250.

Byrom, D. 1994. Polyhydroxyalkanoates. *In:* D. P. Mobley (Ed.) Plastics from Microbes. Hanser Publishers. Munich, Germany. 5–33.

Cameron, D. C., and C. L. Cooney. 1986. A novel fermentation: The production of R(-)-1,2-propanediol and acetol by Clostridium thermosaccharolyticum. Biotechnology 4:651–654.

Cameron, D. C., I. T. Tong, and F. A. Skraly. 1993. Metabolic engineering for the production of 1,3-propanediol. *In:* R. R. Chianelli and B. H. Davison. ACS Symposium on Bioremediation and Bioprocessing. American Chemical Society. Denver, CO. 38:294–295.

Cameron, D. C., N. E. Altaras, M. L. Hoffman, and A. J. Shaw. 1998. Metabolic engineering of propanediol pathways. Biotechnol. Prog. 14:16–125.

Cameron, D. C., A. J. Shaw, and N. E. Altaras. 2000. Microbial production of 1,2-propanediol from sugar. US Patent 6087140.

Cann, I. K. O., P. G. Stroot, K. R. Mackie, B. A. White, and R. I. Mackie. 2001. Characterization of two novel saccharolytic, anaerobic thermophiles, Thermoanaerobacterium polysaccharolyticum sp. nov. and Thermoanaerobacterium zeae sp. nov., and emendation of the genus Thermoanaerobacterium. Int. J. Syst. Evol. Microbiol. 51:293–302.

Carr, N. G. 1966. The occurrence of poly-β-hydroxybutyrate in the blue-green alga, Chloroglea fritschii. Biochim. Biophys. Acta 120:308–310.

Cato, E. P., W. L. George, and S. M. Finegold. 1986. Genus Clostridium Prazmowski 1880. *In:* H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 2:1141–1200.

Cauvin, B., and J. B. Luchansky. 1992. Advances in electrotransformation of Gram-positive bacteria. Bio-rad Laboratories Bulletin 1350A.

Cayol, J.-L., B. Ollivier, B. K. C. Patel, G. Ravot, M. Magot, E. Ageron, P. A. D. Grimont, and J.-L. Garcia. 1995. Description of Thermoanaerobacter brockii subsp. lactiethylicus subsp. nov., isolated from a deep subsurface french oil well, a proposal to reclassify Thermoanaerobacter finnii as Thermoanaerobacter brockii subsp. finnii comb. nov., and an emended description of Thermoanaerobacter brockii. Int. J. Syst. Bacteriol. 45:783–789.

Champluvier, B., J. Decallonne, and P. G. Rouxhet. 1989. Influence of sugar source (lactose, glucose, galactose) on 2,3-butanediol production by Klebsiella oxytoca NRRL-B199. Arch. Microbiol. 152:411–414.

Chang, H. N., Y. C. Kim, S. Y. Lee, and B. S. Kim. 1994. Current status of biodegradable plastics in Korea: Research, commercial production and government policy. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 286–297.

Chang, D.-E., H.-C. Jung, J.-S. Rhee, and J.-G. Pan. 1999. Homofermentative production of D- or L-lactate in metabolically engineered Escherichia coli RR1. Appl. Environ. Microbiol. 65:1384–1389.

Chen, J.-S., and S. F. Hiu. 1986. Acetone-butanol-isopropanol production by Clostridium beijerinckii (synonym, Clostridium butylicum). Biotechnol. Lett. 8:371–376.

Chen, J.-S. 1987. Electron transport in anaerobes. *In:* T. J. Montville (Ed.) Concepts in Physiology and Metabolism. CRC Press. Boca Raton, FL. 1:61–101.

Chen, J.-S. 1993. Properties of acid- and solvent-froming enzymes of clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. Stoneham, MA. 51–76.

Chen, J. S. 1995. Alcohol dehydrogenase: Multiplicity and relatedness in the solvent-producing clostridia. FEMS Microbiol. Rev. 17:263–273.

Chen, C.-K., and H. P. Blaschek. 1999a. Acetate enhances solvent production and prevents degeneration in Clostridium beijerinckii BA101. Appl. Microbiol. Biotechnol. 52:170–173.

Chen, C.-K., and H. Blaschek. 1999b. Effect of acetate on molecular and physiological aspects of Clostridium beijerinckii NCIMB 8052 solvent production and strain degeneration. Appl. Environ. Microbiol. 65: 499–505.

Cheng, P., R. E. Mueller, S. Jaeger, R. Bajpai, and E. L. Iannotti. 1991. Lactic acid production from enzyme-thinned corn starch using Lactobacillus amylovorus. J. Indust. Microbiol. 7:27–34.

Cheryan, M., S. Paretkh, M. Shah, and K. Witjitra. 1997. Production of acetic acid by Clostridium thermoaceticum. Adv. Appl. Microbiol. 43:1–33.

Cheryan, M. 1999. Acetic acid production. *In:* J. Lederberg (Ed.) Encyclopedia of Microbiology. Academic Press. San Diego, CA.

Chiellini, E. 1994. Status of government policy, regulation and standards on the issue of biodegradable plastic materials in Italy. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 273–285.

Choi, J., and S. Y. Lee. 1997. Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. Bioprocess Eng. 17:335–342.

Choi, J., and S. Y. Lee. 1999a. Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals. Biotechnol. Bioeng. 62:546–553.

Choi, J., and S. Y. Lee. 1999b. High -level production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fed-batch culture of recombinant Esherichia coli. Appl. Environ. Microbiol. 65:4363–4368.

Choi, J., and S. Y. Lee. 2000. Economic consideration in the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by bacterial fermentation. Appl. Microbiol. Biotechnol. 53:646–649.

Chotani, G., T. Dodge, A. Hsu, M. Kumar, R. LaDuca, D. Trimbur, W. Weyler, and K. Sanford. 2000. The commercial production of chemicals using pathway engineering. Biochim. Biophys. Acta 1543:434–455.

Chukwu, U. N., and M. Cheryan. 1996. Concentration of vinegar by electrodialysis. J. Food Sci. 61:1223–1226.

Chun, V. H., and P. L. Rogers. 1988. The simultaneous production of sorbitol from fructose and gluconic acid from glucose using an oxidoreductase of Zymomonas mobilis. Appl. Microbiol. Biotechnol. 29:19–24.

Clark, D. P., P. R. Cunningham, S. G. Reams, F. Mat-Jan, R. Mohammedkhani, and C. R. Williams. 1988. Mutants of Escherchia coli defective in acid fermentation. Appl. Biochem. Biotechnol. 17:163–173.

Clark, D. P. 1989. The fermentation pathways of Escherichia coli. FEMS Microbiol. Rev. 63:223–234.

Clark, S. W., G. N. Bennett, and F. B. Rudolph. 1989. Isolation and characterization of mutants of Clostridium acetobutylicum ATCC 824 deficient in acetoacetyl-coenzyme A: Acetate/butyrate:coenzyme A-transferase (EC 2.8.3.9) and in other solvent pathway enzymes. Appl. Environ. Microbiol. 55:970–976.

Clausen, E. C., and J. L. Gaddy. 1996. Ethanol from biomass by gasification/fermentation. *In:* M. R. Khan (Ed.) Conversion and Utilization of Waste Materials. Taylor & Francis. Washington DC, 157–167.

Cocconcelli, P. S., M. J. Gasson, L. Morelli, and V. Bottazzi. 1991. Single-stranded DNA plasmid vector construction and cloning of Bacillus stearothermophilus α-amylase in Lactobacillus. Res. Microbial 142:643–652.

Cocks, G. T., J. Aguilar, and E. C. C. Lin. 1974. Evolution of L-1,2-propanediol catabolism in Escherichia coli by recruitment of enzymes for L-fucose and L-lactate metabolism. J. Bacteriol. 118:83–88.

Colby, G. D., and J.-S. Chen. 1992. Purification and properties of 3-hydroxybutyryl-coenzyme A dehydrogenase from Clostridium beijerinckii ("Clostridium butylicum") NRRL B593. Appl. Environ. Microbiol. 58:3297–3302.

Colby, G. D. 1993. CoA-transferase and 3-hydroxybutyryl-CoA Dehydrogenases: Acetoacetyl-CoA-reacting Enzymes from Clostridium beijerinckii NRRL B593 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Colin, T., A. Bories, and G. Moulin. 2000. Inhibition of Clostridium butyricum by 1,3-propanediol and diols during glycerol fermentation. Appl. Microbiol. Biotechnol. 54:201–205.

Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow. 1994. The phylogeny of the genus Clostridium: Proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44:812–826.

Colomban, A., L. Roger, and P. Boyaval. 1993. Production of propionic acid from whey permeate by sequential fermentation, ultrafiltration, and cell recycling. Biotechnol. Bioeng. 42:1091–1098.

Compere, A. L., and W. L. Griffith. 1979. Evaluation of substrates for butanol production. Devel. Ind. Microbiol. 20:509–517.

Compere, A. L., W. L. Griffith, and J. M. Googin. 1985. Solvents production by clostridia as a function of wood stream organic toxicant concentration. Devel. Ind. Microbiol. 26:535–541.

Conway, T., Y. A. Osman, J. I. Konnan, E. M. Hoffmann, and L. O. Ingram. 1987. Promoter and nucleotide sequences of the Zymomonas mobilis pyruvate decarboxylase. J. Bacteriol. 169:949–954.

Conway, T., and L. O. Ingram. 1989. Similarity of Escherichia coli propanediol oxidoreductase (fucO product) and an unusual alcohol dehydrogenase from Zymomonas mobilis and Saccharomyces cerevisiae. J. Bacteriol. 171:3754–3759.

Cook, G. M., F. A. Rainey, B. K. C. Patel, and H. W. Morgan. 1996. Characterization of a new obligately anaerobic thermophile, Thermanaerobacter wiegelii sp. nov. Int. J. Syst. Bacteriol. 46:123–127.

Cornillot, E., and P. Soucaille. 1996. Solvent-forming genes in clostridia. Nature 380:489.

Cornillot, E., C. Croux, and P. Soucaille. 1997a. Physical and genetic map of the Clostridium acetobutylicum ATCC 824 chromosome. J. Bacteriol. 179:7426–7434.

Cornillot, E., R. V. Nair, E. T. Papoutsakis, and P. Soucaille. 1997b. The genes for butanol and acetone formation in Clostridium acetobutylicum ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. J. Bacteriol. 179:5442–5447.

Croux, C., and J. L. Garcia. 1991. Sequence of the lyc gene encoding the autolytic lysozyme of Clostridium acetobutylicum ATCC 824: Comparison with other lytic enzymes. Gene 104:25–31.

Croux, C., and J. L. Garcia. 1992a. Reconstruction and expression of the autolytic gene from Clostridium acetobutylicum ATCC 824 in Escherichia coli. FEMS Microbiol. Lett. 95:13–20.

Croux, C., B. Canard, G. Goma, and P. Soucaille. 1992b. Autolysis of Clostridium acetobutylicum ATCC 824. J. Gen. Microbiol. 138:861–869.

Croux, C., B. Canard, G. Goma, and P. Soucaille. 1992c. Purification and characterization of an extracellular muramidase of Clostridium acetobutylicum ATCC 824 that acts on non-N-acetylated peptidoglycan. Appl. Environ. Microbiol. 58:1075–1081.

Crow, V. L. 1987. Citrate cycle intermediates in the metabolism of aspartate and lactate by Propionibacterium freudenreichii subsp. shermanii. Appl. Environ. Microbiol. 53:2600–2602.

Cueto, P. H., and B. S. Mendez. 1990. Direct selection of Clostridium acetobutylicum fermentation mutants by a protein suicide method. Appl. Environ. Microbiol. 56:578–580.

Cummins, C., and J. L. Johnson. 1971. Taxonomy of the clostridia: Wall composition and DNA homologies in Clostridium butyricum and other butyric acid-producing clostridia. J. Gen. Microbiol. 67:33–46.

Cummins, C. S., and J. L. Johnson. 1986. The genus Propionibacterium. *In:* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 2:1346–1353.

Currie, J. N., and R. H. Carter (Charles Pfizer, and Co.). 1930. Gluconic Acid. US Patent 1896811. Chem. Abstr. 27:2757.

Currie, J. N., and A. Finlay. 1933. Fermentation such as the production of D-gluconic acid. US Patent 1908225. Chem. Abstr. 27:3774.

Dabrock, B., H. Bahl, and G. Gottschalk. 1992. Parameters affecting solvent production by Clostridium pasteurianum. Appl. Environ. Microbiol. 58:1233–1239.

Daniel, R., and G. Gottschalk. 1992. Growth temperature-dependent activity of glycerol dehydratase in Escherichia coli expressing the Citrobacter freundii dha regulon. FEMS Microbiol. Lett. 100:281–286.

Daniel, R., R. Boenigk, and G. Gottschalk. 1995a. Purification of 1,3-propanediol dehydrogenase from Citrobacter freundii and cloning, sequencing, and overexpression of the corresponding gene in Escherichia coli. J. Bacteriol. 177:2151–2156.

Daniel, R., K. Stuertz, and G. Gottschalk. 1995b. Biochemical and molecular characterization of the oxidative branch of glycerol utilization by Citrobacter freundii. J. Bacteriol. 177:4392–4401.

Daniel, R., T. A. Bobik, and G. Gottschalk. 1999. Biochemistry of coenzyme B12-dependent glycerol and diol dehydratases and organization of the encoding genes. FEMS Microbiol. Rev. 22:553–566.

Datta, R. 1989. Recovery and purification of lactate salts from whole fermentation broth by electrodialysis. US Patent to Michigan Biotech. Inst. 4,885,247.

Datta, R. 1992a. Process for the production of succinic acid by anaerobic fermentation. US Patent. 5,143,833.

Datta, R., D. A. Glassner, M. K. Jain, and J. R. Vick Roy. 1992b. Fermentation and purification process for succinic acid. US Patent. 5,168,055.

Datta, R., S.-P. Tsai, P. Bonsignore, S.-H. Moon, and J. R. Frank. 1995. Technological and economic potential of poly (lactic acid) and lactic acid derivitives. FEMS Microbiol. Rev. 16:221–231.

Davidson, B. E., R. M. Llanos, M. R. Cancilla, N. C. Redman, and A. J. Hillier. 1995. Current research on the genetics of lactic acid production in lactic acid bacteria. Int. Dairy J. 5:763–784.

Dawes, E. A., and P. J. Senior. 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. 10:135–266.

Deckwer, W. D. 1995. Microbial conversion of glycerol to 1,3-propanediol. FEMS Microbiol. Rev. 16:143–149.

de F. S. Barbosa, M., and L. O. Ingram. 1994. Expression of the Zymomonas mobilis alcohol dehydrogenase II (adhB) and pyruvate decarboxylase (pdc) genes in Bacillus. Curr. Microbiol. 28:279–282.

De Koning, G., M. Kellerhals, C. van Meurs, and B. Witholt. 1997. *In:* G. Eggink, A. Steinbuchel, Y. Poirer, and B. Witholt (Eds.) Proceedings of the 1996 International Symposium on Bacterial Polyhdroxyalknoates. NRC Research Press. 137–142.

Demain, A. L., E. L. Rickes, D. Hendlin, and E. C. Barnes. 1961. Nutritional studies on Lactobacillus heterohiochi. J. Bacteriol. 81:147–153.

de Mas, C., N. B. Jansen, and G. T. Tsao. 1988. Production of optically active 2,3-butanediol by Bacillus polymyxa. Biotechnol. Bioengin. 31:366–377.

Demirci, A., and A. L. Pometto 3rd. 1992. Enhanced production of D(-) lactic acid by mutants of Lactobacillus delbrueckii ATCC 9649. J. Ind. Microbiol. 11:23–28.

Demirci, A., and A. L. Pometto 3rd. 1995. Repeated-batch fermentation in biofilm reactors with plastic composite supports for lactic acid production. Appl. Microbiol. Biotechnol. 43:585–589.

Dennis, D., and N. O. Kaplan. 1960. D- and L-lactic acid dehydrogenases in Lactobacillus plantarum. J. Biol. Chem. 235:810–818.

Depasse, E. 1945. Vue d'ensemble d'une production industrielle de cétones. Bull. Assoc. Chim. Sucr. Distill. Fr. 62:317–339.

Desai, R. P., and E. T. Papoutsakis. 1999. Antisense RNA stragies for metabolic engineering of Clostridium acetobytylicum. Appl. Environ. Microbiol. 65:936–945.

DeVos, W. M., and G. F. M. Simons. 1994. Gene cloning and expression systems in Lactococci. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 52–105.

DeWilt, H. G. J. 1972. Oxidation of glucose to gluconic acid. Ind. Eng. Chem. Prod. Res. Dev. 11:370–378.

Diaz-Torres, M., N. S. Dunn-Coleman, M. W. Chase, and D. Trimbur. 2000. Method for the recombinant production of 1,3-propanediol. US Patent 6136576.

Djordjevic, G. M., D. J. O'Sullivan, S. A. Walker, M. A. Conkling, and T. R. Kaenhammer. 1997. Triggered-suicide system designed for bacteriophage defense of Lactococcus lactis. J. Bacteriol. 179:6741–6748.

Doi, Y. 1990a. Microbial Polyesters. VCH. New York, NY.

Doi, Y., A. Segawa, Y. Kawaguchi, and M. Kunioka. 1990b. Cyclic nature of poly(3-hydroxyalkanoate) metabolism in Alcaligenes eutrophus. FEMS Microbiol. Lett. 67:165.

Doi, Y., and K. Fukuda (Eds.). 1994. Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands.

Donnelly, M. I., C. S. Millard, D. P. Clark, M. J. Chen, and J. W. Rathke. 1998a. A novel fermentation pathway in an Escherichia coli mutant producing succinic acid, acetic acid, and ethanol. Appl. Biochem. Biotechnol. 70–72:187–198.

Donnelly, M., C. S. Millard, and L. Stols. 1998b. Mutant *E. coli* strain with increased succinic acid production. US Patent. 5,770,435.

Doran, J. B., and L. O. Ingram. 1993. Fermentation of crystalline cellulose to ethanol by Klebsiella oxytoca containing chromosomally integrated Zymomonas mobilis genes. Biotechnol. Progr. 9:533–538.

Doran, J. B., H. C. Aldrich, and L. O. Ingram. 1994. Saccharification and fermentation of sugar cane bagasse by Klebsiella oxytoca P2 containing chromosomally integrated genes encoding the Zymomonas mobilis ethanol pathway. Biotechnol. Bioengin. 44:240–247.

Drake, H. L. 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: Past and current perspectives. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 3–60.

Dubos, R. J. 1988. Pasteur and Modern Science. Science Tech Publishers. Madison, WI.

Dueresch, R. 1986. Fermentative maufacture of gluconic acid. German Patent DD236, 754. Chem. Abstr. 106:31387.

Dumsday, G. J., B. Zhou, W. Yaqin, G. A. Stanley, and N. B. Pamment. 1999. Comaprative stability of ethanol production by Escherichia coli KO11 in batch and chemostat culture. J. Ind. Microbiol. Biotechnol. 23:701–708.

Dü, P., A. Kuhn, M. Gottwald, and G. Gottschalk. 1987. Enzymatic investigations on butanol dehydrogenase and butyraldhyde dehydrogenase in extracts of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 26:268–272.

Dürre, P., R. J. Fischer, A. Kuhn, K. Lorenz, W. Schreiber, B. Stürzenhofecker, S. Ullmann, K. Winzer, and U. Sauer. 1995. Solventogenic enzymes of Clostridium acetobutylicum, catalytic properties, genetic organization and transcriptional regulation. FEMS Microbiol. Rev. 17:251–262.

Dürre, P., and H. Bahl. 1996. Microbial production of acetone/butanol/isopropanol. *In:* M. Roehr (Ed.) Products of Primary Metabolism, 2nd ed. VCH Publisher. Weinheim, Germany. 6:229–268.

Dürre, P. 1998. New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl. Microbiol. Biotechnol. 49:639–648.

Dürre, P., M. Bohringer, S. Nakotte, S. Schaffer, K. Thormann, and B. Zickner. 2002. Transcriptional regulation of solventogenesis in Clostridium acetobutylicum. J. Molec. Microbiol. Biotechnol. 4:295–300.

Ebner, H., and H. Follmann. 1983. Acetic acid. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. VCH. Weinheim, Germany. 3:387–407.

Ebner, H. 1985. Process for the production of vinegar with more than 12g/100ml acetic acid. US Patent. 4,503,078.

Ebner, H., S. Sellmer, and H. Follmann. 1996. Acetic acid. *In:* M. Roehr (Ed.) VCH. Weinheim, Germany. 6:381–401.

Eden, G., and G. Fuchs. 1982. Total synthesis of acetyl CoA involved in autotrophic CO_2 fixation in Acetobacterium woodii. Arch. Microbiol. 133:66–74.

Eden, G., and G. Fuchs. 1983. Autotrophic CO_2 fixation in Acetobacterium woodii. II: Demonstration of enzymes involved. Arch. Microbiol. 135:68–73.

Eiteman, M. A., and J. H. Miller. 1995. Effect of succinic acid on 2,3-butanediol production by Klebsiella oxytoca. Biotechnol. Lett. 17:1057–1062.

Ennis, B. M., and I. S. Maddox. 1985. Use of Clostridium acetobutylicum P262 for production of solvents from whey permeate. Biotechol. Lett. 7:601–606.

Entani, E., S. Ohmori, H. Masai, and K.-I. Suzuki. 1985. Acetobacter polyoxogenes, sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity. J. Gen. Appl. Microbiol. 31:475–490.

Evans, P. J., and H. Y. Wang. 1990. Effects of extractive fermentation on butyric acid production by Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 32:393–397.

Evans, J. D., and S. A. Martin. 1997. Factors affecting lactate and malate utilization by Selenomonas ruminantium. Appl. Environ. Microbiol. 63:4853–4858.

Evans, V. J., H. Liyanage, A. Ravagnani, M. Young, and E. R. Kashket. 1998. Truncation of peptide deformylase reduces the growth rate and stabilizes solvent production in Clostridium beijerinckii NCIMB 8052. Appl. Environ. Microbiol. 64:1780–1785.

Fayolle, F., R. Marchal, and D. Ballerini. 1990. Effect of controlled substrate feeding on butyric acid production by Clostridium tyrobutyricum. J. Indust. Microbiol. 6:179–183.

Feldmann, S., G. A. Sprenger, and H. Sahm. 1989. Ethanol production from xylose with a pyruvate-formate-lyase mutant of Klebsiella planticola carrying a pyruvate-decarboxylase gene from Zymomonas mobilis. Appl. Microbiol. Biotechnol. 31:152–157.

Fernbach, A., and E. H. Strange. 1911. Acetone and higher alcohols (amyl, butyl or ethyl alcohols and butyric, propionic or acetic acid) from starches, sugars and other carbohydrates. British Patent. 15203–15204.

Fiedler, S., A. Steinbüchel, and B. Rehm. 2000. PhaG-mediated synthesis of poly(3-hydroxyalkanoates) consisting of medium-chain-length constituents from nonrelated carbon sources in recombinant Pseudomonas fragi. Appl. Environ. Microbiol. 66:2117–2124.

Fischer, R. J., J. Helms, and P. Dü. 1993. Cloning, sequencing and molecular analysis of the sol operon of Clostridium acetobutylicum, a chromosomal locus involved in solventogenesis. J. Bacteriol. 175:6959–6969.

Fitz, A. 1878. Über spaltpilzgärungen, IV. Bericht der Deutsch. Chem. Ges. 11:1890.

Flores-Encarnacion, M., and M. Contreras-Zentella. 1999. The respiratory system and diazotrophic activity of Acetobacter diazotrophicus PAL5. J. Bacteriol. 181:6987–6995.

Fond, O., N. B. Jansen, and G. T. Tsao. 1985. A model of acetic acid and 2,3-butanediol inhibition of the growth and metabolism of Klebsiella oxytoca. Biotechnol. Lett. 7:727–732.

Fond, O., J.-M. Engasser, G. Matta-El-Amouri, and H. Petitdemange. 1986a. The acetone butanol fermentation on glucose and xylose. I: Regulation and kinetics in batch cultures. Biotechnol. Bioengin. 28:160–166.

Fond, O., J.-M. Engasser, G. Matta-El-Amouri, and H. Petitdemange. 1986b. The acetone butanol fermentation on glucose and xylose. II: Regulation and kinetics in fed-batch cultures. Biotechnol. Bioengin. 28:167–175.

Fontaine, F., W. H. Peterson, E. McCoy, M. J. Johnson, and G. Ritter. 1942. A new type of glucose fermentation by Clostridium thermoaceticum, n. sp. J. Bacteriol. 43:701–715.

Fontaine, L., I. Meynial-Salles, L. Girbal, X. Yang, C. Croux, and P. Soucaille. 2002. Molecular characterization and transcriptional analysis of adhE2, the gene encoding the NADH-dependent aldehyde/alcohol dehydrogenase responsible for butanol production in alcohologenic cultures of Clostridium acetobutylicum ATCC 824. J. Bacteriol. 184:821–830.

Forage, R. G., and M. A. Foster. 1982a. Glycerol fermentation in Klebsiella pneumoniae: Functions of the coenzyme B12-dependent glycerol and diol dehydratases. J. Bacteriol. 149:413–419.

Forage, R. G., and E. C. C. Lin. 1982b. Dha system mediating aerobic and anaerobic dissimilation of glycerol in Klebsiella pneumoniae NCIB 418. J. Bacteriol. 151:591–599.

Forde, A., C. Daly, and G. F. Fitzgerald. 1999. Identification of four phage resistance plasmids from Lactococcus lactis subsp. cremoris HO2. Appl. Environ. Microbiol. 65:1540–1547.

Frazer, F. R., and T. A. McCaskey. 1991. Effect of components of acid-hydrolyzed hardwood on conversion of D-xylose to 2,3-butanediol by Klebsiella pneumoniae. Enz. Microb. Technol. 13:110–115.

Freier-Schroeder, D., J. Wiegel, and G. Gottschalk. 1989. Butanol formation by Clostridium thermosaccharolyticum at neutral pH. Biotechnol. Lett. 11:831–836.

Fujimaki, T. 1998. Processability and properties of aliphatic polyesters, "BIONELLE," synthesized by polycondensation reaction. Polymer. Degrad. Stabil. 59:209–214.

Fukaya, M., H. Tagami, K. Tayama, H. Okumura, Y. Kawamura, and T. Beppu. 1989. Spheroplast fusion of Acetobacter aceti and its application to the breeding of strains for vinegar production. Agric. Biol. Chem. 53:2435–2440.

Fukaya, M., Y. S. Park, and K. Toda. 1992. Improvement of acetic acid fermentation by molecular breeding. J. Appl. Bacteriol. 73:447–454.

Gabriel, C. L. 1928. Butanol fermentation process. Indust. Engin. Chem. 20:1063–1067.

Gabriel, C. L., and F. M. Crawford. 1930. Development of the butyl-acetonic fermentation indistry. Indust. Engin. Chem. 22:1163–1165.

Gapes, J. R., D. Nimcevic, and A. Friedl. 1996. Long-term continuous cultivation of Clostridium beijerinckii in a two-stage chemostat with on-line solvent removal. Appl. Environ. Microbiol. 62:3210–3219.

Gapes, J. R. 2000a. The economics of the acetone-butanol fermentation: Theoretical and market considerations. J. Molec. Microbiol. Biotechnol. 2:27–32.

Gapes, J. R. 2000b. The history of the acetone-butanol project in Austria. J. Molec. Microbiol. Biotechnol. 2:5–8.

Garg, S. K., and A. Jain. 1995. Fermentative production of 2,3-butanediol: A review. Biores. Technol. 51:103–109.

Gasson, M. J., and W. M. DeVos (Eds.). 1994. Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY.

Gauss, W. F., S. Suzuki, and M. Takagi. 1976. US Patent. 3,990,944.

George, H. A., and J.-S. Chen. 1983a. Acidic conditions are not obligatory for onset of butanol formation by Clostridium beijerinckii (synonym, C. butylicum). Appl. Environ. Microbiol. 46:321–327.

George, H. A., J. L. Johnson, W. E. C. Moore, L. V. Holdeman, and J.-S. Chen. 1983b. Acetone, isopropanol, and butanol production by Clostridium beijerinckii (syn. Clostridium butylicum) and Clostridium aurantibutyricum. Appl. Environ. Microbiol. 45:1160–1163.

Gerischer, U., and P. Dü. 1990. Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from Clostridium acetobutylicum. J. Bacteriol. 172:6907–6918.

Gerngross, T. U., and D. P. Martin. 1995. Enzyme-catalyzed synthesis of poly[R-(-)-3-hydroxybutyrate]: Formation of macroscopic granules in vitro. Proc. Natl. Acad. Sci. USA 92:6279–6283.

Gerngross, T. U. 1999. Can biotechnology move us toward a sustainable society? Nature Biotechnol. 17:541–544.

Ghazvinizadeh, H., G. C. Turtura, and C. Zambonelli. 1972. The fermentation of L-rhamnose by clostridia. Ann. Microbiol. 22:155–158.

Ghommidh, C., J. M. Navarro, and G. Durand. 1981. Acetic acid production by immobilized Acetobacter cells. Biotechnol. Lett. 3:93–98.

Ghose, T. K., and A. Bhadra. 1985. Acetic acid. *In:* M. Moo-Young (Ed.) Biotechnology. Pergamon Press. New York, NY. 3:701–729.

Gibbs, D. F. 1983. The rise and fall (. . . and rise?) of acetone/butanol fermentations. Trends Biotechnol. 1:12–15.

Gilles, M., K. Kerstens, B. Hoste, D. Janssens, R. M. Kroppenstedt, M. P. Stephens, K. R. S. Teixeira, J. Dobereiner, and J. DeLey. 1989. Acetobacter diazotrophicus sp. nov. a nitrogen-fixing acetic acid bacterium associated with sugar cane. Int. J. Syst. Bacteriol. 39:361–364.

Girbal, L., and P. Soucaille. 1994. Regulation of Clostridium acetobutylicum metabolism as revealed by mixed-substrate steady-state continuous cultures: Rle of NADH/NAD ratio and ATP pool. J. Bacteriol. 176:6433–6438.

Girbal, L., C. Croux, I. Vasconcelos, and P. Soucaille. 1995a. Regulation of metabolic shifts in Clostridium acetobutylicum ATCC 824. FEMS Microbiol. Rev. 17:287–297.

Girbal, L., I. Vasoncelos, S. Saint-Amans, and P. Soucaille. 1995b. How neutral red modified carbon and electron flow in Clostridium acetobutylicum grown in chemostat culture at neutral pH. FEMS Microbiol. Rev. 16:151–162.

Girbal, L., and P. Soucaille. 1998. Regulation of solvent production in Clostridium acetobutylicum. Trends Biotechnol. 16:11–16.

Glassner, D. A., and R. Datta. 1992. Process for the production and purification of succinic acid. US Patent. 5,143,834.

Godin, C., and J. M. Engasser. 1990. Two-stage continuous fermentation of Clostridium acetobutylicum: Effects of pH and dilution rate. Appl. Microbiol. Biotechnol. 33:269–273.

Gokarn, R. R., M. A. Eiteman, S. A. Martin, and K. E. L. Eriksson. 1997a. Production of succinate from glucose, cellobiose, and various cellulosic materials by the ruminal anaerobic bacteria Fibrobacter succinogenes and Ruminococcus flavefaciens. Appl. Biochem. Biotechnol. 68:69–80.

Gokarn, R. R., M. A. Eiteman, and J. Sridhar. 1997b. Production of succinate by anaerobic microorganisms. American Chemical Society Symposium Series 666:237–253.

Gokarn, R. R., M. A. Eiteman, and E. Altman. 1998. Expression of pyruvate carboxylase enhances succinate production in Escherichia coli without affecting glucose uptake. Biotechnol. Lett. 20:795–798.

Gokarn, R. R., M. A. Eiteman, and E. Altman. 1999. Pyruvate carboxylase overexpression for enhanced production of oxalacetate-derived biochemicals in microbial cells. PCT International Patent Application. WO 99/53035.

Goldberg, I., K. Lonberg-Holm, E. A. Bagley, and B. Stieglitz. 1983. Improved conversion of fumarate to succinate by Escherichia coli strains amplified for fumarate reductase. Appl. Env. Microbiol. 45:1838–1847.

Goodlove, P. E., P. R. Cunningham, J. Parker, and D. P. Clark. 1989. Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of Escherichia coli. Gene 85:209–214.

Gosalbes, M. J., C. D. Esteban, J. L. Galan, and G. Perez-Martinez. 2000. Integrative food-grade expression system based on the lactose regulon of Lactobacillus casei. Appl. Environ. Microbiol. 66:4822–4828.

Gottschalk, G. 1985. Bacterial Metabolism, 2nd ed. Springer-Verlag. New York, NY. 247.

Gottschalk, G. 1986. Bacterial Metabolism, 2nd ed. Springer-Verlag. New York, NY. 97–98.

Gottschalk, G., and B. Averhoff. 1992. Process for the microbiological preparation of 1,3-propane-diol from glycerol by Citrobacter. US Patent 5164309.

Gottwald, M., H. Hippe, and G. Gottschalk. 1984. Formation of n-butanol from D-gucose by strains of the "Clostridium tetanomorphum" group. Appl. Environ. Microbiol. 48:573–576.

Gottwald, M., and G. Gottschalk. 1985. The internal pH of Clostridium acetobutylicum and its effect on the shift from acid to solvent formation. Arch. Microbiol. 143:42–46.

Goupil-Feuillerat, N., M. Cocaign-Bousquet, J.-J. Godon, S. D. Ehrlich, and P. Renault. 1997. Dual role of a-acetolactate decarboxylase in Lactococcus lactis subsp. lactis. J. Bacteriol. 179:6285–6293.

Green, E. M., and G. N. Bennett. 1996a. Inactivation of an aldehye/alcohol dehydrogenase gene from Clostridium acetobutylicum ATCC 824. Appl. Biochem. Biotechnol. 57/58:213–221.

Green, E. M., Z. L. Boynton, L. M. Harris, F. B. Rudolph, E. T. Papoutsakis, and G. N. Bennett. 1996b. Genetic manipulation of acid formation pathways by gene inactivation in Clostridium acetobutylicum ATCC 824. Microbiology 142:2079–2086.

Greenberg, K. 1999. New PDO technologies create opportunities. Chem. Mark. Rep. 255:4 and 9.

Grethlein, A. J., R. M. Worden, M. K. Jain, and R. Datta. 1990. Continuous production of mixed alcohols and acids from carbon monoxide. Appl. Biochem. Biotechnol. 24–25:875–884.

Grove, L. H. 1982. Process for the production of organic fuel. US Patent. 4326032.

Gruber, P. R., E. S. Hall, J. J. Kolstad, M. L. Iwen, R. D. Benson, and R. L. Borchardt. 1992. Continuous process for manufacture of lactide polymers with controlled optical purity. US Patent to Cargill, Inc. 5,142,023.

Gruber, P. R., E. S. Hall, J. J. Kolstad, M. L. Iwen, R. D. Benson, and R. L. Borchardt. 1994. Continuous process for manufacture of lactide polymers with purification by distillation. US Patent to Cargill, Inc.. 5,357,035.

Grupe, H., and G. Gottschalk. 1992. Physiological events in Clostridium acetobutylicum during the shift from acidogenesis to solventogenesis in continuous culture and

presentation of a model for shift induction. Appl. Environ. Microbiol. 58:3896–3902.

Gu, Z., B. A. Glatz, and C. E. Glatz. 1998. Propionic acid production by extractive fermentation. I: Solvent consideration. Biotechnol. Bioeng. 57:454–461.

Gu, Z., D. A. Rickert, B. A. Glatz, and C. E. Glatz. 1999. Feasibility of propionic acid production by extractive fermentation. Lait 79:137–148.

Guettler, M. V., and M. K. Jain. 1996a. Method for making succinic acid, Anaerobiospirillum succiniciproducens variants for use in process and methods for obtaining variants. US Patent. 5,521,075.

Guettler, M. V., M. K. Jain, and D. Rumler. 1996b. Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. US Patent. 5,573, 931.

Guettler, M. V., M. K. Jain, and B. K. Soni. 1996c. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US Patent. 5,504,004.

Guettler, M. V., M. K. Jain, and B. K. Soni. 1998. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US Patent. 5,723,322.

Guettler, M. V., D. Rumler, and M. K. Jain. 1999. Actinobacillus succinogenes sp. nov., a novel succinic-acid-producing strain from the bovine rumen. Int. J. Syst. Bacteriol. 49:207–216.

Gunzel, B., S. Yonsel, and W. D. Deckwer. 1991. Fermentative production of 1,3-propanediol from glycerol by Clostridium butyricum up to a scale of 2 m^3. Appl. Microbiol. Biotechnol. 36:289–294.

Gupta, A., V. Verma, and G. N. Quazi. 1997. Transposon induced mutation in Gluconobacter oxydans with special reference to its direct glucose oxidation metabolism. FEMS Microbiol. Lett. 147:181–188.

Gupta, A., M. Felder, Y. Yerma, J. Cullum, and G. N. Qazi. 1999. A mutant of Gluconobacter oxydans deficient in gluconic acid dehydrogenase. FEMS Microbiol. Lett. 179:501–506.

Gutierrez, N. A., and I. S. Maddox. 1992. Product inhibition in a nonmotile mutant of Clostridium acetobutylicum. Enz. Microbiol. Technol. 14:101–105.

Hahn, J. J., A. C. Eschenlauer, M. H. Narrol, D. A. Somers, and F. Sriene. 1997. Growth kinetics, nutrient uptake, and expression of the Alcaligenes eutrophus poly(β-hydroxybutyrate) synthesis pathway in transgenic maize cell suspension cultures. Biotechnol. Prog. 13:347–354.

Hamilton, G. A., and F. H. Westheimer. 1959. A crystalline decarboxylase without biotin. J. Am. Chem. Soc. 8:2277.

Han, I. S., and M. Cheryan. 1996. Downstream processing of acetate fermentation broths by nanofiltration. Appl. Biochem. Biotechnol. 57/58:19–28.

Hancock, K. R., E. Rockman, C. A. Young, L. Pearce, I. S. Maddox, and D. B. Scott. 1991. Expression and nucleotide sequence of the Clostridium acetobutylicum β-galactosidase gene cloned in Escherichia coli. J. Bacteriol. 173:3084–3095.

Harlander, S. K. 1987. Transformation of Streptococcus lactis by electroporation. *In:* J. Ferretti and R. Curtiss (Eds.) Streptococcal Genetics. American Society for Microbiology. Washington DC, 229–233.

Harris, J., R. Mulder, D. B. Kell, R. P. Walter, and J. G. Morris. 1986. Solvent production by Clostridium pasteurianum in media of high sugar content. Biotechnol. Lett. 8:889–892.

Harris, L. M., R. P. Desai, N. E. Welker, and E. T. Papoutsakis. 2000. Characterization of recombinant strains of the Clostridium acetobutylicum butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition? Biotechnol. Bioengin. 67:1–11.

Harris, L. M., N. E. Welker, and E. T. Papoutsakis. 2002. Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in Clostridium acetobutylicum ATCC 824. J. Bacteriol. 184:3586–3597.

Hartmanis, M. G. N. 1987. Butyrate kinase from Clostridium acetobutylicum. J. Biol. Chem. 262:617–621.

Hastings, J. J. H. 1971. Development of the fermentation industries in Great Britain. Adv. Appl. Microbiol. 14:1–45.

Hastings, J. J. H. 1978. Acetone-butyl alcohol fermentation. *In:* A. H. Rose (Ed.) Primary Products of Metabolism. Academic Press. London, UK. 31–45.

Haynie, S. L., and L. W. Wagner. 1997. Process for making 1,3-propanediol from carbohydrates using mixed microbial cultures. US Patent 5599689.

Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes. 1988. The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism Alcaligenes eutrophus. FEMS Microbiol. Lett. 52:259–264.

Hein, S., H. Tran, and A. Steinbüchel. 1998. Synechocystis sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. Arch. Microbiol. 170:162–170.

Helbert, J. R. 1982. Beer. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 403–467.

Hermann, M., F. Fayolle, R. Marchal, L. Podvin, M. Sebald, and J.-P. Vandecasteele. 1985. Isolation and characterization of butanol-resistant mutants of Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:1238–1243.

Herrick, H. T., and O. E. May. 1928. The production of gluconic acid by the Penicillium luteum purpurogenum group II: Some optimal conditions for acid formation. J. Biol. Chem. 77:185–195.

Heyndrickx, M., P. De Vos, M. Vancanneyt, and J. De Ley. 1991. The fermentation of glycerol by Clostridium butyricum LMG 1212t2 and 1213t1 and C. pasteurianum LMG 3285. Appl. Microbiol. Biotechnol. 34:637–642.

Himmel, M. E., W. S. Adney, K. Grohmann, and M. P. Tucker. 1994. US Patent. 5,275,944.

Himmel, M. E., W. S. Adney, J. O. Baker, R. Elander, J. D. McMillan, R. A. Nieves, J. J. Sheehan, S. R. Thomas, T. B. Vinzant, and M. Zhang. 1997. Advanced bioethanol production technologies: A perspective. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:2–45.

Himmi, E. H., A. Bories, and F. Barbirato. 1999. Nutrient requirements for glycerol conversion to 1,3-propanediol by Clostridium butyricum. Biores. Technol. 67:123–138.

Himmi, E. H., A. Bories, A. Boussaid, and L. Hassani. 2000. Propionic acid fermentation of glycerol and glucose by Propionibacterium acidipropionici and Propionibacterium freudenreichii ssp. shermanii. Appl. Microbiol. Biotechnol. 53:435–440.

Hippe, H., J. R. Andresen, and G. Gottschalk. 1991. The genus Clostridium, non-medical. *In:* Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.)

The Prokaryotes. Springer-Verlag. New York, NY. 1799–1866.

Hiu, S. F., C.-X. Zhu, R.-T. Yan, and J.-S. Chen. 1987. Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: Different alcohol dehydrogenases in two strains of Clostridium beijerinckii (Clostridium butylicum). Appl. Environ. Microbiol. 53:697–703.

Hlasiwetz, H., and J. Habermann. 1870. Zur Kenntnis einiger Zuckerarten (Glucose, Rhorzucker, Levulose, Sorbin, Phloroglucin). Ann Chem. Pharm. 155:128–144.

Ho, K.-L. G., A. L. Pometto 3rd, and P. N. Hinz. 1997a. Optimization of L-(+)-lactic acid production by ring and disc plastic composite supports through repeated-batch biofilm fermentation. Appl. Environ. Microbiol. 63: 2533–2542.

Ho, K.-L. G., A. L. Pometto 3rd, P. N. Hinz, and A. Demirci. 1997b. Nutrient leaching and end product accumutation in plastic composite supports for L-(+)-lactic acid biofilm fermentation. Appl. Environ. Microbiol. 63:2524–2532.

Ho, K.-L. G., A. L. Pometto 3rd, P. N. Hinz, J. S. Dickson, and A. Demirci. 1997c. Ingredients selection for plastic composite supports for L-(+)-lactic acid biofilm fermentation by Lactobacillus casei subsp. rhamnosus. Appl. Environ. Microbiol. 63:2516–2523.

Hocking, P. J., and R. H. Marchessault. 1994. Biopolymers. *In:* G. J. L. Griffin (Ed.) Chemistry and Technology of Biodegradable Polymers. Chapman and Hall. London, 48–96.

Hoffman, J. 2001. Butadiene hit hard by sharp drop in derivatives demand. Chem. Mark. Rep. 260:1 and 12.

Holland-Staley, C. A., K. Lee, D. P. Clark, and P. R. Cunningham. 2000. Aerobic activity of Escherichia coli alcohol dehydrogenase is determined by a single amino acid. J. Bacteriol. 18(2):6049–6054.

Holt, R. A., G. M. Stephens, and J. G. Morris. 1984. Production of solvents by Clostridium acetobutylicum cultures maintained at neutral pH. Appl. Environ. Microbiol. 48:1166–1170.

Holt, R. A., A. J. Cairns, and J. G. Morris. 1988. Production of butanol by Clostridium puniceum in batch and continuous culture. Appl. Microbiol. Biotechnol. 27:319–324.

Holten, C. M., A. Muller, and D. Rehbinder (Eds.). 1971. Lactic Acid. Weinheim/Bergstr. Germany.

Homann, T., C. Tag, H. Biebl, W. D. Deckwer, and B. Schink. 1990. Fermentation of glycerol to 1,3-propanediol by Klebsiella and Citrobacter strains. Appl. Microbiol. Biotechnol. 33:121–126.

Hong, R. 1999. The Cloning of a Putative Regulatory Gene and the sol Region from Clostridium beijerinckii. M.S. thesis. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Hongo, M., and A. Murata. 1965a. Bacteriophages of Clostridium saccharoperbutylacetonicum. Part I: Some characteristics of the twelve phages obtained from the abnormally fermented broths. Agric. Biol. Chem. 29:1135–1139.

Hongo, M., and A. Murata. 1965b. Bacteriophages of Clostridium saccharoperbutylacetonicum. Part II: Enumeration of phages by the application of the plaque count technique and some factors influencing the plaque formation. Agric. Biol. Chem. 29:1140–1145.

Hoppner, T. C., and H. W. Doelle. 1983. Purification and kinetic characteristics of pyruvate decarboxylase and ethanol dehydrogenase from Zymomonas mobilis in relation to ethanol production. Eur. J. Appl. Microbiol. Biotechnol. 17:152–157.

Houben, M. C. M. 1995. Oxygenated blending components for gasoline-alcohols and ethers. *In:* E. L. Marshall and K. Owen (Ed.) Motor Gasoline: Critical Reports on Applied Chemistry. Royal Society of Chemistry. Cambridge, UK. 34:45–71.

Houge, C. 2000. Getting the MTBE out. Chem Eng. News 6.

Howaldt, M., A. Gottlob, K. D. Kulba, and H. Chmiel. 1988. Simultaneous conversion of glucose/fructose mixtures in a membrane reactor. Ann. NY Acad. Sci. 542:400–405.

Howaldt, M., K. D. Kulbe, and H. Chmiel. 1990. A continous enzyme membrane reactor retaining native nicotinamide cofactor NAD (H). Ann. NY Acad. Sci. 589:253–260.

Howard, W. L. 1991. Acetone. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 1:176–194.

Hrabak, O. 1992. Industrial production of poly-β-hydroxybutyrate. FEMS Microbiol. Rev. 103:251–256.

Hromatka, O., and H. Ebner. 1949. Investigations on vinegar fermentation: Generator for vinegar fermentation and aeration procedures. Enzymologia 13:369.

Hsu, S. T., and S. T. Yang. 1991. Propionic acid fermentation of lactose by Propionibacterium acidipropionici—effects of pH. Biotechnol. Bioeng. 38:571–578.

Huang, Y., and S. T. Yang. 1998a. Acetate production from whey lactose using co-immobilized cells of homolactic and homoacetic bacteria in a fibrous-bed bioreactor. Biotechnol. Bioeng. 60:498–507.

Huang, Y. L., K. Mann, and J. M. Mavak. 1998b. Acetic acid production from fructose by Clostridium formicaceticum immobilized in a fibrous bed bioreactor. Biotechnol. Prog. 800–806.

Huang, K.-X., F. B. Rudolph, and G. N. Bennett. 1999. Characterization of methylglyoxal synthase from Clostridium acetobutylicum ATCC 824 and its use in the formation of 1,2-propanediol. Appl. Environ. Microbiol. 65:3244–3247.

Huang, K.-X., S. Huang, F. B. Rudolph, and G. N. Bennett. 2000. Identification and characterization of a second butyrate kinase from Clostridium acetobutylicum ATCC 824. J. Molec. Microbiol. Biotechnol. 2:33–38.

Huff, G. F., and N. Yata. 1976. US Patent. 3,990,945.

Husemann, M., and E. T. Papoutsakis. 1986. Effect of acetoacetate, butyrate, and uncoupling ionophores on growth and product formation of Clostridium acetobutylicum. Biotechnol. Lett. 8:37–42.

Husemann, M. H. W., and E. T. Papoutsakis. 1988. Solventoggenesis in Clostridium acetobutylicum fermentations related to carboxylic acid and proton concentrations. Biotechnol. Bioengin. 32:843–852.

Husemann, M. H. W., and E. T. Papoutsakis. 1989. Comparison between in vivo and in vitro enzyme activities in continuous and batch fermentations of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 30:585–595.

Ingram, L. O., and T. M. Buttke. 1984. Effects of alcohols on micro-organisms. Adv. Microb. Physiol. 25:253–300.

Ingram, L. O. 1986. Microbial tolerance to alcohols: Role of the cell membrane. Trends Biotechnol. 4:40–44.

Ingram, L. O., T. Conway, D. P. Clark, G. W. Sewell, and J. F. Preston. 1987. Genetic engineering of ethanol production in Escherichia coli. Appl. Environ. Microbiol. 53:2420–2425.

Ingram, L. O., and T. Conway. 1988. Expression of different levels of ethanologenic enzymes from Zymomonas mobilis in recombinant strains of Escherichia coli. Appl. Environ. Microbiol. 54:397–404.

Ingram, L. O., and K. M. Dombek. 1989. Effects of ethanol on Escherichia coli. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 227–237.

Ingram, L. O. 1990. Ethanol tolerance in bacteria. Crit. Rev. Biotechnol. 9:305–319.

Ingram, L. O., F. Alterthum, and T. Conway. 1991. US Patent. 5,000,000.

Ingram, L. O., and D. C. Clark. 1992. US Patent. 5,028,539.

Ingram, L. O., and J. B. Doran. 1995a. Conversion of cellulosic materials to ethanol. FEMS Microbiol. Rev. 16:235–241.

Ingram, L. O., D. S. Beall, G. F. H. Burchhardt, W. V. Guimaraes, K. Ohta, B. E. Wood, K. T. Shanmugam, D. E. Fowler, and B.-B. A. 1995b. US Patent. 5,424,202.

Ingram, L. O., and M. D. F. Barbosa-Alleyne. 1996. US Patent. 5,482,846.

Ingram, L. O., and K. W. Ohta, B. E. 1998a. US Patent. 5821093.

Ingram, L. O., P. F. Gomez, X. Lai, M. Moniruzzaman, B. E. Wood, L. P. Yamano, and S. W. York. 1998b. Metabolic engineering of bacteria for ethanol production. Biotechnol. Bioengin. 58:204–214.

Ingram, L. O., H. C. Aldrich, A. C. C. Borges, T. B. Causey, A. Martinez, F. Morales, A. Saleh, S. A. Underwood, L. P. Yomano, S. W. York, J. Zaldivar, and S. Zhou. 1999. Enteric bacterial catalysts for fuel ethanol production. Biotechnol. Progr. 15:855–866.

Inoue, Y., and A. Kimura. 1995. Methylglyoxal and regulation of its metabolism in microorganisms. Adv. Microb. Physiol. 37:177–227.

Inskeep, G. C., G. G. Taylor, and W. C. Breitzke. 1952. Lactic acid from corn sugar. Ind. Eng. Chem. 44:1955–1966.

Isbell, H. S., H. L. Frush, and F. J. Bates. 1932. Manufacture of calcium gluconate by electrolytic oxidation of glucose. Ind. Eng. Chem. 24:375–378.

Ishizaki, A., N. Taga, T. Takeshita, T. Sugimoto, T. Tsuge, and K. Tanaka. 1997. Microbial production of biodegradable plastics from carbon dioxide and agricultural waste material. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, 295–306.

Ismaiel, A. A., C.-X. Zhu, G. D. Colby, and J.-S. Chen. 1993. Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of Clostridium beijerinckii. J. Bacteriol. 175:5097–5105.

Ismaiel, A. A., and J. S. Chen. 1998. Purification of the aldehyde-alcohol dehydrogenase encoded by the aad gene from Clostridium acetobutylicum ATCC 824. *In:* Abstracts of the 98th General Meeting of the American Society of Microbiology. American Society for Microbiology. Washington, D.C., O-40:400.

Iversen, T.-G., R. Standal, T. Pederson, and D. H. Caucheron. 1994. IS 1032 from Acetobacter xylinum, a new mobile insertion sequence. Plasmid 32:46–54.

Jabalquinto, A. M., M. Laivenieks, J. G. Zeikus, and E. Cardemil. 1999. Characterization of the oxaloacetate decarboxylase and pyruvate kinase-like activities of Saccharomyces cerevisiae and Anaerobiospirillum succiniciproducens phosphoenolpyruvate carboxykinases. J. Prot. Chem. 18:659–664.

Jackson, M. D., and C. B. Moyer. 1991. Alcohol fuels. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 1:826–864.

Jan, G., A. Rouault, and J. L. Maubois. 2000. Acid stress susceptibility and acid adaptation of Propionibacterium freudenreichii subsp. shermanii. Lait 80:325–336.

Jan, G., P. Leverrier, V. Pichereau, and P. Boyaval. 2001. Changes in protein synthesis and morphology during acid adaptation of Propionibacterium freudenreichii. Appl. Environ. Microbiol. 67:2029–2036.

Jansen, N. B., and G. T. Tsao. 1983. Bioconversion of pentoses to 2,3-butanediol by Klebsiella pneumoniae. *In:* A. Fiechter and T. W. Jeffries (Eds.) Advances in Biochemical Engineering/Biotechnology. Springer-Verlag. New York, NY. 27:85–99.

Jansen, N. B., M. C. Flickinger, and G. T. Tsao. 1984. Production of 2,3-butanediol from D-xylose by Klebsiella oxytoca ATCC 8724. Biotechnol. Bioengin. 26:362–369.

Jarvis, L. 2001. Lactic acid outlook up as polylactide nears market. Chemical Market Reporter 259(9):5, 14.

Jendrossek, D., M. Backhaus, and M. Andermann. 1995. Characterization of the Comamonas sp. poly(3-hydroxybutyrate) (PHB) depolymerase and of its structural gene. Can. J. Microbiol. 41:160–169.

Jendrossek, D., A. Schirmer, and H. G. Schlegel. 1996. Biodegradation of polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 46:451–463.

Jin, Z. W., and S. T. Yang. 1998. Extractive fermentation for enhanced propionic acid production from lactose by Propionibacterium acidipropionici. Biotechnol. Prog. 14:457–465.

Joachimsthal, E., K. D. Haggett, J.-H. Jang, and P. L. Rogers. 1998. A mutant of Zymomonas mobilis ZM4 capable of ethanol production from glucose in the presence of high acetate concentrations. Biotechnol. Lett. 20:137–142.

Johansen, L., K. Bryn, and F. C. Stormer. 1975. Physiological and biochemical role of the butanediol pathway in Aerobacter (Enterobacter) aerogenes. J. Bacteriol. 123:1124–1130.

John, M. E., and G. Keller. 1996. Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. USA 93:12768–12773.

Johnson, J. L. 1984. Bacterial classification. III: Nucleic acids in bacterial classification. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:8–11.

Johnson, J. L., and J.-S. Chen. 1995. Taxonomic relationships among strains of Clostridium acetobutylicum and other phenotypically similar organisms. FEMS Microbiol. Rev. 17:233–240.

Johnson, J. L., J. Toth, S. Santiwatanakul, and J.-S. Chen. 1997. Cultures of "Clostridium acetobutylicum" from various collections comprise Clostridium acetobutylicum, Clostridium beijerinckii, and two other distinct types based on DNA-DNA reassociation. Int. J. Syst. Bacteriol. 47:420–424.

Jones, R. P., and P. Greenfield. 1982. Effect of carbon dioxide on yeast growth and fermentation. Enz. Microbiol. Technol. 4:210–223.

Jones, D. T., W. A. Jones, and D. R. Woods. 1985. Production of recombinants after protoplast fusion in Clostridium acetobutylicum P262. J. Gen. Microbiol. 131:1213–1216.

Jones, D. T., and D. R. Woods. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50:484–524.

Jones. D. T. 1993. Mutagenesis and its application to biotechnology. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 77–98.

Jones, D. T., and S. Keis. 1995. Origins and relationships of industrial solvent-producing clostridial strains. FEMS Microbiol. Rev. 17:223–232.

Jones, D. T., M. Shirley, X. Wu, and S. Keis. 2000. Bacteriophage infections in the industrial AB fermentation process. J. Molec. Microbiol. Biotechnol. 2:21–26.

Jossek, R., and A. Steinbüchel. 1998. In vitro synthesis of poly-(3-hydroxybutyric acid) by using an enzymatic coenzyme A recycling system. FEMS Microbiol. Lett. 168:319–324.

Junelles, A.-M., R. Janati-Idrissi, A. El Kanouni, H. Petitdemange, and R. Gay. 1987. Acetone-butanol fermentation by mutants selected for resistance to acetate and butyrate halogen analogues. Biotechnol. Lett. 9:175–178.

Juni, E., and G. A. Heym. 1956. A cyclic pathway for the bacterial dissimilation of 2,3-butanediol, acetylmethylcarbinol, and diacetyl. I: General aspects of the 2,3-butanediol cycle. J. Bacteriol. 71:425–432.

Kandler, O., and N. Weiss. 1986. *In:* P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 2:1208–1234.

Kaneuchi, C., M. Seki, and K. Komagata. 1988. Production of succinic acid from citric acid and related acids by Lactobacillus strains. Appl. Environ. Microbiol. 54: 3053–3056.

Kansiz, M., H. Billman-Jacobe, and D. McNaughton. 2000. Quantitative determination of the biodegradable polymer poly(β-hydroxybutyrate) in a recombinant Escherichia coli strain by use of mid-infrared spectroscopy and multivariative statistics. Appl. Environ. Microbiol. 66:3415–3420.

Kashket, E. R., and Z. Y. Cao. 1995. Clostridial strain degeneration. FEMS Microbiol. Rev. 17:307–315.

Kawaguchi, Y., and Y. Doi. 1990. Structure of native poly (3-hydroxybutyrate) granules characterized by X-ray diffraction. FEMS Microbiol. Lett. 70:151–156.

Kawaguchi, Y., and Y. Doi. 1992. Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in Alcaligenes eutrophus. Macromolecules 25:2324–2329.

Keis, S., C. F. Bennett, V. K. Ward, and D. T. Jones. 1995. Taxonomy and phylogeny of industrial solvent-producing clostridia. Int. J. Syst. Bacteriol. 45:693–705.

Keis, S., R. Shaheen, and D. T. Jones. 2001a. Emended description of Clostridium acetobutylicum and Clostridium beijerinckii and descriptions of Clostridium saccharoperbutylacetonicum sp. nov. and Clostridium saccharobutylicum sp. nov. Int. J. Syst. Evol. Microbiol. 51:2095–2103.

Keis, S., J. T. Sullivan, and D. T. Jones. 2001b. Physical and genetic map of the Clostridium saccharobutylicum (formerly Clostridium acetobutylicum) NCP 262 chromosome. Microbiology 147:1909–1922.

Kelly, F. C. 1936. One Thing Leads To Another. Houghton Mifflin. Boston, MA.

Kennedy, J. F., J. D. Humphreys, S. A. Barker, and R. N. Greenshields. 1980. Application of living immobilized cells to the acceleration of the continuous conversions of ethanol (wort) to acetic acid (vinegar) by hydrous titanium (IV) oxide-immobilized Acetobacter species. Enz. Microbiol. Technol. 2:209–216.

Keshav, K. F., L. P. Yomano, H. An, and L. O. Ingram. 1990. Cloning of the Zymomonas mobilis structural gene encoding alcohol dehydrogenase I (adhA): Sequence comparison and expression in Escherichia coli. J. Bacteriol. 172:2491–2497.

Kessler, D., I. Leibrecht, and J. Knappe. 1991. Pyruvate-formate-lyase-deactivase and acetyl-CoA reductase activities of Escherichia coli reside on a polymeric protein particle encoded by adhE. FEBS Lett. 281:59–63.

Kessler, D., W. Herth, and J. Knappe. 1992. Ultrastructure and pyruvate formate-lyase radical quenching property of the multienzymic AdhE protein of Escherichia coli. J. Biol. Chem. 267:18073–18079.

Kiatpapan, P., Y. Hashimoto, H. Nakamura, Y.-Z. Piao, H. Ono, M. Yamashita, and Y. Murooka. 2000. Characterization of pRG01, a plasmid from Propionibacterium acidipropionici, and its use for development of a host-vector system in propionibacteria. Appl. Environ. Microbiol. 66:4688–4695.

Killeffer, D. H. 1927. Butanol and acetone from corn. Indust. Engin. Chem. 19:46–50.

Kim, D. M., and H. S. Kim. 1992a. Continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an oxido reductase from Zymomonas mobilis and inulinase. Biotechnol. Bioeng. 39:336–342.

Kim, B. S., S. Y. Lee, and H. N. Chang. 1992b. Production of poly-beta-hydroxybutyrate by fed-batch culture of recombinant Escherichia coli. Biotechnol. Lett. 14:811–816.

Kim, B. S., S. C. Lee, S. Y. Lee, H. N. Chang, Y. K. Chang, and S. I. Woo. 1994. Production of poly(3-hydroxybutyric acid) by fed-batch culture of Alcaligenes eutrophus with glucose concentration control. Biotechnol. Bioeng. 43:892–898.

Kim, S. W., P. Kim, H. S. Lee, and J. H. Kim. 1996. High production of poly-beta-hydroxybutyrate (PHB) from Methylobacterium organophilum under potassium limitation. Biotechnol. Lett. 18:25–30.

Kim, G. J., I. Y. Lee, S. C. Yoon, Y. C. Shih, and Y. H. Park. 1997. Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of Pseudomonas putida by combined use of glucose and octanoate. Enz. Microb. Technol. 20:500–505.

Kinoshita, S., T. Kakizono, K. Kadota, K. Das, and H. Taguchi. 1985. Purification of two alcohol dehydrogenases from Zymomonas mobilis and their properties. Appl. Microbiol. Biotechnol. 22:249–254.

Kirk Othmer. 1983. Succinic acid and succinic anhydride. Kirk-Othmer Encyclopedia of Chemical Technology, 3rd ed. 21:848–864.

Kirschner, E. M. 1996. Growth of top 50 chemicals slowed from very high 1994 rate. Chem. Eng. News 74(15):16–19.

Kitahara, K., T. Kaneko, and O. Goto. 1957. Taxonomic studies on the hiochi-bacteria specific saprophytes of sake. I: Isolation and grouping of bacterial strains. J. Gen. Appl. Microbiol. (Japan) 3:102–110.

Klaenhammer, T. R., and G. F. Fitzgerald. 1994. Bacteriophages and bacteriophage resistance. *In:* M. J.Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 106–168.

Klapatch, R. R., M. L. Guerinot, and L. R. Lynd. 1996. Electrotransformation of Clostridium thermosaccharolyticum. J. Ind. Microbiol. 16:342–347.

Klasson, K. T., M. D. Ackerson, E. C. Clausen, and J. L. Gaddy. 1992. Bioconversion of synthesis gas into liquid or gaseous fuels. Enzyme Microbiol. Technol. 14:602–608.

Klinke, S., G. de Roo, B. Witholt, and B. Kessler. 2000. Role of phaD in accumulation of medium-chain-length poly(3-hydroxyalkanoates) in Pseudomonas olevorans. Appl. Environ. Microbiol. 66:3705–3710.

Knauf, H. J., R. F. Vogel, and W. P. Hammes. 1992. Cloning, sequence, and phenotypic expression of KatA which encodes the catalase of Lactobacillus sake LTH677. Appl. Environ. Microbiol. 58:832–839.

Kok, J., and J. M. DeVos. 1994. The proteolytic system of lactic acid bacteria. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 169–210.

Konda, T., and M. Konda. 1996. Efficient production of acetic acid from glucose in a mixed culture of Zymomonas mobilisand Acetobacter sp. J. Ferment. Bioeng. 81:42–46.

Kondo, K., and S. Horinouchi. 1997. A new insertion sequence IS 1452 from Acetobacter pasteurianus. Microbiology 143:539–546.

Korkhin, Y., A. J. Kalb(Gilboa), M. Peretz, O. Bogin, Y. Burstein, and F. Frolow. 1998. NADP-dependent bacterial alcohol dehydrogenases: Crystal structure, cofactor-binding and cofactor specificity of the ADHs of Clostridium beijerinckii and Thermoanaerobacter brockii. J. Molec. Biol. 278:967–981.

Kosaric, N. 1996. Ethanol-potential source of energy and chemical products. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. VCH. New York, NY. 6:123–203.

Kusano, K., H. Yamada, M. Niwa, and K. Yamasoto. 1997. Propionibacterium cyclohexanicum sp nov., a new acid-tolerant omega-cyclohexy/fatty acid-containing propionibacterium isolated from spoiled orange juice. Int. J. Syst. Bacteriol. 47:825–831.

Kutzenok, A., and M. Aschner. 1952. Degenerative processes in a strain of Clostridium butylicum. J. Bacteriol. 64:829–836.

Kutzing, F. T. 1837. Prakt. Chem. J. 11:385.

Kwon, Y. J., R. Kaol, and B. Mattiasson. 1996. Extractive lactic acid fermentation in Poly (ethyleneimine)-based aqueous two-phase system. Biotechnol. Bioeng. 50:280–290.

Ladisch, M. R. 1991. Fermentation-derived butanol and scenarios for its uses in energy-related applications. Enz. Microbiol. Technol. 13:280–283.

Laffend, L. A., V. Nagarajan, and C. E. Nakamura. 1997. Bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism. US Patent. 5686276.

Laffend, L. A., V. Nagarajan, and C. E. Nakamura. 2000. Bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism. US Patent. 6025184.

Laivenieks, M., C. Vielle, and J. G. Zeikus. 1997. Cloning, sequencing, and overexpression of the Anaerobiospirillum succiniciproducens phosphoenolpyruvate carboxykinase (pckA) gene. Appl. Env. Microbiol. 63:2272–2280.

Lamed, R. J., and J. G. Zeikus. 1981. Novel NADP-linked alcohol-aldehyde/ketone oxidoreductase in thermophilic ethanologenic bacteria. Biochem. J. 195:183–190.

Landucci, R., B. Goodman, and C. Wyman. 1994. Methodology for evaluating the economics of biologically producing chemicals and materials from alternative feedstocks. Appl. Biochem. Biotechnol. 45/46:677–696.

Largier, S. T., S. Long, J. D. Santangelo, D. T. Jones, and D. R. Woods. 1985. Immobilized Clostridium acetobutylicum P 262 mutants for solvent production. Appl. Environ. Microbiol. 50:477–481.

Larsen, S. H., and F. C. Stormer. 1973. Diacetyl (Acetoin) reductase from Aerobacter aerogenes. Eur. J. Biochem. 34:100–106.

Larsen, L., P. Nielsen, and B. K. Ahring. 1997. Thermoanaerobacter mathranii sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. Arch. Microbiol. 168:114–119.

Laube, V. M., D. Groleau, and S. M. Martin. 1984. 2,3-butanediol production from xylose and other hemicellulosic components by Bacillus polymyxa. Biotechnol. Lett. 6:257–262.

Layman, P. 1998. Acetic acid industry gears up for possible building boom. Chem. Eng. News 76(31):17–18.

Leaf, T. A., M. S. Peterson, S. K. Stoup, D. Somers, and F. Srienc. 1996. Saccharomyces cerevisiae expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. Microbiol. 142:1169–1180.

Lee, S. F., C. W. Forsberg, and L. N. Gibbins. 1985. Cellulolytic activity of Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:220–228.

Lee, H. K., and I. S. Maddox. 1986. Continuous production of 2,3-butanediolfrom whey permeate using Klebsiella pneumoniae immobilized in calcium alginate. Enz. Microb. Technol. 8:409–411.

Lee, S. Y., G. N. Bennett, and E. T. Papoutsakis. 1992. Construction of Escherichia coli-Clostridium acetobutylicum shuttle vectors and transfromation of Clostridium acetobutylicum strains. Biotechnol. Lett. 14:427–432.

Lee, Y.-E., M. K. Jain, C. Lee, S. E. Lowe, and J. G. Zeikus. 1993. Taxonomic distinction of saccharolytic thermophilic anaerobes: Description of Thermoanaerobacterium xylanolyticum gen. nov., sp. nov., and Thermoanaerobacterium saccharolyticum gen. nov., sp. nov.; reclassification of Thermoanaerobium brockii, Clostridium thermosulfurogenes, and Clostridium thermohydrosulfuricum E100-69 as Thermoanaerobacter brockii comb. nov., and Thermoanaerobacter thermosulfurigenes comb. nov., and Thermoanaerobacter thermohydrosulfuricus comb. nov., respectively; and transfer of Clostridium thermohydrosulfuricum 39E to Thermoanaerobacter ethanolicus. Int. J. Syst. Bacteriol. 43:41–51.

Lee, S. Y., and H. N. Chang. 1995a. Production of poly(hydroxyalkanoic acid). Adv. Biochem. Eng. Biotechnol. 52:27–58.

Lee, S. Y., and H. N. Chang. 1995b. Production of poly(3-hydroxybutyric acid) by recombinant Escherichia coli strains: Genetic and fermentation studies. Can. J. Microbiol. 41, Suppl. 1:207–215.

Lee, S. Y., Y. K. Lee, and H. N. Chang. 1995c. Stimulatory effects of amino acids and oleic acid on poly(3-hydroxybutyric acid) synthesis by recombinant Escherichia coli. J. Ferment. Bioeng. 79:177–180.

Lee, S. Y. 1996a. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49:1–14.

Lee, S. Y. 1996b. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol. 14:431–438.

Lee, I. Y., M. K. Kim, Y. H. Park, and S. Y. Lee. 1996c. Regulatory effects of cellular nicotinamide nucleotides and enzyme activities on poly(3-hydroxybutyrate) synthesis in recombinant Escherichia coli. Biotechnol. Bioeng. 52:707–712.

Lee, S. Y. 1997. E. coli moves into the plastic age. Nature Biotechnol. 15:17–18.

Lee, P. C., W. G. Lee, S. Kwon, S. Y. Lee, and H. N. Chang. 1999a. Succinic acid production by Anaerobiospirillum succiniciproducens: Effects of the H_2/CO_2 supply and glucose concentration. Enz. Microbiol. Technol. 24:549–554.

Lee, P. C., W. G. Lee, S. Y. Lee, and H. N. Chang. 1999b. Effects of medium components on the growth of Anaerobiospirillum succiniciproducens and succinic acid production. Proc. Biochem. 35:49–55.

Lee, S. Y., Y. Lee, and F. L. Wang. 1999c. Chiral compounds from bacterial polyesters: Sugars to plastics to fine chemicals. Biotechnol. Bioeng. 65:363–368.

Lefranc, L. et Cie. 1923. A process for the manufacture of butyric acid and other fatty acids with recovery of the gases of fermentation. British Patent 186-572. Chem Abstr. 17:324.

Leh, M. B., and M. Charles. 1989. The effect of whey protein hydrolyzate average molecular weight on the lactic acid fermentation. J. Ind. Microbiol. 4:77–80.

Leigh, J. A., F. Mayer, and R. S. Wolfe. 1981. Acetogenium kivui a new thermophilic hydrogen-oxidizing, acetogenic bacterium. Arch. Microbiol. 129:275–280.

Lemme, C. J., and J. R. Frankiewicz. 1985. Strains of Clostridium acetobutylicum and process for its preparation. US Patent. 4521516.

Lemmel, S. A. 1985. Mutagenesis in Clostridium acetobutylicum. Biotechnol. Lett. 7:711–716.

Lemmel, S. A., R. Datta, and J. R. Frankiewicz. 1986. Fermentation of xylan by Clostridium acetobutylicum. Enz. Microbiol. Technol. 8:217–221.

Lemoigne, M. 1925. Etudes sur l'autolyze microbienne acidification par formation d'acide β-oxybutyrique. Ann. Inst. Pasteur 39:144–173.

Lemoigne, M. 1926. Produits de déshydration et de polymérisation de l'acide β-oxybutyrique. Bull. Soc. Chim. Biol. 8:770–782.

Lemoigne, M., and H. Girard. 1943. Réserves lipidiques β-hydroxybutyriques chez Azotobacter chroococcum. Comp. Rend. Acad. Sci. 217:557–558.

Lenz, T. G., and A. R. Moreira. 1980. Economic evaluation of the acetone-butanol fermentation. Indust. Engin. Chem. Prod. Res. Devel. 19:478–483.

Lepage, C., F. Fayolle, M. Hermann, and J.-P. Vandecasteele. 1987. Changes in membrane liquid composition of Clostridium acetobutylicum during acetone-butanol fermentation: Effects of solvents, growth temperature and pH. J. Gen. Microbiol. 133:103–110.

Lewis, V. P., and S. T. Yang. 1992. Continuous propionic acid fermentation by immobilized Propionibacterium acidipropionici in a novel packed bed bioreactor. Biotechnol. Bioeng. 40:465–474.

Li, G. 1998. Development of a Reporter System for the Study of Gene Expression for Solvent Production in Clostridium beijerinckii NRRL B582 and Clostridium acetobutylicum ATCC 824 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Liebergesell, M., E. Hustede, A. Timm, A. Steinbüchel, R. C. Fuller, R. W. Lenz, and H. G. Schlegel. 1991. Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. Arch. Microbiol. 155:415–421.

Lin, Y.-L., and H. P. Blaschek. 1984. Transformation of heat-treated Clostridium acetobutylicum protoplasts with pUB110 plasmid DNA. Appl. Environ. Microbiol. 48:737–742.

Lin, R. W., E. E. Atkinson, and D. E. Balhoff. 1996. Process for producing (S,S)-ethylenediamine-N,N′-disuccinic acid. US Patent. 5554791.

Lin, M.-Y., S. Harlander, and D. Saviano. 1999. Construction of an integrative food-grade cloning vector for Lactobacillus acidophilus. Appl. Microb. Biotechnol. 45:484–489.

Linden, J. C., and R. H. Kuhn. 1989. Biochemistry of alcohol effects on Clostridia. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 271–291.

Lindsay, S. E., R. J. Bothast, and L. O. Ingram. 1995. Improved strains of recombinant Escherichia coli for ethanol production from sugar mixture. Appl. Microbiol. Biotechnol. 43:70–75.

Lipinsky, E. S., and R. G. Sinclair. 1986. Is lactic acid a commodity chemical? Chem. Eng. Prog. 82(8):26–32.

Litchfield, J. H. 1996. Microbial production of lactic acid. Adv. Appl. Microbiol. 42:45–95.

Liu, S.-Y., F. A. Rainey, H. W. Morgan, F. Mayer, and J. Wiegel. 1996. Thermoanaerobacterium aotearoense sp. nov., a slightly acidophilic, anaerobic thermophile isolated from various hot springs in New Zealand, and emendation of the genus Thermoanaerobacterium. Int. J. Syst. Bacteriol. 46:388–396.

Liu, S.-J., and A. Steinbüchel. 2000a. Exploitation of butyrate kinase and phosphotransbutyrylase from Clostridium acetobutylicum for the in vitro biosynthesis of poly(hydroxyalkanoic acid). Appl. Microbiol. Biotechnol. 53:545–552.

Liu, S.-J., and A. Steinbüchel. 2000b. A novel genetically engineered pathway for synthesis of poly(hydroxyalkanoic acids) in Escherichia coli. Appl. Environ. Microbiol. 66:739–743.

Ljungdahl, L. G. 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Ann. Rev. Microbiol. 40:415–450.

Ljungdahl, L. G. 1994. The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 63–85.

Lockwood, L. B., D. E. Yoder, and M. Zienty. 1965. Lactic acid. Ann. NY Acad. Sci. 119:854–867.

Logsdon, J. E. 1994. Ethanol. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 9:812–860.

Logsdon, J. E., and R. A. Loke. 1996. Isopropyl alcohol. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 20:216–240.

Logsdon, J. E., and R. A. Loke. 1999. Isopropyl alcohols. *In:* J. I. Kroschwitz (Ed.) Kirk-Othmer Concise Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 1654–1656.

Loos, H., R. Krämer, H. Sahm, and G. A. Sprenger. 1994. Sorbitol promotes growth of Zymomonas mobilis in environment with high concentrations of sugar: Evidence for a physiological function of glucose-fructose

oxidoreductase in osmoprotection. J. Bacteriol. 176: 7688–7693.

Lorowitz, W. H., and M. P. Bryant. 1984. Peptostreptococcus productusstrain that grows rapidly with CO as an energy source. Appl. Environ. Microbiol. 47:961–964.

Lowe, S. E., M. K. Jain, and J. G. Zeikus. 1993. Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. Microbiol. Rev. 57:451–509.

Lowenheim, F. A., and M. K. Moran. 1975. Industrial Chemicals, 4th ed. John Wiley & Sons. New York, NY.

Luchansky, J. B., P. M. Muriana, and T. R. Klaenhammer. 1988. Application of electroporation for transfer of plasmid DNA to Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Bacillus, Staphylococcus, Enterococcus, and Propionibacterium. Molec. Microbiol. 2:637–646.

Luers, F. M., R. Seyfried, R. Daniel, and G. Gottschalk. 1997. Glycerol conversion to 1,3-propanediol by Clostridium pasteurianum: Cloning and expression of the gene encoding 1,3-propanediol dehydrogenase. FEMS Microbiol. Lett. 154:337–345.

Lugar, R. G., and R. J. Woolsey. 1999. The new petroleum. Foreign Affairs 78:88–102.

Lund, B. M., T. F. Brocklehurst, and G. M. Wyatt. 1981. Characterization of strains of Clostridium puniceum sp. nov., a pink-pigmented, pectolytic bacterium. J. Gen. Microbiol. 122:17–26.

Luttik, M. A. H., R. Van Spanning, D. Schipper, J. P. Van Dijken, and J. T. Pronk. 1997. The low biomass yields of the acetic acid bacterium Acetobacter pasteurianus are due to a low stoichiometry of respiration-coupled proton translocation. Appl. Envron. Microbiol. 63:3345–3351.

Lynd, L. R. 1989. Ethanol production from lignocellulosic substrates using thermophilic bacteria. Adv. Biochem. Eng. Biotechnol. 38:1–52.

Lynd, L. R., J. H. Cushman, R. J. Nichols, and C. E. Wyman. 1991. Fuel ethanol from cellulosic biomass. Science 251:1318–1323.

Lynd, L. R., S. Baskaran, and S. Casten. 2001. Salt accumulation resulting from base added for pH control, and not ethanol, limits growth of Thermoanaerobacterium thermosaccharolyticum HG-8 at elevated feed xylose concentrations in continuous culture. Biotechnol. Progr. 17:118–125.

Lyon, W. J., and B. A. Glatz. 1995. Propionibacteria. *In:* Y. H. Hui and G. G. Khachatourians (Eds.) Food Biotechnology, Microorganisms. VCH Publishers. New York, NY. 703–719.

Macis, L., R. Daniel, and G. Gottschalk. 1998. Properties and sequence of the coenzyme B12-dependent glycerol dehydratase of Clostridium pasteurianum. FEMS Microbiol. Lett. 164:21–28.

Mackenzie, K. F., C. K. Eddy, and L. O. Ingram. 1989. Modulation of alcohol dehydrogenase isoenzyme levels in Zymomonas mobilis by iron and zinc. J. Bacteriol. 171:1063–1067.

Maddox, I. S. 1980. Production of n-butanol from whey filtrate using Clostridium acetobutylicum NCIB 2951. Biotechnol. Lett. 2:493–498.

Maddox, I. S. 1996. Microbial production of 2, 3-butanediol. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology, 2nd ed. VCH. New York, NY. 6:270–291.

Maddox, I. S., E. Steiner, S. Hirsch, S. Wessner, N. A. Gutierrez, J. R. Gapes, and K. C. Schuster. 2000. The cause of "acid crash" and "acidogenic fermentations" during the batch acetone-butanol-ethanol (ABE-) fermentation process. J. Molec. Microbiol. Biotechnol. 2:95–100.

Madison, L. L., and G. W. Huisman. 1999. Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic. Microbiol. Molec. Biol. Rev. 63:21–53.

Mai, V., W. W. Lorenz, and J. Wiegel. 1997. Transformation of Thermoanaerobacterium sp. strain JW/SL-YS485 with plasmid pIKM1 conferring kanamycin resistance. FEMS Microbiol. Lett. 148:163–167.

Mai, V., and J. Wiegel. 2000. Advances in development of a genetic system for Thermoanaerobacterium spp.: Expression of genes encoding hydrolytic enzymes, development of a second shuttle vector, and integration of genes into chromosome. Appl. Environ. Microbiol. 66:4817–4821.

Malinowski, J. J. 1999. Evaluation of liquid extraction potentials for downstream separation of 1,3-propanediol. Biotechnol. Tech. 13:127–130.

Malinowski, J. J. 2000. Reactive extraction for downstream separation of 1,3-propanediol. Biotechnol. Prog. 16:76–79.

Marchal, R., M. Ropars, J. Pourquie, F. Fayolle, and J. P. Vandecasteele. 1992. Large-scale enzymatic hydrolysis of agricultural lignocellulosic biomass. Part II: Conversion into acetone-butanol. Biores. Technol. 42:205–217.

Martin, M. E., M. Wayman, and G. Graf. 1961. Fermentation of sulfite waste liquor to produce organic acids. Can. J. Microbiol. 7:341–346.

Martin, M. C., J. C. Alonso, J. E. Suarez, and M. A. Alvarez. 2000. Generation of food-grade recombinant lactic acid bacterium strains by site-specific recombination. Appl. Environ. Microbiol. 66:2599–2604.

Mat-Jan, F., K. Y. Alam, and D. P. Clark. 1989. Mutants of Escherichia coli deficient in the fermentative lactate dehydrogenase. J. Bacteriol. 171:342–348.

Matsusaki, H., H. Abe, K. Taguchi, T. Fukui, and Y. Doi. 2000. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene phaC1 from Pseudomonas sp. 61-3. Appl. Microbiol. Biotechnol. 53:401–409.

Matsushita, K., H. Toyama, and O. Adachi. 1994. Respiratory chains and bioenergetics of acetic acid bacteria. Adv. Microb. Physiol. 36:247–301.

Mattsson, D. M., and P. Rogers. 1994. Analysis of Tn916-induced mutants of Clostridium acetobutylicum altered in solventogenesis and sporulation. J. Ind. Microbiol. 13:258–268.

May, O. E., H. T. Herrick, A. J. Moyer, and P. A. Wells. 1934. Gluconic acid-production by submerged mold growths under increased air pressure. Ind. Eng. Chem. 26:575–578.

McCoy, E., E. B. Fred, W. H. Peterson, and E. G. Hastings. 1926. A cultural study of the acetone butyl alcohol organism. J. Infect. Dis. 39:457–483.

McCoy, E., and L. S. McClung. 1935. Studies on anaerobic bacteria. J. Infect. Dis. 56:333–346.

McCoy, E., and E. B. Fred. 1941. The stability of a culture for industrial fermentation. J. Bacteriol. 41:90–91.

McCoy, M. 1998. Biomass ethanol inches forward. Chem. Eng. News 76:29–32.

McCutchan, W. N., and R. J. Hickey. 1954. The butanol-acetone fermentations. *In:* L. A. Underkofler and R. J.

Hickey (Eds.) Industrial Fermentations. Chemical Publishing. New York, NY. 1:347–388.
McGee, H. 1984 . On Food and Cooking: The Science and Lore of the Kitchen. Scribner's. New York, NY. 36.
McLellan, P. J., A. J. Daugulis, and J. Li. 1999. The incidence of oscillatory behavior in the continuous fermentation of Zymomonas mobilis. Biotechnol. Progr. 15:667–680.
McNeil, B., and B. Kristiansen. 1986. The acetone butanol fermentation. Adv. Appl. Microbiol. 31:61–92.
Meinecke, B., H. Bahl, and G. Gottschalk. 1984. Selection of an asporogenous strain of Clostridium acetobutylicum in continuous culture under phosphate limitation. Appl. Environ. Microbiol. 48:1064–1065.
Menzel, K., A. P. Zeng, and W. D. Deckwer. 1997. High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by Klebsiella pneumoniae. Enz. Microbiol. Technol. 20:82–86.
Mercenier, A., P. H. Pouwels, and B. M. Chassy. 1994. Genetic engineering of lactobacilli, leuconostocs, and Streptococcus thermophilus. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 252–293.
Mercier, P., L. Yerushalmi, D. Rouleau, and D. Dochain. 1992. Kinetics of lactic acid fermentation on glucose and corn by Lactobacillus amylophilus. J. Chem. Tech. Biotechnol. 55:111–121.
Mermelstein, L. D., N. E. Welker, G. N. Bennett, and E. T. Papoutsakis. 1992. Expression of cloned homologous fermentative genes in Clostridium acetobutylicum ATCC 824. Biotechnology 10:190–195.
Mermelstein, L. D., and E. T. Papoutsakis. 1993a. Evaluation of macrolide and lincosamide antibiotics for plasmid maintenance in low pH Clostridium acetobutylicum ATCC 824 fermentations. FEMS Microbiol. Lett. 113:71–76.
Mermelstein, L. D., and E. T. Papoutsakis. 1993b. In vivo methylation in Escherichia coli by the Bacillus subtilis phage 3T I methyltransferase to protect plasmids from restriction upon transformation of Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 59:1077–1081.
Mermelstein, L. D., E. T. Papoutsakis, D. J. Petersen, and G. N. Bennett. 1993c. Metabolic engineering of Clostridium acetobutylicum ATCC 824 for increased solvent production by enhancement of acetone formation enzyme activities using a synthetic acetone operon. Biotechnol. Bioengin. 42:1053–1060.
Mermelstein, L. D., N. E. Welker, D. J. Petersen, G. N. Bennett, and E. T. Papoutsakis. 1994. Genetic and metabolic engineering of Clostridum acetobutylicom. ATCC 824. Ann. NY Acad. Sci. 721:54–68.
Mesa, M. M., I. Caro, and D. Cantero. 1996. Viability reduction of Acetobacter aceti due to the absence of oxygen in submerged culture. Biotech. Progress 12:709–712.
Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990a. Control of the selectivity of butyric acid production and improvement of fermentation performance with Clostridium tyrobutyricum. Appl. Microbiol. Biotechnol. 32:387–392.
Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990b. Butyrate production in continuous culture of Clostridum tyrobutyricum. Appl. Microbiol. Biotechnol. 33: 127–131.
Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990c. Butyric fermentation: Metabolic behavior and production performance of Clostridium tryobutyricum in a continuous culture with cell recycle. Appl. Microbiol. Biotechnol. 34:172–177.
Mickelson, M. N., and C. H. Werkman. 1940. Formation of trimethyleneglycol from glycerol by Aerobacter. Enzymologia 8:252–256.
Millard, C. S., Y. P. Chao, J. C. Liao, and M. I. Donnelly. 1996. Enhanced production of succinic acid by overexpression of phosphoenolpyruvate caboxylase in Escherichia coli. Appl. Env. Microbiol. 62:1808–1810.
Milson, P. E., and J. L. Meers. 1985. Gluconic and itaconic acids. *In:* M. Moo-Young (Ed.) Comprehensive Biotechnology. Pergamon Press. Oxford, 3:681–700.
Minton, N. P., and J. D. Oultram. 1988. Host:vector systems for gene cloning in Clostridium. Microbiol. Sci. 5:310–315.
Minton, N. P., J. K. Brehm, T. J. Swinfield, S. W. Whelan, M. L. Mauchline, N. Bodsworth, and J. D. Oultrum. 1993. Clostridial cloning vectors. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 120–150.
Mishra, P., and S. Kaur. 1991. Lipids as modulators of ethanol tolerance in yeast. Appl. Microbiol. Biotechnol. 34:697–702.
Misoph, M., and H. L. Drake. 1996. Effect of CO_2 on the fermentation capacities of the acetogen Peptostreptococcus productus U-1. J. Bacteriol. 178:3140–3145.
Mitchell, W. J. 1998. Physiology of carbohydrate to solvent conversion by clostridia. Adv. Microb. Physiol. 39:31–130.
Mobley, D. P. (Ed.). 1994. Plastics from Microbes. Hanser. Munich, Germany.
Möller, B., R. Ossmer, B. H. Howard, G. Gottschalk, and H. Hippe. 1984. Sporomusa, a new genus of Gram negative anaerobic bacteria including Sporomusa sphaeroides spec. nov. and Sporomusa ovata spec. nov. Arch. Microbiol. 139:388–396.
Molliard, M. 1922. Survene nouvelle fermentation acide produite par le Sterigmatcystis nigra. C.R. Hebd. Seances Acad. Sci. 174:881–883.
Monot, F., J.-M. Engasser, and H. Petitdemange. 1984. Influence of pH and undissociated butyric acid in the production of acetone and butanol in batch cultures of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 19:422–426.
Montoya, D., C. Arevalo, S. Gonzales, F. Aristizabal, and W. H. Schwarz. 2001. New solvent-producing Clostridium sp. strains, hydrolyzing a wide range of polysaccharides, are closely related to Clostridium butyricum. J. Ind. Microbiol. Biotechnol. 27:329–335.
Moore, S. K. 1999. DuPont, Genencor close in on biological propanediol route. Chem. Week 161:17.
Moreira, A. R., D. C. Ulmer, and J. C. Linden. 1981. Butanol toxicity in butylic fermentation. Biotechnol. Bioengin. Symp. 11:567–579.
Mori, A., and G. Tervi. 1972. Kinetic studies on submerged acetic acid fermentation. III: Efficiency of energy metabolism in acetic acid fermentation using Acetobacter rancens. J. Ferment. Technol. 50:510–517.
Mori, A. 1993. Vinegar production in a fluidized-bed reactor with immobilized bacteria. *In:* A. Tanaka, T. Tosa, and T. Kobayashi (Eds.) Industrial Application of Immobilized Biocatalysts. Dekker. New York, NY. 291–313.
Morris, J. G. 1993. History and future potential of the clostridia in biotechnology. *In:* D. R. Woods (Ed.) The

Clostridia and Biotechnology. Butterworth-Heinemann. London, 1–23.

Moyer, A. J., P. A. Wells, J. J. Stubbs, H. T. Herrick, and O. E. May. 1937. Gluconic acid production-development of innoculum and composition of fermentation solution for gluconic acid production by submerged mold growths under increased air pressure. Ind. Eng. Chem. 29:777–781.

Muller, H. M., and D. Seebach. 1993. Poly(hydroxyfettsaureester) eine funfte Klasse von physiologisch bedeutsamen organischen Biopolymeren? Agnew. Chem. 105:483–459.

Müller, V., and G. Gottschalk. 1994. The sodium ion cycle in acetogenic and methanogenic bacteria: Generation and utilization of a primary electrochemical sodium ion gradient. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 125–156.

Muraoka, H., Y. Watabe, and N. Ogasawara. 1982. Effect of oxygen deficiency on acid production and morphology of bacterial cells in submerged acetic fermentation by Acetobacter aceti. J. Ferment. Technol. 60:171–180.

Muriana, P. M., and T. R. Klaenhammer. 1991. Cloning, phenotypic expression, and DNA sequence of the gene for lactacin F, an antimicrobial peptide produced by Lactobacillus spp. J. Bacteriol. 173:1779–1788.

Nagarajan, V., and C. E. Nakamura. 1998. Production of 1,3-propanediol from glycerol by recombinant bacteria expressing recombinant diol dehydratase. US Patent 5821092.

Nair, R. V., G. N. Bennett, and E. T. Papoutsakis. 1994. Molecular characterization of an alcohol/aldehyde dehydrogenase gene of Clostridium acetobutylicum ATCC 824. J. Bacteriol. 176:871–885.

Nakamura, C. E., A. A. Gatenby, A. K. Hsu, R. D. LaReau, S. L. Haynie, R. Diaz-Torres, D. E. Trimbur, G. M. Whited, A. Nagarajan, M. S. Payne, S. K. Picataggio, and R. V. Nair. 2000. Method for the production of 1,3-propanediol by recombinant microorganisms. US Patent 6013494.

Nakano, M. M., Y. P. Dailly, P. Zuber, and D. P. Clark. 1997. Characterization of anaerobic fermentative growth of Bacillus subtilis: Identification of fermentation end products and genes required for growth. J. Bacteriol. 179:6749–6755.

Nakas, J. P., M. Schaedle, C. M. Parkinson, C. E. Coonley, and S. W. Tanenbaum. 1983. System development for linked-fermentation production of solvents from algal biomass. Appl. Environ. Microbiol. 46:1017–1023.

Nakashimada, Y., K. Kanai, and N. Nishio. 1998. Optimization of dilution rate, pH and oxygen supply on optical purity of 2,3-butanediol produced by Paenibacillus polymyxa in chemostat culture. Biotechnol. Lett. 20:113–1138.

Nakashimada, Y., B. Marwoto, T. Kashiwamura, T. Kakizono, and N. Nishio. 2000. Enhanced 2,3-butanediol production by addition of acetic acid in Paenibacillus polymyxa. J. Biosci. Bioeng. 90:661–664.

Nakotte, S., S. Schaffer, M. Bohringer, and P. Dü. 1998. Electroporation of, plasmid isolation from and plasmid conservation in Clostridium acetobutylicum DSM 792. Appl. Microbiol. Biotechnol. 50:564–567.

Narayan, R. 1994. Impact of governmental policies, regulations, and standards activities on an emerging biodegradable plastics industry. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 261–272.

Nativel, F., J. Pourquie, D. Ballerini, J. P. Vandecasteele, and P. Renault. 1992. The biotechnology facilities at Soustons for biomass conversion. Int. J. Solar Energy 11:219–229.

Nawrath, C., Y. Poirier, and C. Somerville. 1994. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of Arabidopsis thaliana results in high levels of polymer accumulation. Proc. Natl. Acad. Sci. USA 91:12760–12764.

Neale, A. D., R. K. Scopes, J. M. Kelly, and R. E. H. Wettenhall. 1986. The two alcohol dehyrogenases of Zymomonas mobilis: Purification by differential dye ligand chromatography, molecular characterisation and physiological roles. Eur. J. Biochem. 154:119–124.

Neale, A. D., R. K. Scopes, R. E. H. Wettenhall, and N. J. Hoogenraad. 1987. Nucleotide sequence of the pyruvate decarboxylase gene from Zymomonas mobilis. Nucleic Acids Res. 15:1753–1761.

Neale, A. D., R. K. Scopes, and J. M. Kelly. 1988. Alcohol production from glucose and xylose using Escherichia coli containing Zymomonas mobilis genes. Appl. Microbiol. Biotechnol. 29:162–167.

Nghiem, N. P., B. H. Davison, J. E. Thompson, B. E. Suttle, and G. R. Richardson. 1996. The effect of biotin on the production of succinic acid by Anaerobiospirillum succiniciproducens. Appl. Biochem. Biotechnol. 57/58:633–638.

Nghiem, N. P., B. H. Davison, B. E. Suttle, and G. R. Richardson. 1997. Production of succinic acid by Anaerobiospirillum succiniciproducens. Appl. Biochem. Biotechnol. 63–65:565–576.

Nghiem, N. P., M. I. Donnelly, and C. S. Millard. 1999. Method for the production of dicarboxylic acids. US Patent. 5,869,301.

Nickol, G. B. 1979. Vinegar. *In:* H. J. Peppler and D. Pearlman (Eds.) Microbial Technology. Academic Press. New York, NY. 2:155–172.

Nidetzky, B., M. Furlinger, and D. Gollhofer. 1997. Improved operational stability of cell-free glucose-fructose oxidoreductase from Zymomonas mobilis for the efficient synthesis of gluconic acid and sorbitol, in acoutin ultra filtration membrane reactor. Biotechnol. Bioeng. 53: 623–629.

Nilegaonkar, S., S. B. Bhosale, D. C. Kshirsagar, and A. H. Kapadi. 1992. Production of 2,3-butanediol from glucose by Bacillus licheniformis. World J. Microbiol. Biotechnol. 8:378–381.

Nimcevic, D., M. Schuster, and J. R. Gapes. 1998. Solvent production by Clostridium beijerinckii NRRL B592 growing on different potato media. Appl. Microbiol. Biotechnol. 50:426–428.

Nimcevic, D., and J. R. Gapes. 2000. The acetone-butanol fermentation in pilot plant and pre-industrial scale. J. Molec. Microbiol. Biotechnol. 2:15–20.

Nishikawa, M., R. M. R. Brancon, K. L. Pinder, and G. A. Strasdine. 1970. Fermentation of spent sulfite liquor to produce acetic acid, propionic acid and vitamin B12. Pulp and Paper Magazine of Canada 71(3):T59–T64.

Nishioka, M., T. Tuzuki, Y. Wanajyo, H. Oonami, and T. Horiuchi. 1994. Biodegradation of Bionolle. Stud. Polym. Sci. 12:584–590.

Nölling, J., G. Breton, M. V. Omelchenko, K. S. Markarova, Q. Zeng, R. Gibson, H. M. Lee, J. DuBois, D. Qiu, J. Hitti, GTC Sequencing Center Production, Finishing, and Bioinformatics Teams, Y. I. Wolf, R. L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M. J. Daly,

G. N. Bennett, E. V. Koonin, and D. R. Smith. 2001. Genome sequence and comparitive analysis of the solvent-producing bacterium Clostridium acetobutylicum. J. Bacteriol. 183:4823–4838.

Noon, R. 1982. Power-grade butanol. Chemtech 12:681–683.

Northrop, J. H., L. H. Ashe, and J. K. Senior. 1919. Biochemistry of Bacillus acetoethylicum with reference to the formation of acetone. J. Biol. Chem. 39:1–21.

Noury, and N. V. van der Lande. 1962. Process for preparation of gluconic acid monohydrate. British Patent 902609. Chem. Abstr. 57:13020g.

Oben, J. M., M. J. Amagro, and A. Manjon. 1996. Continuous retention of native NADP(H) in an enzyme membrane reactor for gluconate and glutamate production. J. Biotechnol. 50:27–36.

Ogata, S., and M. Hongo. 1979. Bacteriophages of the genus Clostridium. Adv. Appl. Microbiol. 25:241–273.

Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991a. Genetic improvement of Escherichia coli for ethanol production: Chromosomal integration of Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. 57:893–900.

Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991b. Metabolic engineering of Klebsilla oxytoca M5A1 for ethanol production from xylose and glucose. Appl. Environ. Microbiol. 57:2810–2815.

Oiwa, H., M. Naganuma, and S.-I. Ohnuma. 1987. Acetone-butanol production from dahlia inulin by Clostridium pasteurianum var. I-53. Agric. Biol. Chem. 51:2819–2820.

Okumura, H., H. Tagami, M. Fukaya, H. Masai, Y. Kawamura, S. Horinouchi, and T. Beppu. 1988. Cloning of the β-isopropylmalate dehydrogenase gene from Acetobacter aceti and its use for construction of a new host vector system for Acetobacter. Agric. Biol. Chem. 52:3125–3129.

Olijve, W., and J. J. Kok. 1979a. An analysis of growth of Gluconobacter oxydans in chemostat cultures. Arch. Microbiol. 121:291–297.

Olijve, W., and J. J. Kok. 1979b. Analysis of growth of Gluconobacter oxydans in glucose containing medium. Arch. Microbiol. 121:283–290.

O'Mullan, P. J., S. E. Buchholz, T. Chase Jr., and D. E. Eveleigh. 1995. Roles of alcohol dehydrogenases of Zymomonas mobilis (ZADH): Characterization of a ZADH-2-negative mutant. Appl. Microbiol. Biotechnol. 43:675–678.

O'Neill, H., S. G. Mayhew, and G. Butler. 1998. Cloning and analysis of the genes for a novel electron-transferring flavoprotein from Megasphaera elsdenii. J. Biol. Chem. 273:21015–21024.

Osuga, J., A. Mori, and J. Kato. 1984. Acetic acid production by immobilized Acetobacter aceti cells entrapped in a kappa-carrageenan gel. J. Ferment. Technol. 62:139–149.

Osuna, J., X. Soberon, and E. Morett. 1997. A proposed architecture for the central domain of the bacterial enhancer-binding proteins based on secondary structure prediction and fold recognition. Protein Sci. 6:543–555.

Oude Elferink, S. J. W. H., J. Krooneman, J. C. Gorrschal, S. F. Spoelstra, F. Faber, and F. Driehuis. 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by Lactobacillus buchneri. Appl. Environ. Microbiol. 67:125–132.

Ouellette, J. 2000. PO and PG markets face tight margins. Chem. Mark. Rep. 257:5 and 14.

Oultram, J. D., and M. Young. 1985. Conjugal transfer of plasmid pAMβ1 from Streptococcus lactis and Bacillus subtilis to Clostridium acetobutylicum. FEMS Microbiol. Lett. 27:129–134.

Oultram, J. D., A. Davies, and M. Young. 1987. Conjugal transfer of a small plasmid from Bacillus subtilis to Clostridium acetobutylicum by cointegrate formation with plasmid pAMβ1. FEMS Microbiol. Lett. 42:113–119.

Oultram, J. D., M. Loughlin, T.-J. Swinfield, J. K. Brehm, D. E. Thompson, and N. P. Minton. 1988. Introduction of plasmids into whole cells of Clostridium acetobutylicum by electroporation. FEMS Microbiol. Lett. 56:83–88.

Oultram, J. D., I. D. Burr, M. J. Elmore, and N. P. Minton. 1993. Cloning and sequence analysis of the genes encoding phosphotransbutyrylase and butyrate kinase from Clostridium acetobutylicum NCIMB 8052. Gene 131: 107–112.

Owen, K., and T. Coley. 1990. Oxygenated blend components for gasoline. Automotive Fuels Handbook. Society of Automotive Engineers. Warrendale, PA. 221–259.

Page, W. J., and A. Cornish. 1993. Growth of Azotobacter vinelandii UWD in fish peptone medium and simplified extraction of poly-beta-hydroxybutyrate. Appl. Environ. Microbiol. 59:4236–4244.

Paik, H. D., and B. A. Glatz. 1994. Propionic acid production by immobilized cells of a propionate-tolerant strain of Propionibacterium acidipropionici. Appl. Microbiol. Biotechnol. 42:22–27.

Palosaari, N. R., and P. Rogers. 1988. Purification and properties of the inducible coenzyme A-linked butyraldehyde dehydrogenase from Clostridium acetobutylicum. J. Bacteriol. 170:2971–2976.

Pan, J. G., S. A. Soo, C. K. Park, P. Kim, D. E. Chang, and J. E. Kim. 1999. A pta ldhA double mutant Escherichia coli SS373 and the method of producing succinic acid therefrom. PCT Application. WO 99/06532.

Papanikolaou, S., P. Ruiz-Sanchez, B. Pariset, F. Blanchard, and M. Fick. 2000. High production of 1,3-propanediol from industrial glycerol by a newly isolated Clostridium butyricum strain. J. Biotechnol. 77:191–208.

Papoutsakis, E. T., and C. L. Meyer. 1985. Equations and calculations of product yields and preferred pathways for butanediol and mixed-acid fermentations. Biotechol. Bioengin. 27:50–66.

Papoutsakis, E. T., and G. N. Bennett. 1993. Cloning, structure, and expression of acid and solvent pathway genes of Clostridium acetobutylicum. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 157–200.

Papoutsakis, E. T., and G. N. Bennett. 1999. Molecular regulation and metabolic engineering of solvent production by Clostridium acetobutylicum. *In:* E. T. Papoutsakis and S. Y. Lee (Eds.) Bioprocess Technology. Marcel Dekker. New York, NY. 24:253–279.

Paquet, V., C. Croux, G. Goma, and P. Soucaille. 1991. Purification and characterization of the extracellular α-amylase from Clostridium acetobutylicum. Appl. Environ. Microbiol. 57:212–218.

Parekh, S. R., and M. Cheryan. 1991. Production of acetate by mutant strains of Clostridium thermoaceticum. Appl. Microbiol. Biotechnol. 36:384–387.

Parekh, S. R., and M. Cheryan. 1994a. Continuous production of acetate by Clostridium thermoaceticum in a cell-recycle membrane bioreactor. Enz. Microb. Technol. 16:104–109.

Parekh, S. R., and M. Cheryan. 1994b. High concentrations of acetate with a mutant strain of Clostridium thermoaceticum. Biotechnol. Lett. 16:139–142.

Park, Y. S., H. Ohtake, M. Fukaya, H. Okumura, Y. Kawamura, and K. Toda. 1989. Acetic acid production using a fermenter equipped with a hollow fiber filter module. Biotechnol. Bioeng. 33:918–923.

Park, Y. S., H. Ohtake, M. Fukaya, H. Kawamura, and K. Toda. 1991. Production of high concentration of acetic acid by Acetobacter aceti using a repeated fed-batch culture with cell recycling. Appl. Microbiol. Biotechnol. 35:149–153.

Park, D. H., and G. K. Zeikus. 1999. Utilization of electrically reduced neutral red by Actinobacillus succinogenes: Physiological function of netural red in membrane-driven fumarate reduction and energy conservation. J. Bacteriol. 181:2403–2410.

Parrer, G., G. Schroll, J. R. Gapes, W. Lubitz, and K. C. Schuster. 2000. Conversion of solvent evaporation residues from the AB-(acetone-butanol) bioprocess into bacterial cells accumulating thermoplastic polyesters. J. Molec. Microbiol. Biotechnol. 2:81–86.

Pasteur, L. 1861a. Animalcules infusoires vivant sans gaz oxygène libre et déterminant des fermentations. Comptes Rendus 52:344–347.

Pasteur, L. 1861b. Mémoire sur les corpuscles organisés qui éxistent dans l'atmosphere: Esamen de la doctrine des générations spontanées. Annales Sci. Natur., 4th series 16:5–98.

Pasteur, L. 1868. Etudes sur le vinaigre, sa fabrication se maladres, moyens de les préveair nouvelles observations sur la conservation des vinc par la châleur. Gauthier-Villars. Paris, FR.

Pasteur, L. 1879. Studies on Fermentation. London.

Pedroni, P., A. Volpe, G. Galli, G. M. Mura, C. Pratesi, and G. Grandi. 1995. Characterization of the locus encoding the [Ni-Fe] sulfhydrogenase from the archaeon Pyrococcus furiosus: Evidence for a relationship to bacterial sulfite reductases. Microbiology 141:449–458.

Peguin, S., G. Goma, P. Delorme, and P. Soucaille. 1994. Metabolic flexibility of Clostridium acetobutylicum in response to methyl viologen addition. Appl. Microbiol. Biotechnol. 42:611–616.

Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey. 1987. Biosynthetic thiolase from Zoogloea ramigera. III: Isolation and characterization of the structural gene. J. Biol. Chem. 262:97–102.

Peoples, O. P., and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16: Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. 264:15293–15297.

Peretz, M., O. Bogin, S. Tel-Or, A. Cohen, G. Li, J.-S. Chen, and Y. Burstein. 1997. Molecular cloning, nucleotide sequencing, and expression of genes encoding alcohol dehydrogenase from the thermophile Thermoanaerobacter brockii and the mesophile Clostridium beijerinckii. Anaerobe 3:259–270.

Perozich, J., H. Nicholas, B. C. Wang, R. Lindahl, and J. Hempel. 1999. Relationships within the adehyde dehydrogenase extended family. Protein Sci. 8:137–146.

Petersen, D. J., R. W. Welch, F. B. Rudolph, and G. N. Bennett. 1991. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from Clostridium acetobutylicum ATCC 824. J. Bacteriol. 173:1831–1834.

Petersen, D. J., J. W. Cary, J. Vanderleyden, and G. N. Bennett. 1993. Sequence and arrangement of genes encoding enzymes of the acetone-production pathway of Clostridium acetobutylicum ATCC 824. Gene 123:93–97.

Petitdemange, H., C. Cherrier, G. Raval, and R. Gay. 1976. Regulation of NADH and NADPH ferredoxin oxidoreductases in clostridia of the butyric group. Biochem. Biophys. Acta 421:334–347.

Petitdemange, E., C. Durr, S. Abbad-Andalousi, and G. Raval. 1995. Fermentation of raw glycerol to 1,3-propanediol by new strains of Clostridium butyricum. J. Indust. Microbiol. 15:498–502.

Pflugmacher, U., and G. Gottschalk. 1994. Development of an immobilized cell reactor for the production of 1,3-propanediol by Citrobacter freundii. Appl. Microbiol. Biotechnol. 41:313–316.

Philips, J. R., E. C. Clausen, and J. L. Gaddy. 1994. Synthesis gas as substrate for the biological production of fuels and chemicals. Appl. Biochem. Biotechnol. 45–46:145–157.

Phillips, M. A. 1963. Catalytic hydrogenation of glucose to sorbitol using a highly active catalyst. Br. Chem. Eng. 8(11):767–769.

Pierce, S. M., and M. Wayman. 1983. Diesel fuel by fermentation of wastes. US Patent. 4368056.

Piveteau, P. 1999. Metabolism of lactate and sugars by dairy propionibacteria: A review. Lait 79:23–41.

Planas, J., P. Radström, F. Tjerneld, and B. Hahn-Hägerdal. 1996. Enhanced production of lactic acid through the use of a novel aqueous two-phase system as an extractive fermentation system. Appl. Microbiol. Biotechnol. 45:737–743.

Playne, M. J. 1985. Propionic acid and butyric acid. *In:* Moo-Young (Ed.) Comprehensive Biotechnology. Pergamon. New York, NY. 3:731–755.

Podkovyrov, S. M., and J. G. Zeikus. 1993. Purification and characterization of phosphoenolpyruvate carboxykinase, a catabolic CO_2-fixing enzyme, from Anaerobiospirillum succiniciproducens. J. Gen. Microbiol. 139: 223–228.

Poehland, H. D., V. Schierz, and R. Schumann. 1993. Optimization of gluconic acid synthesis by removing limitations and inhibitions. Acta Biotechnol. 13:257–268.

Poirier, Y., D. E. Dennis, K. Klomparens, and C. Somerville. 1992. Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science 256:520–522.

Poirier, Y., C. Nawrath, and C. Somerville. 1995. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Bio/technology 13:142–150.

Posno, M., P. T. H. M. Heuvelmans, M. J. F. van Giezen, B. C. Lokman, R. J. Leer, and P. H. Pouwels. 1991. Complimentation of the inability of Lactobacillus strains to utilize D-xylose with D-xylose catabolism-encoding genes of Lactobacillus pentosus. Appl. Environ. Microbiol. 57:2764–2766.

Potera, C. 1997. Genencor & DuPont create "green" polyester. Genet. Eng. News 17:17.

Prescott, S. C., and C. G. Dunn. 1949. The acetone-butanol fermentation. Industrial Microbiology, 2nd ed. McGraw-Hill. New York, NY. 312–351.

Prescott, S. C., and C. G. Dunn. 1949. The acetone-butanol fermentation. Industrial Microbiology, 2nd ed. McGraw-Hill. New York, NY. 477–486.

Prescott, S. C., and C. G. Dunn. 1959a. The acetone-butanol fermentation. *In:* Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 250–284.

Prescott, S. C., and C. G. Dunn. 1959b. The acetone-ethanol fermentation. *In:* Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 295–298.

Prescott, S. C., and C. G. Dunn. 1959c. The butanol-isopropanol fermentation. Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 285–294.

Pries, A., A. Steinbüchel, and H. G. Schlegel. 1990. Lactose-utilizing and galactose-utilizing strains of poly(hydroxy-alkanoic acid)-accumulating Alcaligenes eutrophus and Pseudomonas saccharophila obtained by recombinant DNA technology. Appl. Microbiol. Biotechnol. 33:410–417.

Prieto, M. A., B. Buhler, K. Jung, B. Witholt, and B. Kessler. 1999a. PhaE, a polyhydroxyalkanoate-granule-associated protein of Pseudomonas oleovorans GPo1 involved in the regulatory expression system for pha genes. J. Bacteriol. 181:858–868.

Prieto, M. A., M. B. Kellerhals, G. B. Bozzato, D. Radnovic, B. Witholt, and B. Kessler. 1999b. Engineering of stable recombinant bacteria for production of chiral medium-chain-length poly-3-hydroxyalkanoates. Appl. Environ. Microbiol. 65:3265–3271.

Pronk, J. T., P. R. Levering, W. Olijve, and J. P. Van Dijken. 1989. The role of NADP-dependent and quino protein glucose dehydrogenase in gluconic acid production by Gluconobacter oxydans. Enz. Microb. Technol. 11:160–164.

Quesada-Chanto, A., A. S. Afschar, and F. Wagner. 1994a. Microbial production of propionic acid and vitamin B-12 using molasses or sugar. Appl. Microbiol. Biotechnol. 41:378–383.

Quesada-Chanto, A., A. S. Afschar, and F. Wagner. 1994b. Optimization of a Propionibacterium acidipropionici continuous culture utilization of sucrose. Appl. Microbiol. Biotechnol. 42:16–21.

Quesada-Chanto, A., J. P. C. L. da Costa, M. M. Silveira, A. G. Schroeder, A. C. Schmidt-Meyer, and R. Jona. 1998a. Influence of different vitamin-nitrogen sources on cell growth and propionic acid production from sucrose by Propionibacterium shermanii. Acta Biotechnologia 18:267–274.

Quesada-Chanto, A., M. M. Silveira, A. C. Schmidt-Meyer, A. G. Schroeder, J. P. C. L. da Costa, J. Lopez, M. F. Carvalho-Jonas, M. J. Artolozaga, and R. Jona. 1998b. Effect of oxygen supply on pattern of growth and corrinoid and organic acid production of Propionibacterium shermanii. Appl. Microbiol. Biotechnol. 49:732–736.

Qureshi, N., and H. P. Blaschek. 2001a. ABE production from corn: A recent economic evaluation. J. Indust. Microbiol. Biotechnol. 27:292–297.

Qureshi, N., A. Lolas, and H. P. Blaschek. 2001b. Soy molasses as fermentation substrate for production of butanol using Clostridium beijerinckii BA101. J. Indust. Microbiol. Biotechnol. 26:290–295.

Ragsdale, S. W. 1994. CO dehydrogenase and the central role of this enzyme in fixation of carbon-dioxide by anaerobic bacteria. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 88–126.

Ragsdale, S. W., and C. G. Riordan. 1996. The role of nickel in acetyl-CoA synthesis by the bifunctional enzyme CO dehydrogenase/acetyl-CoA synthase: Enzymology and model chemistry. J. Biol. Inorg. Chem. 1:489–493.

Rakhley, G., Z. H. Zhou, M. W. W. Adams, and K. L. Kovacs. 1999. Biochemical and molecular characterization of the [NiFe] hydrogenase from the hyperthermophilic archaeon, Thermococcus litoralis. Eur. J. Biochem 266:1158–1165.

Ramachandran, K. B., and G. Goma. 1988. 2,3-Butanediol production from glucose by Klebsiella pneumoniae in a cell recycle system. J. Biotechnol. 9:39–46.

Rani, K. S., and G. Seenayya. 1999. High ethanol tolerance of new isolates of Clostridium thermocellum strains SS21 and SS22. World J. Microbiol. Biotechnol. 15:173–178.

Rao, G., and R. Mutharasan. 1986. Alcohol production by Clostridium acetobutylicum induced by methyl viologen. Biotechnol. Lett. 8:893–896.

Rao, G., and R. Mutharasan. 1987. Altered electron flow in continuous cultures of Clostridium acetobutylicum induced by viologen dyes. Appl. Environ. Microbiol. 53:1232–1235.

Rao, G., and R. Mutharasan. 1988. Altered electron flow in a reduced environment in Clostridium acetobutylicum. Biotechnol. Lett. 10:129–132.

Raspoet, D., B. Pot, D. De Deyn, P. De Vos, K. Kersters, and J. De Ley. 1991. Differentiation between 2,3-butanediol producing Bacillus licheniformis and B. polymyxa strains by fermentation product profiles and whole-cell protein electrophoretic patterns. Syst. Appl. Microbiol. 14:1–7.

Ravagnani, A., K. C. B. Jennert, E. Steiner, R. Grunberg, J. R. Jefferies, S. R. Wilkinson, D. I. Young, E. C. Tidswell, D. P. Brown, P. Youngman, J. G. Morris, and M. Young. 2000. Spo0A directly controls the switch from acid to solvent production in solvent-forming clostridia. Molec. Microbiol. 37:1172–1185.

Rayner, A. 1926. The occurrence, properties, and uses of trimethylene glycol, and the fermentation of glycerin lyes. J. Soc. Chem. Indust. 45:265T–266T.

Reed, G. 1982. Production of fermentation alcohol as a fuel source. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 835–859.

Rehberger, J. L., and B. A. Glatz. 1998. Response of cultures of Propionibacterium to acid and low pH tolerance and inhibition. J. Food Protect. 61:211–216.

Rehm, B. H. A., N. Kruger, and A. Steinbüchel. 1998. A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. J. Biol. Chem. 237:24044–24051.

Rehr, B., C. Wilhelm, and H. Sahm. 1991. The production of sorbitol and gluconic acid by permeablized cells of Zymomonas mobilis. Appl. Microbiol. Biotechnol. 35: 144–148.

Reid, S. J., E. R. Allcock, D. T. Jones, and D. R. Woods. 1983. Transformation of Clostridium acetobutylicum protoplasts with bacteriophage DNA. Appl. Environ. Microbiol. 45:305–307.

Reid, M. F., and C. A. Fewson. 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. 20:13–56.

Reimann, A., and H. Biebl. 1996. Production of 1,3-propanediol by Clostridium butyricum DSM 5431 and product tolerant mutants in fedbatch culture: Feeding strategy for glycerol and ammonium. Biotechnol. Lett. 18:827–832.

Reimann, A., S. Abbad-Andaloussi, H. Biebl, and H. Petitdemange. 1998a. 1,3-propanediol formation with prod-

uct-tolerant mutants of Clostridium butyricum DSM 5431 in continuous culture: Productivity, carbon and electron flow. J. Appl. Microbiol. 84:1125–1130.

Reimann, A., H. Biebl, and W. D. Deckwer. 1998b. Production of 1,3-propanediol by Clostridium butyricum in continuous culture with cell recycling. Appl. Microbiol. Biotechnol. 49:359–363.

Reisch, M. S. 1999. Vision 2020 comes into focus. Chem. Eng. News 77(32):10–12.

Ren, Q., N. Sierro, M. Kellerhals, B. Kessler, and B. Witholt. 2000. Properties of engineered poly-3-hydroxyalkanoates produced in recombinant Esherichia coli strains. Appl. Environ. Microbiol. 66:1311–1320.

Reusch, R. N., and H. L. Sadoff. 1988. Putative structure and functions of a poly-β-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc. Natl. Acad. Sci. USA 85:4176–4180.

Reusch, R. N. 1992a. Biological complexes of poly-β-hydroxybutyrate. FEMS Microbiol. Rev. 103:119–130.

Reusch, R. N., and A. Sparrow. 1992b. Transport of poly-β-hydroxybutyrate in human plasma. Biochim. Biophys. Acta 1123:33–40.

Reynen, M., and H. Sahm. 1988. Comparison of the structural genes for pyruvate decarboxylase in different Zymomonas mobilis strains. J. Bacteriol. 170:3310–3313.

Reysset, G., and M. Sebald. 1985. Conjugal transfer of plasmid-mediated antibiotic resistance from streptococci to Clostridium acetobutylicum. Ann. Inst. Pasteur/Microbiol. 136B:275–282.

Reysset, G., and M. Sebald. 1993. Transformation/electrotransformation of clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 151–158.

Rickert, D. A., C. E. Glatz, and B. A. Glatz. 1998. Improved organic acid production by calcium alginate-immobilized propionibacteria. Enzyme Microb. Technol. 22:409–414.

Ro, H. S., and H. S. Kim. 1991. Continuous production of gluconic acid and sorbitol from sucrose using invertase and an oxidoreductase from Zymomonas mobilis. Enz. Microb. Technol. 13:920–924.

Roehr, M., C. P. Kubicek, and J. Kominek. 1996. Gluconic acid. *In:* M. Roehr (Ed.) Biotechnology, 2nd ed. VCH. New York, NY. 348–362.

Rogers, P. L., K. J. Lee, M. L. Skotnicki, and D. E. Tribe. 1982. Ethanol production by Zymomonas mobilis. Adv. Biochem. Engin. 23:37–84.

Rogers, P. 1986. Genetics and biochemistry of Clostridium relevant to development of fermentation processes. Adv. Appl. Microbiol. 31:1–60.

Rogers, P., and N. Palosaari. 1987. Clostridium acetobutylicum mutants that produce butyraldehyde and altered quantities of solvents. Appl. Environ. Microbiol. 53: 2761–2766.

Rogers, P. L., K. J. Lee, G. M. Smith, and K. D. Barrow. 1989. Ethanol tolerance of Zymomonas mobilis. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 239–256.

Rogers, P., and G. Gottschalk. 1993. Biochemistry and regulation of acid and solvent production in clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. Stoneham, MA. 25–50.

Rogers, P. 1999. Clostridia: Solvent formation. *In:* M. C. Flickinger and S. W. Drew (Eds.) Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation. John Wiley. New York, NY. 2:670–687.

Roos, J. W., J. K. McLaughlin, and E. T. Papoutsakis. 1985. The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of Clostridium acetobutylicum. Biotechnol. Bioengin. 27:681–694.

Rose, N. 1986. Chaim Weizmann. Penguin Books. New York, NY.

Rosenberg, S. L. 1980. Fermentation of pentose sugars to ethanol and other neutral products by microorganisms. Enz. Microb. Technol. 2:185–193.

Ross, D. 1961. The acetone-butanol fermentation. Progr. Indust. Microbiol. 3:71–90.

Rudolph, F. B., D. L. Purich, and H. J. Fromm. 1968. Coenzyme A-linked aldehyde dehydrogenase from Escherichia coli. I: Partial purification, properties, and kinetic studies of the enzyme. J. Biol. Chem. 243:5539–5545.

Ryden, R. 1958. Development of anaerobic fermentation processes: Acetone-butanol. *In:* R. Steel (Ed.) Biochemical Engineering. Macmillan. New York, NY. 125–148.

Sablayrolles, J. M., and G. Goma. 1984. Butanediol production by Aerobacter aerogenes NRRL B199: Effects of initial substrate concentration and aeration agitation. Biotechnol. Bioengin. 26:148–155.

Saeki, A., M. Taniguchi, K. Matsushita, H. Yoyama, G. Theeragol, N. Lotong, and O. Adachi. 1997a. Microbial aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. Biosci. Biotech. Biochem. 61:317–323.

Saeki, A., G. Theeragol, and K. Matsushita. 1997b. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at high temperatures. Biosci. Biotech. Biochem. 61:138–145.

Saha, B. C., and R. J. Bothast. 1999. Production of 2,3-butanediol by newly isolated Enterobacter cloacae. Appl. Microbiol. Biotechnol. 52:321–326.

Saint-Amans, S., P. Perlot, G. Goma, and P. Soucaille. 1994. High production of 1,3-propanediol from glycerol by Clostridium butyricum VPI 3266 in a simply controlled fed-batch system. Biotechnol. Lett. 16:831–836.

Saint-Amans, S., L. Girbal, J. Andrade, K. Ahrens, and P. Soucaille. 2001. Regulation of carbon and electron flow in Clostridium butyricum VP 13266g frown on glucose-glycerol mixtures. J. Bacteriol. 183:1748–1754.

Sakura, H., H. W. Lee, S. Sato, S. Mukataka, and J. Takahashi. 1989. Gluconic acid production by Aspergillus niger immobilized on nonwoven fabric. J. Ferment. Bioeng. 67:404–408.

Samuelov, N. S., R. Lamed, S. Lowe, and J. G. Zeikus. 1991. Influence of CO_2-HCO_3-levels and pH on growth, succinate production, and enzyme activities of Anaerobiospirillum succiniciproducens. Appl. Environ. Microbiol. 57:3013–3019.

Samuelov, N. S., R. Datta, M. K. Jain, and J. G. Zeikus. 1999. Whey fermentation by Anaerobiospirillum succiniciproducens for production of a succinate-based animal feed additive. Appl. Env. Microbiol. 65:2260–2263.

Sanchez-Riera, F., D. C. Cameron, and C. L. Cooney. 1987. Influence of environmental factors in the production of R(-)-1,2-propanediol by Clostridium thermosaccharolyticum. Biotechnol. Lett. 9:449–454.

Sass, C., J. Walter, and G. N. Bennett. 1993. Isolation of mutants of Clostridium acetobutylicum ATCC 824. Curr. Microbiol. 26:151–154.

Sauer, E. T. 1991. Carboxylic acids (economic aspects). *In:* Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 5:179–187.

Sauer, U., and P. Dürre. 1995. Differential induction of genes related to solvent formation during the shift from acidogenesis to solventogenesis in continuous culture of Clostridium acetobutylicum. FEMS Microbiol. Lett. 125:115–120.

Sauer, U., J. D. Santangelo, A. Trever, M. Bucholz, and P. Dürre. 1995. Sigma factor and sporulation genes in Clostridium. FEMS Microbiol. Rev. 17:331–340.

Scheirlinck, T., J. Mahillon, H. Joos, P. Dahaese, and F. Michiels. 1989. Integration and expression of α?amylase and endoglucanase genes in the Lactobacillus plantarum chromosome. Appl. Environ. Microbiol. 55:2130–2137.

Schilling, L. B. 1995. Chemicals from alternative feedstocks in the United States. FEMS Microbiol. Rev. 16:101–110.

Schlegel, H. G., G. Gottschalk, and R. Von Vartha. 1961. Formation and utilization of poly-β-hydroxybutyric acid by Knallgas bacteria (Hydrogenomonas). Nature 191:463–465.

Schneider, Z., E. G. Larsen, G. Jacobson, B. C. Johnson, and J. Pawelkiewicz. 1970. Purification and properties of glycerol dehydrase. J. Biol. Chem. 245:3388–3396.

Schoutens, G. H., M. C. H. Nieuwenhuizen, and N. W. F. Kossen. 1984. Butanol from whey ultrafiltrate: Batch experiments with Clostridium beyerinckii LMD 27.6. Appl. Microbiol. Biotechnol. 19:203–206.

Schoutens, G. H., and W. J. Groot. 1985a. Economic feasibility of the production of iso-propanol-butanol-ethanol fuels from whey permeate. Process Biochem. 20:117–121.

Schoutens, G. H., M. C. H. Nieuwenhuizen, and N. W. F. Kossen. 1985b. Continuous butanol production from whey permeate with immobilized Clostridium beyerinckii LMD 27.6. Appl. Microbiol. Biotechnol. 21:282–286.

Schubert, P., A. Steinbüchel, and H. G. Schlegel. 1988. Cloning of the Alcaligenes eutrophus gene for synthesis of poly-β-hydroxybutyric acid and synthesis of PHB in Escherichia coli. J. Bacteriol. 170:5837–5847.

Schuster, K. C., R. Goodacre, J. R. Gapes, and M. Young. 2001. Degeneration of solventogenic Clostridium strains monitored by Fourier transform infrared spectroscopy of bacterial cells. J. Ind. Microbiol. Biotechnol. 27:314–321.

Scopes, R. K. 1983. An iron-activated alcohol dehydrogenase. FEBS Lett. 156:303–306.

Seifert, C., S. Bowien, G. Gottschalk, and R. Daniel. 2001. Identification and expression of the genes and purification nd characterization of the gene products involved in reactivation of coenzyme B12-dependent glycerol dehydratase of Citrobacter freundii. Eur. J. Biochem. 268:2369–2378.

Senthuran, A., V. Senthuran, B. Mathasson, and R. Kaul. 1997. Lactic acid fermentation in a recycle batch reactor using immobilized Lactobacillus casei. Biotechnol. Bioeng. 55:841– 853.

Seyfried, M., R. Daniel, and G. Gottschalk. 1996. Cloning, sequencing, and overexpression of the genes encoding coenzyme B12-dependent glycerol dehydratase of Citrobacter freundii. J. Bacteriol. 178:5793–5796.

Shah, M. M., and M. Cheryan. 1995a. Acetate production by Clostridium thermoaceticum in corn steep liquor media. J. Ind. Microbiol. 15:424–428.

Shah, M. M., and M. Cheryan. 1995b. Improvement of productivity in acetic acid fermentation with Clostridium thermoaceticum. Appl. Biochem. Biotechnol. 51/52: 418–422.

Sheehan, J. 2000. The road to bioethanol: A strategic perspective of the U.S. Department of Energy's national ethanol program. *In:* H. M. E., J. O. Baker, and J. N. Saddler (Ed.) Glycosyl Hydrolases for Biomass Conversion. American Chemical Society. Washington DC, ACS Symposium Series 769:2–25.

Shelley, S., and R. L. D'Aquino. 1999. Three routes vie for the 1,3-propanediol market. Chem. Engin. 106:56.

Shen, G. J., B. A. Annons, R. W. Lovitt, M. K. Jain, and J. G. Zeikus. 1996. Biochemical route and control if butyrate synthesis in Butyribacterium methylotropicum. Appl. Microbiol. Biotechnol. 45:355–362.

Sherman, J. M., and R. H. Shaw. 1923. Process for the production of propionates and propionic acid. US Patent 1,470,885. Chem Abstr. 18:146.

Shiraishi, F., K. Kawakami, S. Kono, and A. Tamura. 1989a. Continous production of free gluconic acid by Gluconobacter suboxydans IFO 3290 immobilized by adsorption on ceramic honeycomb monolith: Effect of reactor configuration on further oxidation of gluconic acid to ketogluconic acid. Appl. Microbiol. Biotechnol. 31:445–447.

Shiraishi, F., K. Kawakami, S. Kono, A. Tamura, S. Tsuruta, and K. Kosmoki. 1989b. Characterization of production of free gluconic acid by Gluconobacter suboxydans absorbed on ceramic honeycomb monolith. Biotechnol. Bioeng. 33:1413–1418.

Shoham, Y., R. Lamed, and E. A. Bayer. 1999. The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol. 7:275–281.

Shone, C. C., and H. J. Fromm. 1981. Steady-state and presteady-state kinetics of coenzyme A linked aldehyde dehydrogenase from Escherichia coli. Biochemistry 20:7494–7501.

Sievers, M., S. Sellmer, and M. Teuber. 1992. Acetobacter europaeus sp. nov., a main component of industrial vinegar fermenters in central Europe. Syst. Appl. Microbiol. 15:386–392.

Sievers, M., W. Ludwig, and M. Teuber. 1994a. Phylogenetic positioning of Acetobacter, Gluconobacter, Rhodophila, and Acidiphilium species as a branch of acidophilic bacteria in the α-subclass of proteobacteria based on 16S ribosomal DNA sequences. Syst. Appl. Microbiol. 17:189–196.

Sievers, M., W. Ludwig, and M. Teuber. 1994b. Phylogenetic positioning of Acetobacter, Gluconobacter, Rhodophila and Acidiphilium species as a branch of acidophilic bacteria in the α subclass of Proteobacteria based on 16S RNA sequences. Syst. Appl. Microbiol. 17:352–354.

Sievers, M., W. Ludwig, and M. Teuber. 1994c. Revival of the species Acetobacter methanolicus (ex: Uhlig et al., 1986) nom. rev. Syst. Appl. Microbiol. 17:352–354.

Silva-Martinez, M., D. Haltrich, S. Novalic, K. D. Kobe, and B. Nidetzky. 1998. Simultaneous enzymatic syntheses of gluconic acid and sorbitol. Appl. Biochem. Biotechnol. 70–72:863–875.

Silveira, M. M., E. Wisbeck, and C. Lemmel. 1999. Bioconversion of glucose and fructose to sorbitol and gluconic acid by untreated cells of Zymomonas mobilis. J. Biotechnol. 75:99–103.

Silveira, M. M., E. Wisbeck, I. Hoch, and R. Jonas. 2001. Production of glucose-fructose oxidoreductase and ethanol by Zymomonas mobilis ATCC 29191 in medium containing corn steep liquor as a source of vitamins. Appl. Microbiol. Biotechnol. 55:442–445.

Sinskey, A. J., M. Akedo, and C. L. Cooney. 1981. Acrylate fermentations. *In:* A. Hollaender (Ed.) Trends in the Biology of Fermentations. Plenum Press. New York, NY. 473–492.

Skraly, F. A., B. L. Lytle, and D. C. Cameron. 1998. Construction and characterization of a 1,3-propanediol operon. Appl. Env. Microbiol. 64:98–105.

Slapack, G. E., I. Russell, and G. G. Stewart. 1987. Thermophilic Microbes in Ethanol Production. CRC Press. Boca Raton, FL.

Slater, S., K. L. Houmiel, M. Tran, T. A. Mitsky, N. B. Taylor, S. R. Padgette, and K. J. Gruys. 1998. Multiple β-ketothiolase mediate poly(β-hydroxyalkanoate) copolymer synthesis in Ralstonia eutropha. J. Bacteriol. 180:1979–1987.

Slater, S., T. A. Mitsky, K. L. Houmiel, M. Hao, S. E. Reiser, N. B. Taylor, M. Tran, H. E. Valentin, J. Rodriguez, D. A. Stone, S. R. Padgette, G. Kishore, and K. J. Gruys. 1999. Metabolic engineering of Arabidopsis and Brassica for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production. Nature Biotechnol. 17:1011–1016.

Smith, W. P. 1993. Soap/Cosmetic Specialties 9:54–58.

Société Lefranc et Cie. 1925. An improved process for the manufacture of dipropylketone. British Patent 216-120. Chem. Abstr. 19:77.

Sokollek, S. J., and W. P. Hammes. 1997. Description of a starter culture preparation for vinegar fermentation. Syst. Appl. Microbiol. 20:481–491.

Sokollek, S. J., C. Hertel, and W. P. Hammes. 1998. Cultivation and preservation of vinegar bacteria. J. Biotechnol. 60:195–206.

Solichien, M. S., D. O'Brien, E. G. Hammond, and C. E. Glatz. 1995. Membrane-based extractive fermentation to produce propionic and acetic acids: toxicity and mass transfer considerations. Enzyme Microb. Technol. 17:23–31.

Solomon, B. O., A. P. Zeng, H. Biebl, A. O. Ejiofor, C. Posten, and W. D. Deckwer. 1994. Effects of substrate limitation on product distribution and H_2/CO_2 ratio in Klebsiella pneumoniae during anaerobic fermentation of glycerol. Appl. Microbiol. Biotechnol. 42:222–226.

Solomon, B. O., A.-P. Zeng, H. Biebl, H. Schlieker, C. Posten, W.-D. Deckwer. 1995. Comparison of the energetic efficiencies of hydrogen and oxychemicals formation in Klebsiella pneumoniae and Clostridium butyricum during anaerobic growth on glycerol. J. Biotechnol. 39:107–117.

Solomons, G. L. 1976. Solvents from carbohydrates: Some economic considerations. Process Biochem. 11:32–37.

Somrutai, W., M. Takagi, and T. Yoshida. 1996. Acetone-butanol fermentation by Clostridium aurantibutyricum ATCC 17777 from a model medium for palm oil mill effluent. J. Ferment. Bioengin. 81:543–547.

Spivey, M. J. 1978. The acetone/butanol/ethanol fermentation. Process Biochem. 13:2–25.

Sprenger, G. A., B. A. Hammer, E. A. Johnson, and E. C. Lin. 1989. Anaerobic growth of Escherichia coli on glycerol by importing genes of the dha regulon from Klebsiella pneumoniae. J. Gen. Microbiol. 135:1255–1262.

Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846–849.

Steinbüchel, A. 1991. Polyhydroxyalkanoic acids. *In:* D. Byrom (Ed.) Biomaterials: Novel Materials from Biological Sources. Macmillan.

Steinbüchel, A., and G. Schmack. 1995. Large-scale production of poly(3-hydroxyvaleric acid) by fermentation of Chromobacterium violaceleum, processing, and characterization of the homopolyester. J. Environ. Polym. Degrad. 3:243–258.

Steinbüchel, A. 1996. PHB and other polyhydroxyalkanoic acids. *In:* M. Roehr (Ed.) Biotechnology, 2nd edn. VCH. Weinheim, Germany. 6:403–464.

Steinbüchel, A., and V. Gorenflo. 1997. Biosynthetic and biodegradable polyesters from renewable resources: Current state and prospects. Macromol. Symp. 123:61–66.

Steinbüchel, A., and B. Füchtenbusch. 1998. Bacterial and other biological systems for polyester production. Trends Biotechnol. 16:419–427.

Stephens, G. M., R. A. Holt, J. C. Gottschal, and J. G. Morris. 1985. Studies on the stability of solvent production by Clostridium acetobutylicum in continuous culture. J. Appl. Bacteriol. 59:597–605.

Stevens, D., S. Alam, and R. Bajpai. 1988. Fermentation of cheese whey by a mixed culture of Clostridium beijerinckii and Bacillus cereus. J. Ind. Microbiol. 3:15–19.

Stieb, M., and B. Schink. 1984. A new 3-hydroxybutyrate fermenting anaerobe, Ilyobacter polytropus, gen. nov.sp.nov., possessing various fermentation pathways. Arch Microbiol. 140:139–146.

Stim-Herndon, K. P., D. J. Petersen, and G. N. Bennett. 1995. Molecular characterization of the acetyl coenzyme A acetyltransferase (thiolase) from Clostridium acetobutylicum ATCC 824. Gene 154:81–85.

St. Martin, E. J., W. B. Freedberg, and E. C. C. Lin. 1977. Kinase replaceent by a dehydrogenase for Escherichia coli glycerol utilization. J. Bacteriol. 131:1026–1028.

Stols, L., and M. I. Donnelly. 1997a. Production of succinic acid through overexpression of NAD^+-dependent malic enzyme in an Escherichia coli mutant. Appl. Env. Microbiol. 63:2695–2701.

Stols, L., G. Kulkarni, B. G. Harris, and M. I. Donnelly. 1997b. Expression of Ascaris suum malic enzyme in a mutant Escherichia coli allows production of succinic acid from glucose. Appl. Biochem. Biotechnol. 63–65:153–158.

Stormer, F. C. 1975. 2,3-Butanediol biosynthetic system in Aerobacter aerogenes. *In* W. A. Wood (Eds.) Methods in Enzymology. Academic Press. New York, NY. 41:518–533.

Strecker, A. 1854. Über eine eigentümliche Bildungsweise der Propionsaüre und einige Salze derselben. Ann. Chem. 92:80.

Streekstra, H., M. J. Teixera de Mattos, O. M. Neijssel, and D. W. Tempest. 1987. Overflow metabolism during anaerobic growth of Klebsiella aerogenes NCTC 418 on glycerol and dihydroxyacetone in chemostat culture. Arch. Microbiol. 147:268–275.

Suzuki, T., and H. Onishi. 1968. Aerobic dissimilation of L-rhamnose and the production of L-rhamnonic acid and 1,2-propanediol by yeasts. Agric. Biol. Chem. 32:888–893.

Suzuki, T., T. Yamane, and S. Shimizu. 1986. Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. Appl. Microbiol. Biotechnol. 24:370–374.

Swick, R. W., and H. G. Wood. 1960. The role of transcarboxylation in propionic acid fermentation. Proc. Natl. Acad. Sci. USA 46:28–41.

Swings, J., and J. De Ley. 1977. The biology of Zymomonas. Bacteriol. Rev. 41:1–46.

Swings, J., and J. De Ley. 1984. Genus Zymomonas. *In:* N. R. Krieg and J. G. Holt (Ed.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:576–580.

Swings, J. 1992. The genera Acetobacter and Gluconobacter. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) {The Prokaryotes}. Springer-Verlag. New York, NY. 3:2268–2286.

Syu, M. J. 2001. Biological production of 2,3-butanediol. Appl. Microbiol. Biotechnol 55:10–18.

Szmant, H. H. 1989. Organic Building Blocks of the Chemical Industry. John Wiley. New York, NY. 347–348.

Taguchi, H., and T. Ohata. 1991. D-Lactate dehydrogenase is a member of the D-isomer- specific 2-hydrox acid dehydrogenase family. J. Biol. Chem. 266:12588–12594.

Takahashi, R., N. Fujimoto, M. Suzuki, and T. Endo. 1997. Biodegradabilities of ethylenediamine-N,N'-disuccinic acid (EDDS) and other chelating agents. Biosci. Biotech. Biochem. 61:1957–1959.

Takemura, H., S. Horinouchi, and T. Beppu. 1991. Novel insertion sequence IS1380 from Acetobacter pasteurianus is involved in loss of ethanol-oxidizing ability. J. Bacteriol. 173:7070–7076.

Takiyama, E., and T. Fujimaki. 1994. "BIONELLE" biodegradable plastic through chemical synthesis. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 150–174.

Tanaka, K., A. Ishizaki, T. Kanamaru, and T. Kawano. 1995. Production of poly(D-3-hydroxybutyrate) from CO_2, H_2, and O_2 by high cell-density autotrophic cultivation of Alcaligenes eutrophus. Biotechnol. Bioeng. 45:268–275.

Tang, J. C. T., F. E. Ruch, and E. C. C. Lin. 1979. Purification and properties of a nicotinamide adenine dinucleotide-linked dehydrogenase that serves an Escherichia coli mutant for glyerol catabolism. J. Bacteriol. 140:182–187.

Tang, J. C. T., R. G. Forage, and E. C. C. Lin. 1982. Immunochemical properties of NAD^+-linked glycerol dehydrogenases from Escherichia coli and Klebsiella pneumoniae. J. Bacteriol. 152:1169–1174.

Tanner, R. S., L. M. Miller, and D. Yang. 1993. Clostrium ljungdahlii sp. nov., an acetogenic species in clostridial rRNA homology group I. Int. J. Syst. Bacteriol. 43:232–236.

Taroncher-Oldenburg, G., K. Nishia, and G. Stephanopoulos. 2000. Identification and analysis of polyhydroxyalkanoate-specific β-ketothiolase and acetoacetyl coenzyme A reductase genes in cyanobacterium Synechocystis sp. strain PCC6803. Appl. Environ. Microbiol. 66:4440–4448.

Taylor, M. B., and E. Juni. 1960. Stereoisomeric specificities of 2,3-butanediol dehydrogenases. Biochim. Biophys. Acta 39:448–457.

Terracciano, J. S., and E. R. Kashket. 1986. Intracellular conditions required of initiation of solvent production by Clostridium acetobutylicum. Appl. Environ. Microbiol. 52:86–91.

Thauer, R. K., K. Jungerman, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100–180.

Thauer, R. K. 1989. Biochemistry of acetic acid metabolism in anaerobic chemotropic bacteria. Ann. Rev. Microbiol. 43:43–67.

Thayer, A. 2000. Challenges of a biobased economy. C & EN May 29:40.

Thomas, S. R., W. S. Adney, J. O. Baker, Y.-C. Chou, and M. E. Himmel. 1997. US Patent. 5,712,142.

Thompson, D. K., and J.-S. Chen. 1990. Purification and properties of an acetoacetyl coenzyme A-reacting phosphotransbutyrylase from Clostridium beijerinckii ("Clostridium butylicum") NRRL B593. Appl. Environ. Microbiol. 56:607–613.

Thomson, A. W., J. G. O'Neill, and J. F. Wilkinson. 1976. Acetone production by methlobacteria. Arch. Microbiol. 109:243–246.

Thormann, K., L. Feustel, K. Lorenz, S. Nakotte, and P. Dü. 2002. Control of butanol formation in Clostridium acetobutylicum by transcriptional activation. J. Bacteriol. 184:1966–1973.

Tobimatsu, T., T. Hara, M. Sakaguchi, Y. Kishimoto, Y. Wada, M. Isoda, T. Sakai, and T. Toraya. 1995. Molecular cloning, sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of Klebsiella oxytoca. J. Biol. Chem. 270:712–7148.

Tobimatsu, T., M. Azuma, H. Matsubara, H. Takatori, T. Niida, K. Nishinoto, H. Satoh, R. Hayashi, and T. Toraya. 1996. Cloning, sequencing, and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydrase of Klebsiella pneumoniae. J. Biol. Chem. 271:22352–22357.

Tobimatsu, T., H. Kjiura, M. Yunoki, M. Azuma, and T. Toraya. 1999. Identification and expression of the genes encoding a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. J. Bacteriol. 181:4110–4113.

Toda, K., Y. S. Park, T. Asakura, C. Y. Cheng, and H. Ohtake. 1989. High rate acetic acid production in a shallow flow bioreacter. Appl. Microbiol. Biotechnol. 30:559–563.

Tolan, J. S., and R. K. Finn. 1987a. Fermentation of D-xylose and L-arabinose to ethanol by Erwinia chrysanthemi. Appl. Environ. Microbiol. 53:2033–2044.

Tong, I.-T., H. H. Liao, and D. C. Cameron. 1991. 1,3-propanediol production by Escherichia coli expressing genes from the Klebsiella pneumoniae dha regulon. Appl. Env. Microbiol. 57:3541–3546.

Tong, I. T., and D. C. Cameron. 1992. Enhancement of 1,3-Propanediol Production by Cofermentation in Escherichia coli expression Klebsiella pneumoniae dha regulon genes. Appl. Biochem. Biotechnol. 34/35:149–159.

Toth, J., A. A. Ismaiel, and J.-S. Chen. 1999. Purification of a coenzyme A-acylating aldehyde dehydrogenase and cloning of the structural gene from Clostridium beijerinckii NRRL B593. Appl. Environ. Microbiol. 65:4973–4980.

Traeger, M., G. N. Qazi, N. Gulan, R. Buse, and V. Onken. 1992. Comparison of direct glucose oxidation by Gluconobacter oxydans ssp. suboxydans and Aspergillus niger in a pilot scale airlift reactor. J. Ferment. Bioeng. 74:274–281.

Tran-Din, K., and G. Gottschalk. 1985. Formation of D(-)-1,2-propanediol and D(-)-lactate from glucose by Clostridium sphenoides under phosphate limitation. Arch. Microbiol. 142:87–92.

Truffaut, N., J. Hubert, and G. Reysset. 1989. Construction of shuttle vectors useful for transforming Clostridium acetobutylicum. FEMS Microbiol. Lett. 58:15–20.

Trunger, V., and W. Boos. 1994. Mapping and cloning of gldA, the structural gene of the Escherichia coli glycerol dehydratase. J. Bacteriol. 176:1796–1800.

Tsai, S. P., and S.-H. Moon. 1998. An integrated bioconversion process for the production of L-lactic acid from

starchy potato feed stocks. Appl. Biochem. Biotechnol. 70–72:417–428.

Tsao, G. T., N. J. Cao, J. Du, and C. S. Gong. 1999. Production of mutifunctional organic acids from renewable resources. Adv. Biochem. Eng. Biotechnol. 65:243–280.

Tullo, A. 1999. BDO market embraces new technologies. Chemical Market Reporter April 26.

Tummala, S. B., N. E. Welker, and E. T. Papoutsakis. 1999. Development and characterization of a gene expression reporter system for Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 65:3793–3799.

Turk, R. 1993. Metal free and low metal salt substitutes containing lysine. US Patent. 5229161.

Turner, K. W., and A. M. Roberton. 1979. Xylose, arabinose, and rhamnose fermentation by Bacteroides ruminicola. Appl. Environ. Microbiol. 38:7–12.

Uchida, K. 1974. Lipids of alcoholophilic lactobacilli. II: Occurrence of polar lipids with unusually long acyl chains in Lactobacillus heterochiochii. Biochim. Biophys. Acta 369:146–155.

Uchida, K. 1975a. Alteration of the unsaturated to saturated ratio of fatty acids in bacterial lipids by alcohols. Agric. Biol. Chem. 39:1515–1516.

Uchida, K. 1975b. Effects of cultural conditions on the cellular fatty acid composition of Lactobacillus heterohiochii, an alcoholophilic bacterium. Agric. Biol. Chem. 39:837–842.

Uhlig, H., K. Kahrbaum, and A. Steudel. 1986. Acetobacter methanolicus sp. nov., an acetophilic facultatively methylotrophic bacterium. Int. J. Syst. Bacteriol. 36:317–322.

Ui, S., H. Masuda, and H. Muraki. 1983. Laboratory-scale production of 2,3-butanediol isomers (D(−),L(+), and Meso) by bacterial fermentations. J. Ferment. Technol. 61:253–259.

Ui, S., T. Masuda, H. Masuda, and H. Muraki. 1986. Mechanism for the formation of 2,3-butanediol stereoisomers in Bacillus polymyxa. J. Ferment. Technol. 64:481–486.

Ui, S., M. Odagiri, A. Mimura, H. Kanai, T. Kobayashi, and T. Kudo. 1996. Preparation of a chiral acetoinic compound using transgenic Escherichia coli expressing the 2,3-butandiol dehydrogenase gene. J. Ferment. Bioeng. 81:386–389.

Ui, S., Y. Okajima, A. Mimura, H. Kanai, and T. Kudo. 1997. Molecular generation of an Escherichia coli strain producing only the meso-isomer of 2,3-butanediol. J. Ferment. Bioeng. 84:185–189.

Ui, S., T. Hosaka, K. Watanabe, and A. Mimura. 1998. Discovery of a new mechanism of 2,3-butanediol stereoisomer formation in Bacillus cereus YUF-4. J. Ferment. Bioeng. 85:79–83.

Valentin, H. E., T. A. Mitsky, D. A. Mahadeo, M. Tran, and K. J. Gruys. 2000a. Application of a propionyl coenzyme A synthetase for poly(3-hydroxypropionate-co-3-hydroxybutyrate) accumulation in recombinant Esherichia coli. Appl. Environ. Microbiol. 66:5253–5258.

Valentin, H. E., S. Reiser, and K. J. Gruys. 2000b. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) formation from γ-aminobutyrate and glutamate. Biotechnol. Bioeng. 67:291–299.

Vandak, D., M. Telgarsky, and E. Sturkik. 1995a. Influence of growth factor components on butyrate production form sucrose by Clostridum butyricum. Folia Microbiol. 40:32–42.

Vandak, D., T. M. Zigova, and E. Sturdik. 1995b. Effect of growth supplements and whey pretreatment on butyric acid production by Clostridium butyricum. World J. Microbiol. Biotechnol. 11:363.

Vandak, D., J. Zigova, E. Sturdik, and S. Schlosser. 1997. Evaluation of solvent and pH for extractive fermentation of butyric acid. Process Biochem. 32:245–251.

Van der Werf, M. J., M. V. Guettler, M. K. Jain, and J. G. Zeikus. 1997. Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by Actinobacillus sp. 130Z. Arch. Microbiol. 167:332–342.

van der Westhuizen, A., D. T. Jones, and D. R. Woods. 1982. Autolytic activity and butanol tolerance of Clostridium acetobutylicum. Appl. Environ. Microbiol. 44:1277–1281.

Van Niel, C. B. 1928. The Propionic Acid Bacteria (thesis). Labortorium voor Microbiologie der Techische Hoogeschool Delft, N.V. Uitgeversaak, J. W. Boissevain & Co. Haarlem, The Netherlands.

Varadarajan, S., and D. J. Miller. 1999. Catalytic upgrading of fermentation-derived organic acids. Biotechnol. Prog. 15:845–854.

Vasconcelos, I., L. Girbal, and P. Soucaille. 1994. Regulation of carbon and electron flow in Clostridium acetobutylicum grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J. Bacteriol. 176:1443–1450.

Vega, J. L., S. Prieto, B. B. Elmore, E. C. Clausen, and J. L. Gaddy. 1989. The biological production of ethanol from synthesis gas. Appl. Biochem. Biotechnol. 20/21:781–797.

Verhasselt, P., F. Poncelet, K. Vits, A. van Gool, and J. Vanderleyden. 1989. Cloning and expression of a Clostridium acetobutylicum α-amylase gene in Escherichia coli. FEMS Microbiol. Lett. 59:135–140.

Verhave, T. H. 1930. Verfahren zur bacteriellen oxydation organisher Verbindugen zwecks Herstellungen von Oxydationsprodukten wie Dioxyacton usw. German Patent 563, 758. Chem. Abstr. 27:1085.

Vick Roy, T. B. 1985. Lactic acid. *In:* M. Moo Young (Ed.) Comprehensive Biotechnology. Pergamon Press. Oxford, New York, NY. 761–776.

Voget, C. E., C. F. Mignone, and R. J. Ertola. 1985. Butanol production from apple pomace. Biotechnol. Lett. 7:43–46.

Vollbrecht, and El Nawawy. 1980. Restricted oxygen supply and excretion of metabolites. Euro. J. Appl. Microbiol. Biotechnol. 9:1–8.

Voloch, M., M. R. Ladisch, V. W. Rodwell, and G. T. Tsao. 1983. Reduction of acetoin to 2,3-butanediol in Klebsiella pneumoniae: A new model. Biotechnol. Bioengin. 25:173–183.

Von Freudenreich, E., and O. Jensen. 1906. Über die im Emmentaler Käse stattfindende Propionsauregärung. Zentralblatt für Bacteriologie, Parasiten Kunde Infectious Krankheiten und Hygiene Abt. II, 17:529–546.

Wagner, T. O., D. S. Gray, B. Y. Zarah, and A. A. Kozinski. 1980. Practicality of alcohols as motor fuel. *In:* Alcohols as Motor Fuels. Society of Automotive Engineers. Warrendale, PA. 249–265.

Walker, S. A., and T. R. Klaenhammer. 2000. An explosive antisense RNA strategy for inhibition of a lactococcal bacteriophage. Appl. Environ. Microbiol. 66:310–319.

Walter, K. A., G. N. Bennett, and E. T. Papoutsakis. 1992. Molecular characterization of two Clostridium acetobutylicum ATCC 824 butanol dehydrogenase isozyme genes. J. Bacteriol. 174:7149–7158.

Walter, K. A., R. V. Nair, J. W. Cary, G. N. Bennett, and E. T. Papoutsakis. 1993. Sequence and arrangement of two genes of the butyrate-synthesis pathway of Clostridium acetobutylicum ATCC 824. Gene 134:107–111.

Walton, M. T., and J. L. Martin. 1979. Production of butanol-acetone by fermentation. *In:* H. J. Peppler and D. Perlman (Eds.) Microbial Technology, 2nd ed. Academic Press. New York, NY. 1:187–209.

Wang, T.-T., and B. H. Lee. 1997a. Plasmids in Lactobacillus. Crit. Rev. Biotechnol. 17:227–272.

Wang, F. L., and S. Y. Lee. 1997b. Poly(3-hydroxybutyrate) production with high productivity and high polymer content by a fed-batch culture of Alcaligenes latus under nitrogen limitation. Appl. Environ. Microbiol. 63:3703–3706.

Wang, F., and S. Y. Lee. 1998a. High cell density culture of metabolically engineered Escherichia coli for the production of poly(3-hydroxybutyrate) in a defined medium. Biotechnol. Bioeng. 58:325–328.

Wang, X., C. S. Gong, and G. T. Tsao. 1998b. Bioconversion of fumaric acid to succinic acid by recombinant E. coli. Appl. Biochem. Biotechnol. 70–72:919–928.

Waterson, R. M., F. J. Castellino, G. M. Hass, and R. L. Hill. 1972. Purification and characterization of crotonase from Clostridium acetobutylicum. J. Biol. Chem. 247: 5266–5271.

Wayman, M., M. E. Martin, and G. Graf. 1962. Propionic Acid Fermentation. US Patent 3,067,107. Chem. Abstr. 58:7337.

Weber, G. H., and W. A. Broich. 1986. Shelf life extension of cultured dairy foods. Cult. Dairy Prod. J. 21(4):19–23.

Webster, J. R., S. J. Reid, D. T. Jones, and D. R. Woods. 1981. Purification and characterization of an autolysin from Clostridium acetobutylicum. Appl. Environ. Microbiol. 41:371–374.

Weimer, P. J. 1984a. Control of product fromation during glucose fermentation by Bacillus macerans. J. Gen. Microbiol. 130:103–111.

Weimer, P. J. 1984b. Fermentation of 6-deoxyhexoses by Bacillus macerans. Appl. Environ. Microbiol. 47:263–267.

Weiss, N., U. Schillinger, and O. Kandler. 1983. Lactobacillus trichodes and Lactobacillus heterochiochii, subjective synonym of Lactobacillus fruitivorans. Syst. Appl. Microbiol. 4:507–511.

Weizmann, C. 1915. Improvements in bacterial fermentation of carbohydrates and in bacterial cultures for the same. British Patent. 4845.

Welch, R. W., F. B. Rudolph, and E. T. Papoutsakis. 1989. Purification and characterization of the NADH-dependent butanol dehydrogenase from Clostridium acetobutylicum (ATCC 824). Arch. Biochem. Biophys. 273:309–318.

Welch, R. W. 1991. Purification and studies of two butanol (ethanol) dehydrogenases and the effects of rifampicin and chloramphenicol on other enzymes important in the production of butyrate and butanol in Clostridium acetobutylicum ATCC 824 [PhD thesis]. Rice University. Houston, TX.

Welling, H. 1998. Fibers industry get ready. Apparel Industry Magazine 59:65–66.

Weusthuis, R. A., G. N. M. Huijberts, and G. Eggink. 1997. *In:* G. Eggink, A. Steinbuchel, Y. Poirer, and B. Witholt (Eds.) Proceedings of the 1996 International Symposium on bacterial Polyhydroxyalkanoates. NRC Research Press. 102–109.

Weyer, E. R., and L. F. Rettger. 1927. A comparative study of six different strains of the organism commonly concerned in large-scale production of butyl alcohol and acetone by the biological process. J. Bacteriol. 14:399–424.

White, D. 1995. The Physiology and Biochemistry of Prokaryotes. Oxford University Press. New York, NY.

Whitfield, C. D., and S. G. Mayhew. 1974. Purification and properties of electron-transferring flavoprotein from Peptostreptococcus elsdenii. J. Biol. Chem. 249:2801–2810.

Wiegel, J. 1980. Formation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 36:1434–1446.

Wiegel, J., M. Braun, and G. Gottschalk. 1981. Clostridium thermoautotrophicum species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. Curr. Microbiol. 5:255–260.

Wiegel, J., and L. G. Ljungdahl. 1986. The importance of thermophilic bacteria in biotechnology. Crit. Rev. Biotechnol. 3:39–108.

Wiegel, J. 1992. The obligately anaerobic thermophilic bacteria. *In:* J. K. Kristjansson (Ed.) Thermophilic Bacteria. CRC Press. Boca Raton, FL. 105–184.

Wiegel, J. 1994. Acetate and the potential of homoacetogenic bacteria for industrial applications. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 484–504.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1988. Thiolase from Clostridium acetobutylicum ATCC 824 and its role in the synthesis of acids and solvents. Appl. Environ. Microbiol. 54:2717–2722.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1989a. Coenzyme A transferase from Clostridium acetobutylicum ATCC 824 and its role in the uptake of acids. Appl. Environ. Microbiol. 55:323–329.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1989b. Phosphotransbutyrylase from Clostridium acetobutylicum ATCC 824 and its role in acidogenesis. Appl. Environ. Microbiol. 55:317–322.

Wilke, D. 1995. What should and what can biotechnology contribute to chemical bulk production? FEMS Microbiol. Rev. 16:89–100.

Wilke, D. 1999. Chemicals from biotechnology: Molecular plant genetics will challenge the chemical and the fermentation industry. Appl. Microbiol. Biotechnol. 52:135–145.

Wilkinson, S. R., and M. Young. 1994. Targeted integration of genes into the Clostridium acetobutylicum chromosome. Microbiology 140:89–95.

Wilkinson, S. R., and M. Young. 1995. Physical map of the Clostridium beijerinckii (formerly Clostrdium acetobutylicum) NCIMB 8052 chromosome. J. Bacteriol. 177:439–448.

Wilkinson, S. R., D. I. Young, J. G. Morris, and M. Young. 1995. Molecular genetics and the initiation of solventogenesis in Clostridium beijerinckii (formerly Clostridium acetobutylicum) NCIMB 8052. FEMS Microbiol. Rev. 17:275–285.

Williams, D. R., D. I. Young, and M. Young. 1990. Conjugative plasmid transfer from Escherichia coli to Clostridium acetobutylicum. J. Gen. Microbiol. 136:819–826.

Wills, C., P. Kratofil, D. Londo, and T. Martin. 1981. Characterization of the two alcohol dehydrogenases of

Zymomonas mobilis. Arch. Biochem. Biophys. 210:775–785.

Winston, S. J. 1981. Ethanol Fuels: Use, Production & Economics. Solar Energy Information Data Bank, 1st ed. US Government Printing Office.

Winzer, K., K. Lorenz, and P. Dü. 1997. Acetate kinase from Clostridium acetobutylicum: A highly specific enzyme that is actively transcribed during acidogenesis and solventogenesis. Microbiology 143:3279–3286.

Winzer, K., K. Lorenz, B. Zickner, and P. Dü. 2000. Differential regulation of two thiolase genes from Clostridium acetobutylicum DSM 792. J. Molec. Microbiol. Biotechnol. 2:531–541.

Wittlich, P., A. Themann, and K.-D. Vorlop. 2001. Conversion of glycerol to 1,3-propanediol by a newly isolated thermophilic strain. Biotechnol. Lett. 23:463–466.

Wong, H. H., and S. Y. Lee. 1998. Poly(3-hydroxybutyrate) production from whey by high density cultivation of recombinant Escherichia coli. Appl. Microbiol. Biotechnol. 50:30–33.

Wood, H. G., and C. H. Werkman. 1938. The utilization of CO_2 by the propionic acid bacteria. Biochem. J. 32:1262–1271.

Wood, H. G., and C. H. Werkman. 1940. The relationship of bacterial utilization of CO_2 to succinic acid formation. Biochem. J. 34:129–138.

Wood, B. E., and L. O. Ingram. 1992. Ethanol production from cellobiose, amorphous cellulose, and crystalline cellulose by recombinant Klebsiella oxytoca containing chromosomally integrated Zymomonas mobilis genes for ethanol production and plasmids expressing thermostable cellulase genes from Clostridium thermocellum. Appl. Environ. Microbiol. 58:2103–2110.

Wood, A. 2001. DuPont, Genencor extend alliance. Chem. Week 163:38.

Woods, D. R. 1995. The genetic engineering of microbial solvent production. Trends Biotechnol. 13:259–264.

Woolley, R. C., A. Pennock, R. J. Ashton, A. Davies, and M. Young. 1989. Transfer of Tn1545 and Tn916 to Clostridium acetobutylicum. Plasmid 22:169–174.

Woolley, R. C., and J. G. Morris. 1990. Stability of solvent production by Clostridium acetobutylicum in continuous culture: Strain differences. J. Appl. Bacteriol. 69:718–728.

Worden. R. M., A. J. Grethlein, J. G. Zeikus, and R. Datta. 1989. Butyrate production from carbonmonoxide by Butyribacterium methylotrophicum. App. Biochem. Biotechnol. 20/21:687–698.

Worden, R. M., G. A. J., M. K. Jain, and R. Datta. 1991. Production of butanol and ethanol from synthesis gas via fermentation. Fuel 70:615–619.

Worden, R. M., M. D. Bredwell, and A. J. Grethlein. 1997. Engineering issues in synthesis-gas fermentations. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:320–335.

Woskow, S. A., and B. A. Glatz. 1991. Propionic acid production by a propionic acid tolerant strain of Propionibacterium acidipropionici in batch and semicontinuous fermentation. Appl. Environ. Microbiol. 57:2821–2828.

Wyman, C. E. 1994. Ethanol from lignocellulosic biomass: Technology, economics, and opportunities. Bioresource Technol. 50:3–16.

Wyman, C. E. (Ed.). 1996. Handbook on Bioethanol: Production and Utilization. Taylor and Francis. Washington DC.

Wyman, C. E. 2001. Twenty years of trials, tribulations and research progress in bioethanol technology. Appl. Biochem. Biotechnol. 91–93:5–21.

Xavier, A. M. R. B., L. M. D. Gonsalvas, and J. L. Moreira. 1995. Operation patterns affecting lactic acid production in ultrafiltration cell recycle bioreactor. Biotechnol. Bioeng. 45:320–327.

Xue, Y., Y. Xu, Y. Liu, Y. Ma, and P. Zhou. 2001. Thermoanaerobacter tengcongensis sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tongcong, China. Int. J. Syst. Evol. Microbiol. 51:1335–1341.

Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi. 1995. Transfer of two Burkholderia and an Alcaligenes species to Ralstonia gen. nov.: Proposal of Ralstonia pickettii (Ralston, Palleroni and Doudoroff 1973) comb. nov., Ralstonia salanacearum (Smith 1896) comb. nov. and Ralstonia eutropha (Davis 1969) comb. nov. Microbiol. Immunol. 39:897–904.

Yamada, K. 1977. Recent advances in industrial fermentation in Japan. Biotech. Bioeng. 19:1563–1621.

Yamamoto, I., T. Saiki, S.-M. Liu, and L. G. Ljungdahl. 1983. Purification and properties of NADP-dependent formate dehydrogenase from Clostridium thermoaceticum, a tungsten-selenium-iron protein. J. Biol. Chem. 258: 1826–1832.

Yan, R.-T., C.-X. Zhu, C. Golemboski, and J.-S. Chen. 1988. Expression of solvent-forming enzymes and onset of solvent production in batch cultures of Clostridium beijerinckii ("Clostridium butylicum"). Appl. Environ. Microbiol. 54:642–648.

Yan, R.-T., and J.-S. Chen. 1990. Coenzyme A-acylating adlehyde dehydrogenase from Clostridium beijerinckii NRRL B592. Appl. Environ. Microbiol. 56:2591–2599.

Yan, R.-T. 1991. Enzymology of butanol formation in Clostridium beijerinckii NRRL B592 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Yang, S. T., H. Zhu, Y. Li, and G. Hong. 1994. Continuous propionate production from whey permeate using a novel fibrous bed bioreactor. Biotech. Bioeng. 43:1124–1130.

Yang, S. T., Z. W. Jin, and B. H. Chollar. 1997. Production of low-cost acetate deicers from biomass and industrial wastes. Snow Removal and Ice Control Technology. Transportation Research Board, National Research Council. Washington DC, 60–69.

Yang, S. T., Y. L. Huang, Z. Jin, Y. Huang, H. Zhu, and W. Qin. 1999. Calcium Magnesium Acetate at Power-production Cost: Production of CMA Deicer from Cheese Whey. US DOT. Publication No. FHWA-RD-98-174.

Yasui, Y. 1958. Process for continuous production of vinegar. Japanese Patent 244,905. Publication No. Sho. 33-3798/1958.

Yeh, C.-S. 1955. Butanol-acetone fermentation [in Chinese]. *In:* Petroleum Communications. China Petroleum Corporation. Taiwan, China. December, Issue 54:10–19.

Yomano, L. P., S. W. York, and L. O. Ingram. 1998. Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production. J. Ind. Microbiol. Biotechnol. 20:132–138.

Yoon, K.-H., and M. Y. Pack. 1990. Nucleotide sequence of the Zymomonas mobilis alcohol dehydrogenase II gene. Nucleic Acids Res. 18:187.

Yoon, K.-H., J.-K. Lee, and B. H. Kim. 1991. Construction of a Clostridium acetobutylicum-Escherichia coli shuttle vector. Biotechnol. Lett. 13:1–6.

Yoshino, S., T. Yoshino, S. Hara, S. Ogata, and S. Hayashida. 1990. Construction of shuttle vector plasmid between Clostridium acetobutylicum and Escherichia coli. Agric. Biol. Chem. 54:437–441.

Young, M., N. P. Minton, and W. L. Staudenbauer. 1989. Recent advances in the genetics of the clostridia. FEMS Microbiol. Rev. 63:301–326.

Young, M. 1993. Development and exploitation of conjugative gene transfer in clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 99–118.

Youngleson, J. S., J. D. Santangelo, D. T. Jones, and D. R. Woods. 1988. Cloning and expression of a Clostridium acetobutylicum alcohol dehydrogenase gene in Escherichia coli. Appl. Environ. Microbiol. 54:676–682.

Youngleson, J. S., D. T. Jones, and D. R. Woods. 1989. Homology between hydroxybutyryl and hydroxyacyl coenzyme A dehydrogenase enzymes from Clostridium acetobutylicum fermentation and vertebrate fatty acid β-oxidation pathways. J. Bacteriol. 171:6800–6807.

Youngleson, J. S., F.-P. Lin, S. J. Reid, and D. R. Woods. 1995. Structure and transcription of genes within the β-hbd-adh1 region of Clostridium acetobutylicum P262. FEMS Microbiol. Lett. 125:185–192.

Yu, E. K. C., and J. N. Saddler. 1982a. Enhanced production of 2,3-butanediol by Klebsiella pneumoniae grown on high sugar concentrations in the presence of acetic acid. Appl. Environ. Microbiol. 44:777–784.

Yu, E. K. C., N. Levitin, and J. N. Saddler. 1982b. Production of 2,3-butanediol by Klebsiella pneumoniae grown on acid-hydrolyzed wood hemicellulose. Biotechnol. Lett. 4:741–746.

Yu, E. K. C., and J. N. Saddler. 1983. Fed-batch approach to production of 2,3-butanediol by Klebsiella pneumoniae grown on high substrate concentrations. Appl. Environ. Microbiol. 46:630–635.

Yu, E. K. C., and J. N. Saddler. 1985a. Biomass conversion to butanediol by simultaneous saccharification and fermentation. Trends Biotechnol. 3:100–104.

Yu, E. K. C., L. Deschatelets, G. Louis-Seize, and J. N. Saddler. 1985b. Butanediol production from cellulose and hemicellulose by Klebsiella pneumoniae grown in sequential coculture with Trichoderma harzianum. Appl. Environ. Microbiol. 50:924–929.

Yu, P.-L., and L. E. Pearce. 1986. Conjugal transfer of streptococcal antibiotic resistance plasmids into Clostridium acetobutylicum. Biotechnol. Lett. 8:469–474.

Zachariou, M., and R. K. Scopes. 1986. Glucose-fructose oxidoreductase, a new enzyme isolated from Zymomonas mobilis that is responsible for sorbitol production. J. Bacteriol. 167:863–869.

Zakpaa, H. D., A. Ishizaki, and K. Shimizu. 1997. Computer-mediated addition of fresh medium in continuous culture of Zymomonas mobilis by monitoring weight changes. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:143–154.

Zanin, G. M., C. C. Santana, E. P. S. Bon, R. C. L. Giordano, F. F. de Moraes, S. R. Andrietta, C. C. de Carvalho Neto, I. C. Macedo, D. L. Fo, L. P. Ramos, and J. D. Fontana. 2000. Brazilian bioethanol program. Appl. Biochem. Biotechnol. 84–86:1147–1161.

Zappe, H., D. T. Jones, and D. R. Woods. 1986. Cloning and expression of Clostridium acetobutylicum endoglucanase, cellobiase and amino acid biosynthesis genes in Escherichia coli. J. Gen. Microbiol. 132:1367–1372.

Zappe, H., D. T. Jones, and D. R. Woods. 1987. Cloning and expression of a xylanase gene from Clostridium acetobutylicum P262 in Escherichia coli. Appl. Microbiol. Biotechnol. 27:57–63.

Zappe, H., W. A. Jones, D. T. Jones, and D. R. Woods. 1988. Structure of an endo-β-1,4-glucanase gene from Clostridium acetobutylicum P262 showing homology with endoglucanase genes from Bacillus spp. Appl. Environ. Microbiol. 54:1289–1292.

Zeikus, J. G. 1980. Chemical and fuel production by anaerobic bacteria. Ann. Rev. Microbiol. 34:423–464.

Zeikus, J. G., M. K. Jain, and P. Elankoven. 1999. Biotechnology of succinic acid production and markets for derived industrial products. Appl. Microbiol. Biotechnol. 51:545–552.

Zeng, A.-P., H. Biebl, and W.-D. Deckwer. 1990. Effect of pH and acetic acid on growth and 2,3-butanediol production of Enterobacter aerogenes in continuous culture. Appl. Microbiol. Biotechnol. 33:485–489.

Zeng, A.-P., and W.-D. Deckwer. 1991. A model for multiproduct-inhibited growth of Enterobacter aerogenes in 2,3-butanediol fermentation. Appl. Microbiol. Biotechnol. 35:1–3.

Zeng, A.-P., H. Biebl, H. Schlieker, and W.-D. Deckwer. 1993. Pathway analysis of glycerol fermentation by Klebsiella pneumoniae: Regulation of reducing equivalent balance and product formation. Enz. Microbol. Technol. 15:770–779.

Zeng, A.-P., T.-G. Byun, C. Posten, and W.-D. Deckwer. 1994a. Use of respiratory quotient as a control parameter for optimum oxygen supply and scale-up of 2,3-butanediol production under microaerobic conditions. Biotechnol. Bioengin. 44:1107–1114.

Zeng, A.-P., A. Ross, H. Biebl, C. Tag, B. Gunzel, and W.-D. Deckwer. 1994b. Multiple product inhibition and growth modeling of Clostridium butyricum and Klebsiella pneumoniae in glycerol fermentation. Biotechnol. Bioengin. 44:902–911.

Zeng, A.-P. 1996. Pathway and kinetic analysis of 1,3-propanediol production from glycerol fermentation by Clostridium butyricum. Bioproc. Engin. 14:169–175.

Zeng, A.-P., H. Biebl, and W. D. Deckwer. 1997. Microbial conversion of glycerol to 1,3-propanediol: Recent progress. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. Oxford Press. Oxford, UK. ACS Symposium Series 666:264–279.

Zhang, D. X., and M. Cheryan. 1994a. Starch to lactic acid in a continuous membrane reactor. Process Biochem. 29(2):145–150.

Zhang, H., V. Obias, K. Gonyer, and D. Dennis. 1994b. Production of polyhydroxyalkanoates in sucrose-utilizing recombinant Escherichia coli and Klebsiella strains. Appl. Environ. Microbiol. 60:1198–1205.

Zhang, M., C. Eddy, K. Deanda, M. Finkelstein, and S. Picataggio. 1995. Metabolic engineering of a pentose metabolism pathway in ethanologenic Zymomonas mobilis. Science 267:240–243.

Zhou, X., and R. W. Traxler. 1992. Enhanced butanol production and reduced autolysin activity after chloramphenicol treatment of Clostridium acetobutylicum ATCC 824. Appl. Microbiol. Biotechnol. 37:293–297.

Ziffer, J. A., S. Gaffney, S. Rothenberg, and T. J. Cairney. 1971. Aldonic acid and aldonate compositions and production thereof. British Patent 1,249,347. Chem. Abstr. 79:113.

Zigova, J., E. Sturdik, D. Vandak, and S. Schlosser. 1999. Butyric acid production by Clostridium butyricum with integrated extraction and pertraction. Process Biochem. 34:835–843.

Zigova, J., and E. Sturdik. 2000. Advances in biotechnological production of butyric acid. J. Indust. Microb. Biotechnol. 24:153–160.

Organic Acid and Solvent Production Part III: Butanol, Acetone and Isopropanol; 1,3- and 1,2-Propanediol Production; and 2,3-Butanediol Production

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Section 8: Butanol, Acetone, and Isopropanol

Introduction

Butanol (also known as *n*-butanol or 1-butanol), acetone and isopropanol (2-propanol) are major fermentation products of several species of clostridia. As microbial products, butanol, acetone and isopropanol are usually studied together because their production involves shared metabolic pathways. These three compounds are important industrial chemicals with a broad range of uses. They also have potential uses as additives in automobile fuels because of their oxygen content, octane rating, heating value, and capacity to increase water tolerance of ethanol-gasoline blends. At present, butanol and isopropanol are commercially produced by synthesis using petroleum-derived propylene as the starting material. Acetone is coproduced during the synthesis of phenol, and this route satisfies the bulk of the market need for acetone. Therefore, isopropanol is a useful by-product from a biomass-based process for the production of butanol, whereas acetone is not as desirable unless the manufacturing method for phenol or the relative market need for acetone and phenol changes.

Because acetone and butanol were traditionally used as solvents, the industrial fermentation for the production of acetone, butanol and isopropanol is known as the "solvent fermentation." Industrial solvent fermentation is unique: it was the first industrial fermentation utilizing pure cultures and aseptic techniques, its large scale was unprecedented, and it was the first method for the production of butanol as a commodity chemical. In addition, modern industrial microbiology is based on the serendipitous history behind the development of this industrial fermentation (Kelly, 1936), the pivotal role played by the scientist-turned-statesman Chaim Weizmann in this endeavor (Rose, 1986), and the intricate relationship between enterprising scientists and businessmen who turned this fermentation into a successful industry (Rose, 1986). With its preeminent past and the continued improvements through research, solvent fermentation may serve to demonstrate the practicality of changing a petroleum-based economy back to a biomass-based (or bio-based) economy, which will utilize biomass-derived feedstocks to produce liquid fuels, organic chemicals, and materials. For the United States, a bio-based economy has been advocated as the primary route toward a sustainable and secure future (Thayer, 2000).

Butanol-acetone fermentation was first developed to produce butanol for use in the manufacture of synthetic rubber. However, it was the need for acetone as a solvent during World War I that provided the impetus for developing this fermentation into a successful industrial process. After World War I, the established fermentation process was used commercially to produce butanol needed to make butyl acetate (a solvent for the rapidly growing lacquer industry; Gabriel, 1928). The industrial process went through significant changes in the ensuing fifteen years as both the organisms and the raw materials for the fermentation were changed as a result of continued efforts to improve this fermentation (Beesch, 1952; Beesch, 1953).

Although most known solvent-producing clostridia produce both acetone and butanol, the discovery of these chemicals as products of microbial fermentation was reported half a century apart and with different organisms. Pasteur in 1861–62 (McCoy et al., 1926; Dürre and Bahl, 1996) reported the production of butanol by butyric acid-producing microorganisms, whereas Schardinger in 1905 (Prescott and Dunn, 1959b) reported the production of acetone by *Bacillus macerans*, which is not a butanol or butyric acid-producing organism. It was Fernbach who observed the production of both acetone and butanol by a bacillus he isolated for the production of butanol, which was used in the production of butadiene for the manufacture of synthetic rubber (Fernbach and Strange, 1911). Industrial solvent fermentation began in 1913 as a process

for producing butanol from potatoes (Gabriel, 1928).

The outbreak of World War I in 1914 changed the course of industrial solvent fermentation. To make large amounts of acetone needed in Britain as a solvent for the production of the smokeless explosive cordite, the Fernbach and Strange process was used at first (Gabriel, 1928). However, when the Weizmann bacterium or BY (which was later named "*Clostridium acetobutylium*") was found to produce from corn more acetone and butanol than the Fernbach bacillus produced, it replaced the Fernbach bacillus for the production of acetone, first in Britain and then in North America, during World War I (Gabriel, 1928). After the Armistice in 1919, acetone was no longer in demand and the government-operated fermentation system was terminated. However, butanol was soon found to be extremely valuable for the manufacture of the fast drying lacquer used by the automobile industry. Thus, beginning in 1920, solvent fermentation (used to produce butanol and acetone or isopropanol and other products) was a major commercial operation for four decades. For about a decade, the industry used the patented Weizmann process exclusively, with strains of *C. acetobutylicum* developed against phage infections and with corn as the raw material. But by 1933, the raw material was switched to molasses, and *C. acetobutylicum* (which is not productive in a molasses-based medium) was replaced by an organism known as "No. 8" (Kelly, 1936). This switch in raw materials and organisms occurred before the expiration of the Weizmann patent in 1936.

When the raw material was switched from corn to molasses, many new patents were issued for the molasses-based fermentation processes, and many newly isolated bacteria for these processes were named in the patents (Beesch, 1952). Public culture collections have maintained some of these molasses-fermenting solvent-producing clostridia, most of which were referred to as strains of *C. acetobutylicum* either by the culture collections or by the investigators. When research on solvent fermentation became active again in the late 1970s, several strains of "*C. acetobutylicum*" from public and private culture collections were extensively studied, and these "*C. acetobutylicum*" strains displayed surprisingly different properties (Johnson and Chen, 1995; Keis et al., 1995).

A systematic study of these cultures revealed nine groups (Keis et al., 1995), whereas analysis of DNA-DNA reassociation identified these cultures as strains of four species (Johnson and Chen, 1995; Johnson et al., 1997). These four *Clostridium* species, *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum* can now be differentiated by their genetic and phenotypic traits (Johnson et al., 1997; Keis et al., 2001a). Several other characterized species of *Clostridium* produce butanol but in lower concentrations than that produced by these four *Clostridium* species (see the subsection Solvent-Producing Bacteria in this Chapter).

In addition to the correct identification of the solvent-producing organisms, much progress has been made since the early 1980s in determining the properties of the solvent-producing enzymes (see the subsection Metabolic Pathways and Enzymology of Solvent Production in this Chapter) and their structural genes (see the subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter). Manipulation of the genes encoding the acid- and solvent-producing enzymes in *C. acetobutylicum* has demonstrated the feasibility of reaching a butanol concentration higher than any previously reported values (see the subsection Metabolic Engineering in this Chapter). The genome of *C. acetobutylicum* ATCC 824 has now been sequenced (Nolling et al., 2001), which should facilitate the characterization of the regulatory mechanisms for solvent production.

Since the mid-1980s, a number of reviews have been written on microbial production of butanol, acetone, and isopropanol (Jones and Woods, 1986; McNeil and Kristiansen, 1986; Dürre and Bahl, 1996; Dürre, 1998; Rogers, 1999). There are also reviews that cover all aspects of industrial solvent fermentation (Gabriel, 1928; McCutchan and Hickey, 1954; Ryden, 1958; Prescott and Dunn, 1959c; Ross, 1961; Hastings, 1978). The following reviews address in detail specific aspects of solvent fermentation or the solvent-producing clostridia: microbiology (Beesch, 1952; Beesch, 1953; Prescott and Dunn, 1959c; Johnson and Chen, 1995; Jones and Keis, 1995), biochemistry and physiology (Rogers, 1986; Chen, 1993; Chen, 1995; Bennett and Rudolph, 1995; Dürre et al., 1995; Girbal and Soucaille, 1998; Mitchell, 1998), genetics (Rogers, 1986; Minton and Oultram, 1988; Young et al., 1989; Woods, 1995; Dürre, 1998), the industrial processes (Killeffer, 1927; Gabriel, 1928; Beesch, 1952; Beesch, 1953; McCutchan and Hickey, 1954; Ryden, 1958; Spivey, 1978; Walton and Martin, 1979), and history (Gabriel, 1928; Gabriel and Crawford, 1930; Kelly, 1936; McCutchan and Hickey, 1954; Jones and Woods, 1986).

Scientific Background

PHYSICOCHEMICAL PROPERTIES The physicochemical properties of acetone, butanol, and isopropanol are listed in Table 31. Ethanol is included in the table because it is a product of all known

Table 31. Physical properties of the neutral products of the solvent-producing bacteria.

	Acetone	*n*-Butanol	Isopropanol	Ethanol
Formula	$(CH_3)_2CO$	$CH_3(CH_2)_3OH$	$(CH_3)_2CHOH$	C_2H_5OH
Mol. wt.	58.08	74.12	60.09	46.07
Boiling point (°C)	56.5	117.7	82.5	78.5
Specific gravity	0.791 (20°/20°)	0.811 (20°/4°)	0.785 (20°/4°)	0.789 (20°/4°)
Pounds/gallon	6.58	6.75	6.55	6.578
Heating value				
Btu/gallon	93,028	103,849	87,400	76,000
MJ/liter	26	29	24	21
Oxygen content	27.6	21.6	26.6	34.7
RON[a]		113	121[b]	106, 130[b]
MON[a]	93	94	96[b]	92, 96[b]

Abbreviations: Btu, British thermal units; MJ, megajoules; RON, research octane number, the number used in predicting antiknock performance during low-speed acceleration; and MON, motor octane number, the number used in predicting antiknock performance at high constant speed.

[a]In gasoline blends, these and other measurements of octane numbers are influenced by the composition of the base fuel and other factors, which makes it difficult to give absolute values for the octane quality of the various alcohols and oxygenates (Owen and Coley, 1990; Houben, 1995).

[b]Blending octane numbers (Houben, 1995).

solvent-producing bacteria and because these four compounds share properties which make them useful as oxygenates and as octane-rating enhancers in gasoline (see "Ethanol" in Organic Acid and Solvent Production, Part II in this Volume). These compounds, because of their similar fuel properties, can be used as a mixture in gasoline or diesel. Besides increasing the oxygen content and the octane rating, acetone and isopropanol can increase the water tolerance of the ethanol-gasoline mixture (see the subsection Uses in this Chapter).

Solvent-Producing Bacteria The early history (1861–1909) of the discovery of the butanol-, acetone-, and isopropanol-producing bacteria has been reviewed by Dürre and Bahl (1996), who listed 22 references (all in either German or French) documenting the contribution of Pasteur, Fitz, Beijerinck, Schardinger, and Pringsheim to the field. Pasteur, Schardinger and Pringsheim, respectively, were the first to observe or report the production of, respectively, butanol, acetone and isopropanol by anaerobic bacteria. Pasteur coined the term "anaerobic" to describe butyric acid- and butanol-producing bacteria that were killed in air.

All known butanol-producing and most acetone-producing organisms are Gram-positive, spore-forming bacteria, and all produce ethanol in addition to butanol and acetone or isopropanol. Processes using butanol-producing bacteria yield less ethanol than other neutral products, whereas processes using bacteria that do not produce butanol yield more ethanol than acetone, which can be explained by the fact that the latter organisms (such as *B. macerans*) dispose of excess reducing power mainly through ethanol production.

The correct identification of the butanol- and acetone-producing clostridia is difficult because they are phenotypically similar. In addition, some useful criteria for bacterial identification are not reliable when applied to this group of organisms. The fatty acid composition of the membrane lipids (Lepage et al., 1987) and the pattern of fermentation products change when cells switch from the acid-producing stage to the solvent-producing stage of growth or when solventogenesis occurs under slightly different conditions. Although the phylogeny of the genus *Clostridium* can be deduced from a comparison of 16S rRNA sequences (Collins et al., 1994), data of chromosomal DNA-DNA hybridization are necessary to establish the species status (Stackebrandt and Goebel, 1994). Most of the recognized species of solvent-producing clostridia have been examined by the DNA-DNA hybridization technique (Cummins and Johnson, 1971; George et al., 1983b; Johnson et al., 1997).

The solvent-producing bacteria may be divided into four groups (Table 32) according to their major end products: 1) Those producing acetone but not butanol or isopropanol, 2) those producing butanol and acetone, 3) those producing isopropanol in addition to acetone and butanol, and 4) those producing butanol but not acetone. The salient properties of species within each group are described below.

Acetone-Producing Bacteria Acetone as a product of bacterial fermentation was first reported by Schardinger in 1905 (Prescott and Dunn, 1959b) when the species *Bacillus macerans* was named. Ethanol, acetone, and acetic and formic acids are the major end products from the fermentation of potatoes or potato starch in media

Table 32. Four groups of solvent-producing bacteria[a] on the basis of their neutral end products.

Neutral end products			
Acetone	Butanol and acetone	Butanol, isopropanol and acetone	Butanol
Bacillus macerans *Methylosinus trichosporium*	*Clostridium acetobutylicum* *Clostridium beijerinckii* (some strains) *Clostridium saccharoperbutylacetonicum* *Clostridium saccharobutylicum* *Clostridium puniceum* *Clostridium pasteurianum*	*Clostridium aurantibutyricum* *Clostridium beijerinckii* (some strains) "*Clostridium toanum*"	*Clostridium tetanomorphum* *Clostridium thermosaccharolyticum*

[a]All produce ethanol.

also containing peptone and calcium carbonate. Subsequently, an organism named "*Bacillus acetoethylicum*" (later changed to "*Bacillus acetoethylicus*" and is now considered synonymous to *B. macerans*) was isolated (Northrop et al., 1919) and used in the study of factors influencing acetone formation (Arzberger et al., 1920). "*Bacillus acetoethylicum*" produced ethanol (12–26% of the weight of the carbon substrate) and acetone (4–10% of the weight of the carbon substrate) from mono-, di- and polysaccharides; however, it produced ethanol (40–43% of the weight of the carbon substrate) but no acetone when glycerol was the fermentation substrate (Northrop et al., 1919). The lack of acetone production has also been observed with *Clostridium acetobutylicum* when it is grown under the alcohologenic conditions (see the subsection Physiology of Solvent Production in this Chapter), which include the use of a mixture of glucose and glycerol in the growth medium (Girbal and Soucaille, 1994; Vasconcelos et al., 1994).

Acetone production by *B. macerans* occurs late in the fermentation and is enhanced by a low culture pH (Prescott and Dunn, 1959b). A significant increase in acetone (from 12 to 40 mM) and ethanol (from 44 to 154 mM) production by *B. macerans* ATCC 7068 was observed when $CaCO_3$ (100 mM) and sodium acetate (24 mM) were added to the CM5 medium containing 2% glucose and 0.2% yeast extract (Weimer, 1984a).

In addition to strains maintained at national culture collections, the culture collection of the United States Department of Agriculture (USDA) at the Midwest Area National Center for Agricultural Utilization Research in Peoria, Illinois, maintains many strains of *B. macerans*, which carry the designation "NRRL." These NRRL strains of *B. macerans* include the NRS strains originally maintained by N. R. Smith. The following NRRL strains of *B. macerans* produced 20–40 mM acetone and >150 mM ethanol at 35°C in the CM5 medium (see above) containing 2% glucose and 0.2% yeast extract without the addition of $CaCO_3$ or sodium acetate: B-392 (NRS-646), B-433 (NRS-1098), B-434 (NRS-1099), and B-3185 (NRS-649; P. Moldenhawer and J.-S. Chen, unpublished result).

Acetone is also produced from poly-3-hydroxybutyrate by the methylobacterium *Methylosinus trichosporium* OB3B (Thomson et al., 1976). It was proposed that poly-3-hydroxybutyrate is metabolized to 3-hydroxybutyrate and acetoacetate, whereas acetone is produced from acetoacetate via acetoacetate decarboxylase (reaction 17 in Fig. 26).

Butanol- and Acetone-Producing Bacteria

Clostridium acetobutylicum is the best known butanol- and acetone-producing organism, and some misconception about this species persists in the literature. Elizabeth McCoy and coworkers at the University of Wisconsin proposed the name "*Clostridium acetobutylicum*" in 1926 for "the acetone butyl alcohol organism" represented by the 11 strains they used in a cultural study (McCoy et al., 1926). Seven of the 11 strains were received from the Commercial Solvents Corporation of Terre Haute (Indiana, United States), and the other four (strains 6, 8, 10, and 11) were from the Department of Agricultural Bacteriology at the University of Wisconsin (McCoy et al., 1926; McCoy and McClung, 1935). Strain 1 of this study was believed to be a Weizmann culture although its history was not fully known (McCoy and McClung, 1935). In 1927, Weyer and Rettger (1927) reported the result of a comparative study of six cultures of the acetone butyl alcohol organism, one of which was isolated from Connecticut (United States) garden soil in October 1924 and was designated "strain *So*," which eventually became ATCC 824, the type strain for the species *C. acetobutylicum* (McCoy and McClung, 1935). Weyer and Rettger (1927) supported the use of the name *Clostridium acetobutylicum* for this group of organisms. In a study on "the serological agglutination of *Clostridum acetobutylicum*

and related species" (McCoy and McClung, 1935), 22 cultures were used and they included "Strain W_{17}" as well as "Strain 1" (believed to be a Weizmann culture) and "Strain W" (the Weyer and Rettger Strain *So*; now ATCC 824). Strain W_{17} was an original Weizmann culture, which was "preserved in a sealed spore stock for seventeen years by Weizmann, and received by us through one intermediate, the late Sir Frederick Andrews of London" (McCoy and McClung, 1935). Results of these cultural and serological studies support the conclusion that these strains are sufficiently similar among themselves and different from other known species to be considered *C. acetobutylicum*.

Some publications incorrectly credited Chaim Weizmann for proposing the name *Clostridium acetobutylicum*. McCoy and McClung (1935) clearly stated "discovery of the organism and certainly discovery of its usefulness in industrial fermentation is attributed to Weizmann, who, though he never fully described or named his organism, succeeded in differentiating it from formerly known butyl-alcohol-producing anaerobes." For several reasons, some investigators thought that Weizmann named the species *C. acetobutylicum*. McCoy and coworkers (McCoy et al., 1926) proposed the name "Clostridium acetobutylicum (Weizmann)" [*sic*] for the species, whereas Weyer and Rettger (1927) gave the name "*Clostridium acetobutylicum*, Weizmann" [*sic*] in the description chart for their Strain *So* (now ATCC 824). Statements such as "the butyl alcohol organism of Weizmann, *Clostridium acetobutylicum*" (McCoy and McClung, 1935) and "*Clostridium acetobutylicum Weizmann* [*sic*]" in the biography for Weizmann (Rose, 1986) probably also contributed to the misconception. That the strain *So* (now ATCC 824), which was isolated by Weyer and Rettger, was designated "strain W" by McCoy and McClung (1935) may also have misled some investigators to think that strain ATCC 824 was the Weizmann strain.

Weizmann did not fully describe the isolation of the organism that was successfully used in industrial production of acetone and butanol from corn, which may have prompted John J. H. Hastings to suggest "Weizmann obtained such an organism, though it is possible that he did not isolate it himself" (Hastings, 1971) and "there is some doubt whether he in fact isolated his own culture or obtained it from an existing source" (Hastings, 1978). However, in the Weizmann biography (p. 120; Rose, 1986), it is stated that "...Weizmann turned to a relatively new field: microbiology. From the spring of 1909 he began to spend his vacations at the Pasteur Institute in Paris." Weizmann studied microbiology under Professor Auguste Fernbach, the director of the Fermentation Laboratories of the Pasteur Institute, and was considered by contemporary microbiologists as "a student of Fernbach's" (Arzberger et al., 1920). It is thus conceivable that Weizmann was proficient in anaerobic bacteriological techniques and capable of isolating from corn the strain used in his industrial process awarded the 1915 British Letters patent no. 4845 (Weizmann, 1915). Fernbach isolated an organism (bacillus of the type of Fitz or BF) that produced acetone and butanol from potatoes but not corn, and the industrial performance of the Fernbach's patented process (Fernbach and Strange, 1911) was inferior to the Weizmann process using *C. acetobutylicum* and corn mash (see the subsection Industrial Solvent Fermentation in this Chapter). After the outbreak of World War I, the Fernbach process was replaced by the Weizmann process for better acetone production (Gabriel, 1928; Gabriel and Crawford, 1930).

As an obligate anaerobe, *C. acetobutylicum* is relatively insensitive to O_2, which was considered somewhat paradoxical because

"...it can be cultured in open tubes of fresh carbohydrate media.... Germination of spores in air is not possible as in the case of ordinary facultative organisms. And yet after a culture is well started in corn mash, oxygen may be bubbled through it intermittently without seriously disturbing its fermentation. Likewise, colonies developed in an anaerobic jar may on removal to air continue to grow by piling up of the bottom layers of growth." (McCoy et al., 1926).

The oxygen tolerance of *C. acetobutylicum* may have contributed to its commercial success and is a useful property.

In 1926, the name "*Clostridium acetonigenum*" was proposed by H. J. L. Donker, a Dutch microbiologist at Delft, for the species now known as "*C. acetobutylicum*." To circumvent the need to determine the priority of the names and to avoid confusion, the Dutch scientist later withdrew the name "*Clostridium acetonigenum*" (McCoy and McClung, 1935).

Clostridium acetobutylicum can use a wide range of carbohydrates ranging from pentoses to starch for growth and is strongly proteolytic. Production of acetone and butanol from sugars by *C. acetobutylicum*, however, requires close control of the pH of the medium. Corn mash, on the other hand, has the right buffering capacity so that this species can without pH control produce acetone and butanol in corn mash. An inability to produce high levels of solvents from molasses distinguishes *C. acetobutylicum* from the other solvent-producing clostridia. *Clostridium acetobutylicum* can now be clearly differentiated from the other solvent-producing species by measuring DNA-DNA reassociation (Johnson et al., 1997) or by comparing several genetic and phenotypic traits (Keis et al., 2001a).

Although acetone, butanol, and ethanol are the characteristic end products of *C. acetobutylicum* during the well known solventogenic mode of growth, there is another solventogenic mode of growth, dubbed alcohologenesis, during which *C. acetobutylicum* produces butanol and ethanol but little or no acetone. See the subsection Physiology of Solvent Production in this Chapter for growth conditions leading to alcohologenesis.

Clostridium beijerinckii (one of the molasses-fermenting butanol-producing clostridia) is less well known than *C. acetobutylicum* because many strains of this species were previously given different species names. *Clostridium beijerinckii* replaced *C. acetobutylicum* for commercial solvent production after the corn-based Weizmann process was phased out in the late 1930s (Johnson and Chen, 1995; Jones et al., 2000). Many extant strains of *C. beijerinckii* have been definitively identified by DNA-DNA reassociation or a combination of traits and are available from culture collections (Cummins and Johnson, 1971; Johnson et al., 1997; Keis et al., 2001a).

Clostridium acetobutylicum and *C. beijerinckii* are among the many species of butyric acid-producing clostridia that are difficult to differentiate on the basis of morphological and growth characteristics alone. The seventh edition of *Bergey's Manual of Determinative Bacteriology* listed 19 species of nonpathogenic, butyric acid-producing clostridia (Cummins and Johnson, 1971). Measurements of DNA-DNA reassociation and cell wall composition allowed the identification of a species (Homology group II) of butyric acid-producing clostridia (e.g., *C. beijerinckii*; Cummins and Johnson, 1971). This group initially consisted of 20 cultures previously designated as "*C. butyricum*," "*C. multifermentans*," "*C. amylolyticum*," "*C. rubrum*," "*C. lactoacetophilum*" and "*C. aurantibutyricumi*" (Cummins and Johnson, 1971). Notably, only some of the strains previously labeled as members of these species as well as "*Clostridium butylicum*" (George et al., 1983b), "*C. madisonii*" (Keis et al., 2001a), several strains of *C. acetobutylicum*, and others (Johnson et al., 1997) were found to belong to *C. beijerinckii*.

The species *C. beijerinckii* was named after the Dutch bacteriologist M. W. Beijerinck by Donker in his 1926 thesis (George et al., 1983b). At that time, *C. beijerinckii* was thought to be unable to ferment starch and hence differed from "*Clostridium butylicum*" (originally "*Granulobacter butylicum Beijerinck 1893*"). However, many strains of *C. beijerinckii* have since been found to ferment starch (George et al., 1983b; Nimcevic et al., 1998), and *C. beijerinckii* NRRL B592 has been used in pilot-plant studies to produce solvents from potatoes (Nimcevic and Gapes, 2000). Although the name "*C. butylicum*" would have priority over *C. beijerinckii*, an apparent oversight during the preparation of the *1980 Approved Lists of Bacterial Names* resulted in the use of *C. beijerinckii* as the name for this solvent-producing species (George et al., 1983b). *Clostridium beijerinckii* also contains strains that produce isopropanol, instead of acetone, as a major end product. When methyl viologen is added to the medium, *C. beijerinckii* NRRL B591 (formerly labeled as "*C. acetobutylicum*") produces either ethanol (pH 6.8) or butanol and ethanol (pH 5), but with little or no acetone (Rao and Mutharasan, 1986).

Clostridium aurantibutyricum is a species distinguishable from *C. beijerinckii* and *C. butyricum* on the basis of DNA-DNA reassociation (Cummins and Johnson, 1971). Two strains (including the type strain) of *C. aurantibutyricum* have been examined for solvent production, and both produce butanol, acetone and isopropanol from glucose (George et al., 1983b). It is not known whether isopropanol production is a general property of this species; however, many strains of *C. beijerinckii* produce acetone but not isopropanol, and some do not produce solvents under conditions that are solventogenic for the producing strains (Chen and Hiu, 1986).

Clostridium saccharoperbutylacetonicum was isolated from Japanese soil by Hongo and Nagata in 1959 (Hongo and Murata, 1965a). Derivatives of *C. saccharoperbutylacetonicum* strain N1 were used in industrial production of acetone and butanol from molasses in Japan between 1959 and 1960 (Ogata and Hongo, 1979). This species was chosen for industrial application because it produced a high proportion of butanol (Hongo and Murata, 1965a). During the period that *C. saccharoperbutylacetonicum* was used in industrial butanol production, a series of phage-resistant strains (such as *C. saccharoperbutylacetonicum* strains N1-120, N1-508, and N1-621) were selected to counter the frequent occurrence (12 times in a year) of phage contamination of the fermentation (Hongo and Murata, 1965a; Ogata and Hongo, 1979), and a record of their development is available.

"*Clostridium saccharobutylicum*" is the name proposed (Keis et al., 2001a) for the species represented by strains NCP 262 (also known as P262 or NCP P262) and NRRL B-643 (Johnson and Chen, 1995; Johnson et al., 1997). Strain NCP 262 was one of the production strains used at the National Chemical Products (NCP) plant in Germiston, South Africa (Keis et al., 1995), whereas strain NRRL B-643 was provided by Commercial Solvents Co. to Northern Regional Research Laboratory (now Midwest Area National Center for Agricultural Utilization Research) of the USDA (L. K. Nakamura, personal communica-

tion). The Commercial Solvents Co. utilized a number of molasses-fermenting clostridia for solvent production after the expiration of the Weizmann patent (see the subsection Industrial Solvent Fermentation in this Chapter).

Clostridium puniceum produces butanol and acetone from glucose or starch, and a molar ratio of butanol (179 mM) to acetone (16.8 mM) reached above 10 in a fed-batch fermentation with a total of 7.2% (w/v) of glucose (Holt et al., 1988). *Clostridium puniceum* is a pink-pigmented, pectinolytic bacterium (Lund et al., 1981), while *C. beijerinckii* NRRL B592 produces a pink-lavender pigment (J.-S. Chen, unpublished observation). On the basis of 16S rRNA sequences, *C. puniceum* is phylogenetically close to *C. beijerinckii* and three not validly published clostridial species "*C. corinoforum*," "*C. favososporum*" and "*C. caliptrosporum*" (Collins et al., 1994). *Clostridium puniceum* has not been examined by the DNA-DNA reassociation technique.

Clostridium pasteurianum ATCC 6013 produces acetone (90 mM), butanol (135 mM) and a low level of ethanol in a mineral salts medium with 12.5% (w/v) of glucose (Harris et al., 1986). In media containing 3.5% (w/v) or less of glucose, *C. pasteurianum* produced only low levels of butanol and little or no acetone (George et al., 1983b; Harris et al., 1986; Dabrock et al., 1992). A newly isolated strain of *C. pasteurianum* produced acetone and butanol from 3% dahlia inulin (Oiwa et al., 1987). *Clostridium pasteurianum* produces butanol, 1,3-propanediol, and ethanol from glycerol (Nakas et al., 1983; Heyndrickx et al., 1991; Dabrock et al., 1992).

Butanol- and Isopropanol-Producing Bacteria Some strains of *Clostridium aurantibutyricum* and *C. beijerinckii* have the capacity to reduce acetone to isopropanol, and in these organisms, isopropanol may supercede acetone as the second most abundant neutral end product after butanol (George et al., 1983b). The butanol- and isopropanol-producing organism "*Clostridium toanum* Baba" (Prescott and Dunn, 1959c) was used in large-scale production of butanol and isopropanol in Taiwan between 1942 and 1958 (see the subsection Industrial Solvent Fermentation in this Chapter), but this organism is not available from any major culture collection.

Butanol-Producing Bacteria When a viologen dye or one of the other additives is present in the growth medium and the pH is maintained above a certain value, *C. acetobutylicum* and *C. beijerinckii* produce butanol and ethanol but not acetone (see the subsection Physiology of Solvent Production in this Chapter). There are other species of clostridia that produce butanol but not acetone or isopropanol in growth media without these additives. They include *Clostridium tetanomorphum* (Gottwald et al., 1984) and *Clostridium thermosaccharolyticum* (Freier-Schroeder et al., 1989). The amount of butanol produced by these species is much less than that produced by the acetone- and butanol-producing species.

Taxonomic Studies For over 60 years, many strains of solvent-producing clostridia were used in large-scale commercial production. Other strains that were developed for patented processes and documented in the literature were not actually used in commercial production. Some of these strains are still available from culture collections, but many of them have been assigned incorrect species names. Historically, strains that are similar enough to qualify as members of the same species were often given different species or genus names when they were part of patented processes (Beesch, 1952). After the expiration of the patents, culture collections and individual laboratories may do the reverse, i.e., lump genuinely different organisms into a single species. The latter practice is especially problematic because seemingly contradictory properties would be reported for cultures labeled with the same name but actually belonging to different species (Johnson and Chen, 1995).

When the species *C. acetobutylicum* was proposed by McCoy and coworkers (McCoy et al., 1926) for the 11 strains of acetone- and butanol-producing clostridia that include a strain believed to have come from Weizmann (McCoy and McClung, 1935), the comprehensive cultural study provided a detailed description of the organism's growth characteristics. For many of the solvent-producing clostridia that were isolated later for the molasses (sucrose)-based fermentation, there are no published records of comparably comprehensive cultural studies, but some of these cultures were labeled as *C. acetobutylicum* after they were maintained in the culture collections (Johnson and Chen, 1995; Keis et al., 1995; Keis et al., 2001a; Johnson et al., 1997). The loose use of species names plagued the early investigators (McCoy and McClung, 1935), and it remains a problem now. Attention to the current definition for a bacterial species (Johnson, 1984) and the proper procedure for differentiating species (Stackebrandt and Goebel, 1994) should be helpful.

For phenotypically similar bacteria, provided they share a similar DNA base composition (mol% G+C), which is a characteristic of most solvent-producing clostridia, the DNA-DNA reassociation technique is required to separate them to the species level (Stackebrandt and Goebel, 1994). On the basis of percent DNA relatedness as measured by DNA-DNA reassociation, cultures of "*C. acetobutylicum*" from var-

ious collections can be clearly separated into four species (Johnson and Chen, 1995; Johnson et al., 1997): *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* (Keis et al., 2001a). On average, *C. acetobutylicum* strains have a 10% DNA sequence similarity with the other three species, whereas the other three species share a 20% sequence similarity. Within each species, the level of DNA sequence similarity among strains is between 70 and 100%, except for strain NCIMB 6444, which has a similarity level between 54 and 80% (average 69.6%) with 16 strains of *C. beijerinckii*. The cutoff value for strains to be considered as belonging to the same species is 70% (Johnson, 1984).

Using DNA fingerprinting and biotyping (susceptibility to rifampin, inhibition by bacteriocins, and cell lysis by bacteriophages), 55 cultures of solvent-producing clostridia (most of them having been labeled as "*C. acetobutylicum*") were separated into nine groups (Keis et al., 1995). Sequence analyses of the partial and almost complete 16S rRNA genes of strains representing the nine groups clearly separated *C. acetobutylicum* strain ATCC 824 from the others; however, these other strains of solvent-producing clostridia shared a 16S rRNA sequence similarity above 98% and could not be further divided into groups. In fact, some of these strains are more similar to each other (such as *C. saccharobutylicum* strain NCP 262 and *C. saccharoperbutylacetonicum*) than to *Clostridium puniceum* on the basis of 16S rRNA sequence (Keis et al., 1995). That bacterial species within a genus cannot generally be differentiated on the basis of the 16S rRNA sequence has been emphatically pointed out (Stackebrandt and Goebel, 1994).

Industrial Solvent Fermentation Solvent fermentation was a significant industrial process between the late 1910s and early 1960s, and it was used for commercial purposes for most of this period. After the early 1960s, use of this biomass-based fermentation process remained practical at only a few places. The continued operation of this fermentation in those localities as well as the operation before the fermentation was commercialized shows that the usefulness of solvent fermentation is not solely dictated by commercial or economic factors; the bacterial process enables a nation to use locally generated biomass, instead of imported or strategically more important raw materials, to produce needed chemicals. Examples of such strategic operations are the production of acetone for the British Navy during World War I (Jones and Woods, 1986), the production of butanol for Japanese armed forces during World War II (Anonymous, 1956), and the production of acetone and butanol in South Africa, until the early 1980s (Jones and Woods, 1986). The future usefulness of this fermentation will continue to be affected by national security needs.

All aspects of industrial solvent fermentation have been reviewed, both during the years of peak production (Killeffer, 1927; Gabriel, 1928; Gabriel and Crawford, 1930; Kelly, 1936; Prescott and Dunn, 1949; Beesch, 1952; Beesch, 1953; McCutchan and Hickey, 1954) and thereafter when industrial solvent fermentation was viewed from a different perspective (Ryden, 1958; Ross, 1961; Hastings, 1971; Hastings, 1978; Spivey, 1978; Walton and Martin, 1979; Jones and Woods, 1986; McNeil and Kristiansen, 1986). Therefore, only a brief history of industrial solvent fermentation is given here.

Industrial interests in butanol fermentation began during the first decade of the 20th century because of a drive to make synthetic rubber. Butadiene, a leading monomer being considered for polymerization into synthetic rubber, can be manufactured from butanol, but at that time, butanol had not been produced commercially. Auguste Fernbach, director of the Fermentation Laboratories at the Pasteur Institute (Paris), was then a principal investigator of fermentation studies, and since the spring of 1909, Chaim Weizmann began to spend his vacations with Fernbach to study fermentations (Rose, 1986). In 1910, E. Halford Strange of the English firm Strange and Graham, Ltd., contracted Sir William Perkin, professor of chemistry at Manchester University (who in turn contracted his assistant Chaim Weizmann) to conduct research on synthetic rubber. From his study with Fernbach, Weizmann gained the expertise in anaerobic microbiology that he applied to his investigation in Manchester on the fermentation processes related to synthetic rubber. Through Weizmann, Fernbach was also under contract with Strange by December 1910 to work on butanol fermentation (Rose, 1986).

Fernbach and Strange (1911) were issued the British patents 15,203–15,204 on "Acetone and high alcohols (amyl, butyl, or ethyl alcohols and butyric, propionic or acetic acid) from starches, sugars, and other carbohydrates." In 1913, the first plant for the production of butanol from potatoes began in Rainham, England, using the bacillus isolated by Fernbach (Gabriel and Crawford, 1930). In the meantime, Weizmann was conducting research on butanol fermentation on his own, after he had broken his association with Perkin, Strange and Fernbach in 1912 (Rose, 1986). It was during this period that Weizmann isolated the acetone- and butanol-producing anaerobic bacterium that was later named "*Clostridium acetobutylicum*" by McCoy and coworkers (McCoy et al., 1926). *Clostridium*

acetobutylicum produces useful quantities of butanol and acetone from corn, which is a property not possessed by the bacillus used in the patented process of Fernbach and Strange.

When World War I broke out in 1914, the British government recognized the usefulness of the fermentation process of Weizmann for the production of acetone, which was needed as a solvent for the production of the smokeless explosive cordite for the British Navy. The process was initially used in England to produce acetone, but a shortage of corn there forced the transfer of the operation to Canada (between August 1916 and November 1918), where 3,000 tons of acetone were produced for the British government. After the entry of the United States into World War I in 1917, two distilleries (the Commercial Distillery and the Majestic Distillery) in Terra Haute, Indiana, were purchased, respectively, by the British and United States governments to produce acetone, and they were in operation between May and November 1918 (Gabriel, 1928). These government-operated plants were closed after Armistice in November, 1918. However, commercial solvent fermentation began in the United States two years later, and by the end of 1927, a total of 148 fermentors were in operation to produce solvents.

The commercial success of solvent fermentation was driven by the fast-growing automobile industry, which needed a fast-drying, low-viscosity paint for finishing the automobile body. Butyl acetate, which is produced from butanol, has the desired properties for the manufacturing of the fast-drying nitrocellulose lacquer. This potential use of butanol resulted in the formation of the Commercial Solvents Corporation of Maryland, which purchased from the Allied War Board the two plants at Terre Haute in 1919. Operations at the Majestic Distillery started in 1920, which is the real beginning of the commercial use of the Weizmann process and the associated organism *C. acetobutylicum* (Gabriel, 1928).

The rapid growth in demand for butanol is illustrated by the rate at which the plant capacity was expanded between 1920 and 1927. The Terre Haute plant had 40 fermentors initially, and it was increased to 52 during 1923. When the plant in Peoria began operation at the end of 1923, it had 32 fermentors (50,000 gallons each). A year later, the Peoria plant had 48 fermentors, and toward the end of 1927, the number increased to 96 (50,000 gallons each), for a total of 148 fermentors for both plants (Gabriel, 1928). By 1927, the two plants together produced more than 100 tons of solvents per day (Killeffer, 1927). After the expiration of the Weizmann patent in 1936, industrial solvent fermentation was practiced around the world as other companies built plants in the United States, Puerto Rico, the United Kingdom, Japan, India, Australia, South Africa and elsewhere (Jones and Woods, 1986). Fermentors with a volume up to 15,000 barrels (472,500 gallons) were used for acetone-butanol fermentation (Beesch, 1953). Although molasses replaced corn as the predominant raw material in the United States, new processes utilizing starchy materials or admixtures of starchy and sugary materials were used where it is economical to do so (Beesch, 1953).

The Weizmann process is based on the fermentation of corn by *C. acetobutylicum*. The kernel is ground into a coarse meal (Prescott and Dunn, 1949) or a fine powder (Beesch, 1953; McCutchan and Hickey, 1954) and mixed with water and stillage (distillation slop), and the cooked corn mash does not require any other nutrients to be added for the fermentation. When it was economical, the germ of corn was removed for oil extraction, but the oil-cake meal may be returned to the mash to increase the feed value of the recovered solids at the end of the fermentation. Besides *C. acetobutylicum*, an unusual variant of "*Clostridium saccharo-butyl-acetonicum-liquefaciens*" (known as Code C-12) was also used for the corn-based fermentation, but it required the addition of ammonia to the corn mash because of its poor proteolytic power (Walton and Martin, 1979).

The concentration of corn in the mash is about 8.5% (based on the original dried corn), and this concentration was used to ensure that the final butanol concentration in the broth (beer) did not exceed 13 gm/liter (1.3% or 175 mM) and to ensure complete utilization of the starch by the end of the fermentation (Walton and Martin, 1979). The normal fermentation of starches gives a final total solvent concentration of about 2.2% (22 gm/liter), with an approximate 6 : 3 : 1 weight ratio of butanol:acetone:ethanol. This corresponds to a yield of 1 lb (0.4536 kg) of mixed solvents from 4.3 lb (1.95 kg) of corn or 2.9 lb (1.315 kg) of starch (Beesch, 1953). In addition, hydrogen, carbon dioxide, and the dried stillage (which contained proteins and vitamins B_2 and B_{12} and was used in animal feeds) were recovered and sold (Walton and Martin, 1979).

An interesting practice that was part of the corn- and the molasses-based fermentation is the reutilization of the distillation slop or stillage, i.e., the fermentation broth from which the solvents have been removed. This practice was known as "slop-back," and the volume of the spent medium reutilized amounted to 25–50% of the total mash (Walton and Martin, 1979). The advantages of slopback include an increase in yields of solvents, a decrease in the amount of nutrients required (for the molasses-based process), and savings in heat, cooling water, and steam for the various steps. Considering the

physiological effects of added acetate and butyrate on the solvent-producing bacteria (see the subsection Physiology of Solvent Production in this Chapter), slop-back could be an important aspect of the industrial process. A more detailed description of the industrial fermentation can be found in several reviews (Beesch, 1953; Walton and Martin, 1979).

Molasses was the most widely used raw material for solvent fermentation, and the use of corn or *C. acetobutylicum* was phased out by 1933 (Kelly, 1936; Walton and Martin, 1979). However, other starch-based processes were later developed and used for industrial production while the molasses-based processes were in use (Beesch, 1953), and some of the saccharolytic organisms are capable of fermenting starch directly under suitable conditions and producing almost full yields of solvents (Beesch, 1952). Two types of molasses, the blackstrap molasses and the high-test or invert molasses, were predominantly used in industrial solvent fermentation (McCutchan and Hickey, 1954; Walton and Martin, 1979), although other types of molasses and sugary materials were also used (Beesch, 1952).

Blackstrap molasses is the concentrated mother liquor remaining after the crystallization of sucrose from sugar cane juice and contains on average 52% total sugars, consisting of about 30% sucrose and 22% invert sugar (glucose plus fructose). It contains salts that are added to aid in the recovery of crystalline sucrose. Batch-to-batch variations in the composition of blackstrap molasses caused wide variation in yields and nutrient requirements for fermentation based on this raw material (McCutchan and Hickey, 1954).

High-test molasses contains about 50% invert sugar and 25% sucrose and is a better raw material than blackstrap molasses because it contains less nonfermentable solids, including salts, and has a more consistent composition. High-test molasses is produced from excess sugar cane. The juice is concentrated to about 70–75% sugar in the presence of a small amount of mineral acid, which hydrolyzes (inverts) about two-thirds of the sucrose to glucose and fructose to avoid crystallization on standing (McCutchan and Hickey, 1954). As in the corn fermentation, the concentration of molasses in the fermentor medium was calibrated so that it was not more than what was needed to give a final butanol concentration of 13 gm/liter. The sugar concentration used in the fermentor medium varied from 5.5 to 7.5% depending upon the solvent ratio produced by the culture selected (Walton and Martin, 1979).

Although the patent literature contains the names of many organisms for the molasses-based processes (Beesch, 1952), information is scarce as to the strains actually used by the industry. It is now known that the "*C. madisonii*" strain used in Puerto Rico in the 1940s (Jones et al., 2000) and the two strains (P265 and P270) used at National Chemical Products in South Africa in the late 1970s (Spivey, 1978) are *C. beijerinckii* (Keis et al., 2001a). Before solvent fermentation was terminated in Japan in the early 1960s, the organisms used were strains of *C. saccharoperbutylacetonicum* (Hongo and Murata, 1965a; Hongo and Murata, 1965b), a species now recognizable by molecular methods (Johnson et al., 1997; Keis et al., 2001a).

Molasses-based media are generally deficient in available nitrogen and phosphate, which may be responsible for poor solvent production when *C. acetobutylicum* was tested in unfortified media in early studies, although the low buffering capacity of molasses may also be a factor. Good growth and solvent production in molasses-based media require the addition of a pH-control agent, vitamins, and mineral nutrients in addition to nitrogen and phosphorus compounds. In practice, ammonia (1.2–1.3% NH_3 based on sugar concentration) was added both as a pH-control agent (when used in the form of ammonium hydroxide) and as a nitrogen source. Although ammonia alone gave satisfactory results, the use of ammonia plus a source of complex nitrogen (such as yeast water, corn-steep liquor or stillage) was usually preferred in the fermentor medium to ensure a maximum yield of solvents (Beesch, 1952). Superphosphate (depending on the manufacturing process, different preparations of superphosphate contain different amounts of the key ingredient, monobasic calcium phosphate, and other phosphorus compounds; the available amount of phosphorus in superphosphate is measured as P_2O_5) was used to give 0.05–0.2% of P_2O_5 based on sugar (Walton and Martin, 1979). Table 33 shows the result of the molasses-based industrial fermentation used in South Africa in the 1970s.

Fermentable Substrates Corn and molasses (sugar cane) were the principal raw materials for industrial solvent fermentation. Corn was primarily used during the era of the Weizmann process, which was based on *C. acetobutylicum*, whereas molasses was used when other bacteria replaced *C. acetobutylicum* in the industrial fermentation (see the subsection Industrial Solvent Fermentation in this Chapter). Besides corn and molasses, other starchy substrates were also used in industrial solvent fermentation outside North America. For example, the raw materials for solvent production by "*Clostridium toanum* Baba" in Taiwan (Formosa) between 1947 and 1957 included sweet potato, casava, and wheat starch (a by-product of gluten production; Yeh, 1955; Anonymous, 1956; Anonymous, 1958). In addi-

Table 33. Production of acetone, butanol, and ethanol from molasses in 90,000-liter batch fermentation in South Africa.[a,b]

Product	Amount (kg)	Percent (wt/wt) of sugar fermented
Butanol	1,053	18
Acetone	526	9
Ethanol	175	3
Carbon dioxide	2,900	50
Hydrogen	117	2

[a]The 90,000-liter batch contains 5,850 (approx.) kgm of fermentable sugars from molasses.
[b]*Clostridium beijerinckii* strains NCP265 and NCP270 were used (Spivey, 1978; Keis et al., 2001a). The fermentation medium was supplemented with corn steep liquor, in addition to liquid ammonia, as a source of nitrogen and other growth stimulants to enhance solvent production. The starting pH was adjusted to 5.8–6.0 with ammonia, and calcium carbonate was added for its pH regulatory effect. The total cycle of cleaning, sterilization, filling, fermentation, and emptying the fermentor took 48 hours, of which 30–34 hours was fermentation time.
Data from Spivey (1978).

tion, a diverse range of raw materials has been tested as alternative substrates for solvent production (McCutchan and Hickey, 1954; Prescott and Dunn, 1959a; Jones and Woods, 1986; Dürre and Bahl, 1996).

The succession of the corn- and the molasses-based fermentations implies that the molasses (sugar)-fermenting clostridia are not suitable for fermenting starchy substrates. In fact, starch is a fermentable substrate for strains of *C. aurantybutyricum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*, besides *C. acetobutylicum* (Cato et al., 1986; Keis et al., 2001a). Potatoes have been used successfully as a substrate for solvent production by *C. beijerinckii* NRRL B592 (Nimcevic et al., 1998), although partially hydrolyzed (liquefied) starch is preferred for operational reasons (Nimcevic and Gapes, 2000). It is economically attractive to produce solvents from alternative substrates that are considered waste and normally present disposal problems. Besides potato waste (which includes industrial residues, low-grade potatoes, and spoiled potatoes; Nimcevic et al., 1998), other waste materials including cheese whey (Maddox, 1980; Schoutens et al., 1984; Schoutens et al., 1985b; Ennis and Maddox, 1985; Stevens et al., 1988), hydrolyzed lignocellulosic and hemicellulosic materials (Compere and Griffith, 1979; Compere et al., 1985; Fond et al., 1986a; Fond et al., 1986b; Lemmel et al., 1986), palm oil mill effluent (Somrutai et al., 1996), apple pomace (Voget et al., 1985), and soy molasses (Qureshi et al., 2001b) can be substrates for solvent production. The range of fermentable substrates for the solvent-producing clostridia is thus much broader than starch, sucrose, and their component sugars. A comprehensive review of the physiology of carbohydrate utilization by the solvent-producing clostridia is available (Mitchell, 1998). A brief review of the properties of the polymers starch, cellulose and xylan is given below, as these polymers represent potential substrates for future industrial solvent fermentation.

Starch is composed of amylose (a linear polymer of α-1,4-linked D-glucose residues) and amylopectin, which has α-1,6 linkages to connect branching linear chains. Endo-acting α-amylase randomly hydrolyzes α-1,4-D-glucosidic linkages in a chain containing three or more glucose units, with the reducing groups liberated in the α-configuration, whereas β-amylase hydrolyzes α-1,4-D-glucosidic linkages to remove successive β-maltose units from the nonreducing end. Glucoamylase hydrolyzes the terminal 1,4-linked α-D-glucose residues successively from the nonreducing ends, releasing β-D-glucose. The α-1,6 linkages at the branch points are hydrolyzed by pullulanase. Although starch was a major substrate for industrial solvent production, little is known about the amylolytic system in *C. acetobutylicum* or *C. beijerinckii*, and a consistent picture of the regulation of starch hydrolysis and metabolism is yet to emerge (Dürre and Bahl, 1996; Mitchell, 1998). An 84-kDa α-amylase has been purified from *C. acetobutylicum* ATCC 824 (Paquet et al., 1991), yet a cloned amylase gene from the organism encodes a 53.9-kDa polypeptide (Verhasselt et al., 1989), indicating that *C. acetobutylicum* ATCC 824 produces different amylases for the utilization of starch. Two α-amylase genes (CAP0098 and CAP0168) have been identified on the plasmid pSOL1 (Nolling et al., 2001), and they encode polypeptides of about 60 and 80 kDa, respectively.

Solvent-producing clostridia have not been reported to grow on cellulose, but the disaccharide cellobiose is metabolized by *C. acetobutylicum*, *C. aurantibutyricum*, *C. beijerinckii*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum* (Cato et al., 1986; Keis et al., 2001a). Cellobiose is an excellent substrate for solvent production by *C. beijerinckii* NRRL B592 but not by *C. acetobutylicum* NRRL B527 (= ATCC 824) or *C. beijerinckii* NRRL B593 (Compere and Griffith, 1979). Efficient degradation of crystalline cellulose requires cellulosomes (extracellular supramolecular structures composed of scaffold-bound endo- and exoglucosidases and cellulose-binding proteins; Mitchell, 1998). Genes encoding at least 11 cellulosome component proteins have been identified in the genome of *C. acetobutylicum* ATCC 824 (Nolling et al., 2001). Inducible activities of endoglucanase (endoglucanase is endo-1,4-β-glucanase or

1,4-[1,3; 1,4]-β-D-glucan 4-glucanohydrolase) and cellobiase were in *C. acetobutylicum* NRRL B527 and ATCC 824 (Lee et al., 1985) and *C. beijerinckii* NCP270 (Allcock and Woods, 1981a). The endoglucanase hydrolyzes carboxymethyl cellulose, acid-swollen cellulose, and microcrystalline cellulose (Avicel) but not crytalline cellulose. Genes encoding an endoglucanase and a cellobiase have been cloned from *C. saccharobutylicum* NCP 262 (Zappe et al., 1986; Zappe et al., 1988).

The solvent-producing clostridia can grow on xylan and pentoses (Cato et al., 1986; Mitchell, 1998; Keis et al., 2001a) and produce moderate levels of solvents (Compere and Griffith, 1979). Arabinose is an excellent substrate for solvent production by *C. beijerinckii* NRRL B592, but not for *C. acetobutylicum* NRRL B527 (= ATCC 824) or *C. beijerinckii* NRRL B593 (Compere and Griffith, 1979). Pentoses are broken down by the transaldolase-transketolase pathway to produce fructose 6-phosphate and glyceraldehyde 3-phosphate, which are further metabolized via glycolysis to pyruvate before entering the acid- and solvent-producing pathways. A predicted operon (genes CAP0114 to CAP0120) consisting mostly of genes for xylan degradation has been identified on the pSOL1 plasmid of *C. acetobutylicum* ATCC 824 (Nolling et al., 2001). The gene encoding a xylanase has been cloned from *C. saccharobutylicum* NCP262 (Zappe et al., 1987).

For both hexoses and pentoses, the mono-, di- and oligosaccharides and their derivatives are taken up via specific membrane-bound transport proteins. Few detailed studies of sugar uptake by the clostridia have been reported, but observations made to date indicate that the transport mechanisms are similar to those found in a wide variety of other bacteria (Mitchell, 1998). A large number of genes for different types of transporters has been found in the genome of *C. acetobutylicum* ATCC 824 (Nolling et al., 2001).

Bacteriophage Infections, Autolysins, and Cell Lysis Infections by bacteriophages were a recurrent problem for industrial solvent fermentation. The first phage contamination incident on record occurred in 1923 (Ogata and Hongo, 1979), in the Terre Haute (Indiana, United States) plant, and resulted in the halving of fermentation yields for about a year (Gabriel, 1928). Many cases of such "sluggish fermentation" experienced in the ensuing years were presumed to be caused by phage infections. However, no proven case of phage contamination occurred until 1943 when McCoy and Sylvester reported the isolation of phages from fermentation broth from a plant in Puerto Rico that used "*Clostridium madisonii*," a strain now recognized as *C. beijerinckii* (Ogata and Hongo, 1979; Jones et al., 2000). A specific abnormal fermentation, called "sleeping sickness of acetone-butanol fermentation," occurred at several factories in Japan around 1943, and incidences of such sleeping sickness or sluggish fermentation continued to occur for years. The review by Ogata and Hongo (1979) describes the isolation and characterization of phages from the abnormal fermentation broth as well as the selection of phage-resistant strains of clostridia for industrial uses.

By examining previously unreleased company reports and records, a review (Jones et al., 2000) provided information about phage infections at the National Chemical Products (NCP) factory, which produced solvents by fermentation from 1936 until 1982 in Germiston, South Africa. Confirmed or presumed phage infections of 1943, 1947, 1960, 1976 and 1980 at the NCP factory were described in this review, and the properties of the phage CA1 that caused the relatively mild 1980 phage infection were reported.

The characteristics of phage infections in solvent fermentation may be summarized as follows (Jones et al., 2000): Regardless of the bacterial strains or substrates used, the typical symptoms of phage-infected batches are slow or sluggish fermentations with extended fermentation times and reduced solvent yields. Because of a decrease in the metabolic and growth rates, the infected cultures produce less gas and leave more substrates unused. There are changes in the cell population and morphology that include a marked reduction in cell numbers, a loss of motility, and the presence of etched cells. In some cases, elongated cells or protoplasts may be observed. Lysis of infected cells can be observed in the laboratory but may not occur at the factory. Virulent, lysogenic, and pseudo-lysogenic phages have been identified, and they are quite strain-specific. Of the seven phages that were tested against the strains of *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum*, all have restricted host ranges (Keis et al., 1995). At present, the selection of phage-resistant variants and the maintenance of good plant practice and hygiene remain the effective measures against phage infections. The selection and isolation of phage-resistant mutants or variants that retain solvent-producing capabilities are referred to as "phage immunization." One danger of producing resistant strains is the possibility of generating lysogenic cultures, which may later release phage particles and continue to cause phage infections (Ryden, 1958). It has been suggested that no matter how good the plant practice and hygiene, phage infections are bound to occur from time to time. It is therefore important that companies operating this fermen-

tation process develop strategies to minimize the impact of such infections (Jones et al., 2000).

Solvent-producing clostridia are also known to undergo autolysis as a result of lytic enzyme activity or bacteriocins with lytic activity (Barber et al., 1979; Allcock et al., 1981b; Webster et al., 1981; van der Westhuizen et al., 1982; Roos et al., 1985; Croux et al., 1992b). Lysis of *C. acetobutylicum* ATCC 824 is stimulated by environmental conditions (e.g., pH 6.3, 55°C and the presence of monovalent cations; Croux et al., 1992b) and can be reduced by treatment with chloramphenical (Zhou and Traxler, 1992). The *lyc* gene (CAC0554) of *C. acetobutylicum* ATCC 824, which encodes an autolytic lysozyme (a muramidase acting on non-*n*-acetylated peptidoglycan; Croux et al., 1992c), has been cloned and characterized (Croux and Garcia, 1991; Croux and Garcia, 1992a). When expressed in a recombinant strain of *Escherichia coli* under the control of the *lac* promoter, the autolytic lysozyme was secreted although it does not contain a cleavable signal peptide, suggesting that like other autolysins this lytic enzyme is also secreted through a specific mechanism (Croux and Garcia, 1992a). The use of autolytic deficient mutants of solvent-producing clostridia may be a useful improvement for the industrial fermentation.

Physiology of Solvent Production Butyric acid is a characteristic fermentation product of many anaerobic bacteria. In the carbohydrate-fermenting clostridia, the key metabolic intermediate pyruvate is cleaved by the pyruvate : ferredoxin oxidoreductase to acetyl-CoA and CO_2, with ferredoxin serving as the electron acceptor (Chen, 1993). Two molecules of acetyl-CoA are combined by thiolase to form acetoacetyl-CoA, which is metabolized through the reactions catalyzed by enzymes 4 through 9 (Fig. 26) to form a molecule of butyric acid, with the concomitant consumption of two molecules of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the production of an ATP. During the exponential phase of growth, acetic acid is produced in addition to butyric acid, and the acetate-producing pathway could be viewed as a branch of the butyrate-producing pathway (Fig. 26). Acetyl-CoA is thus the branch-point between the acetate- and the butyrate-producing pathways. The production of butanol and acetone/isopropanol, on the other

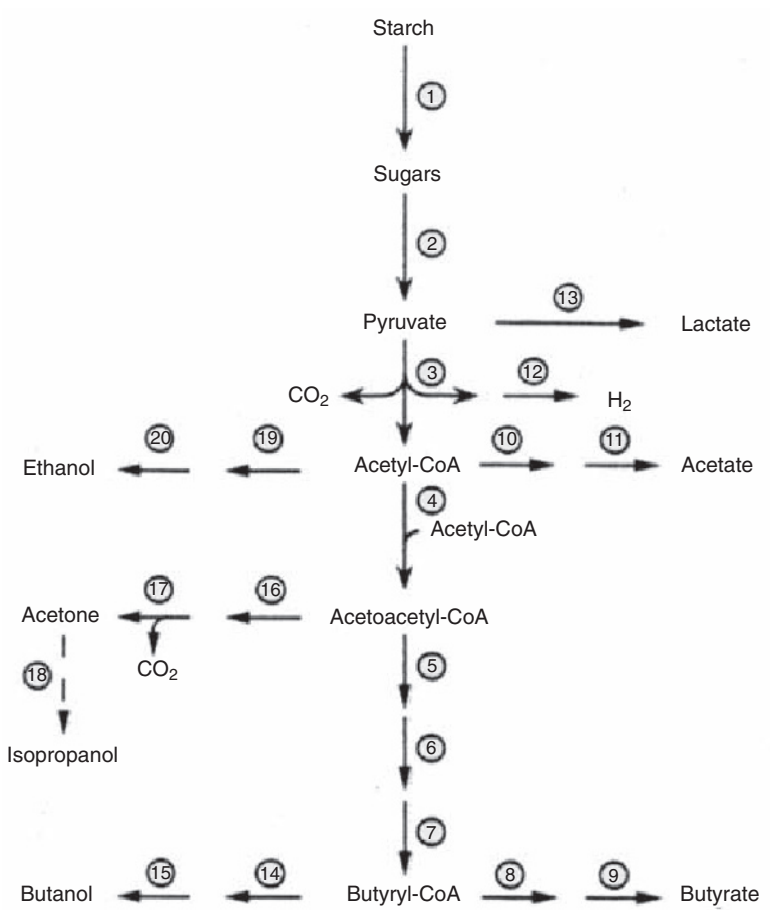

Fig. 26. Metabolic pathways for the fermentation of starch and sugars to acids, solvents, hydrogen and carbon dioxide in the clostridia. Arrows indicate the direction of carbon or electron flow as well as the major steps of the pathways. The enzyme(s) catalyzing the reaction(s) represented by an arrow is numbered: 1) amylase; 2) phosphoenolpyruvate phosphotransferase systems and glycolytic enzymes of the Embden-Meyerhof-Parnas pathway; 3) pyruvate:ferredoxin oxidoreductase; 4) thiolase; 5) 3-hydroxybutyryl-CoA dehydrogenase; 6) crotonase; 7) butyryl-CoA dehydrogenase; 8) phosphotransbutyrylase; 9) butyrate kinase; 10) phosphotransacetylase; 11) acetate kinase; 12) hydrogenase; 13) lactate dehydrogenase; 14) aldehyde (butyraldehyde) dehydrogenase and aldehyde/alcohol dehydrogenase; 15) alcohol (butanol) dehydrogenase and aldehyde/alcohol dehydrogenase; 16) acetoacetate: acetate/butyrate CoA-transferase; 17) acetoacetate decarboxylase; 18) primary/secondary alcohol dehydrogenase; 19) aldehyde (acetaldehyde) dehydrogenase, and 20) alcohol (ethanol) dehydrogenase. The dashed line to isopropanol indicates that only some strains of a species are able to perform this reaction. From Dürre (1998).

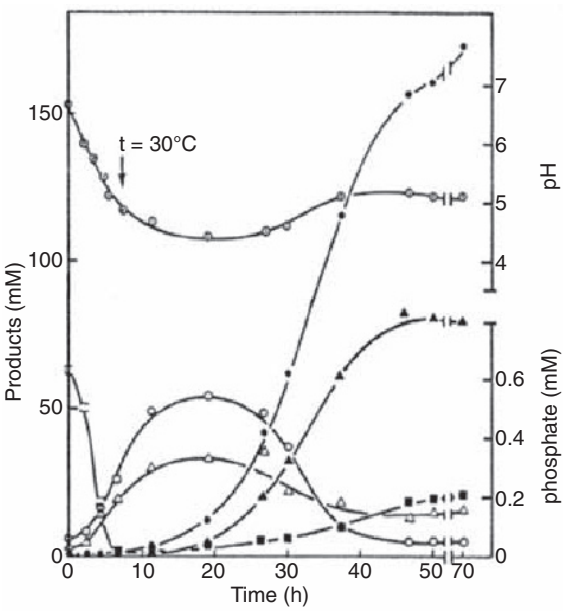

Fig. 27. Time course of acid production and subsequent solvent accumulation with concurrent acid reutilization in a batch culture of *C. acetobutylicum* DSM 1731. The mineral medium contained 60 gm/liter of glucose and a low concentration (0.62 mM) of phosphate. The temperature was 37°C during the exponential phase of growth, and the pH was regulated to allow it to decrease linearly from 6.6 to 5.0. When phosphate was exhausted in the medium and the pH was below 5.0, the temperature was lowered to 30°C and the pH regulation was switched off. The final concentrations (mM) after three days were: butanol, 175; acetone, 77; ethanol, 22; butyrate, 9; acetate, 18; acetoin, 9; glucose, 0; and phosphate, 0. Symbols: butanol, ●; acetone, ▲; ethanol, ■; butyrate, ○; acetate, △; phosphate, □; and pH, ○. From Bahl et al. (1982a).

hand, involves regulated metabolic pathways that branch off the butyrate-producing pathways at the C_4-intermediates butyryl-CoA and acetoacetyl-CoA, respectively (Fig. 26). The accumulation of solvents in the culture medium occurs late during growth of *C. acetobutylicum* as shown in Fig. 27. Other solvent-producing species exhibit a similar time course (Rogers, 1986; Yan et al., 1988), suggesting that solvent production is a programmed response to either specific environmental conditions or growth-stage related signals.

Initiation of Solvent Production In batch cultures, the start of solvent production by wildtype cells requires a variety of environmental conditions, including a high concentration of butyric acid, a low culture pH, and a growth-limiting concentration of phosphate or sulfate. These conditions are associated with changes in carbon and electron flux and in concentrations of high-energy intermediates in the cell, and the growth rate decreases under such environmental conditions. One or several of the environmental conditions determine the accompanying cellular conditions present shortly before the start of active solvent production, and the extracellular and intracellular conditions directly or indirectly serve as signals for activating the transcription of the solvent-production genes and result in a metabolic switch from acid to solvent production. Although the solventogenic switch clearly involves events at the transcriptional level (Dürre et al., 2002), the molecular mechanism for activating the transcription of solvent-production genes remains to be elucidated. It has been suggested that because the environmental conditions required for solvent production can affect DNA topology directly, a relaxation of the degree of negative DNA supercoiling might be the trigger for solvent-production gene activation (Dürre et al., 1995; Dürre, 1998). However, specific regulatory (effector) proteins apparently play important roles in the transcriptional activation of solvent-production genes inasmuch as conserved motifs have been identified in the promoter region of solvent-production operons (Ravagnani et al., 2000; Thormann et al., 2002). In addition, different carboxylic acids affect the initiation of transcription of the solvent-production genes differently, suggesting a specificity of the sensor proteins toward the solventogenic signals.

In a glucose-limited continuous culture maintained at pH 4.3 and with an excess of phosphate, the presence of butyric acid at 20 mM or above allowed *C. acetobutylicum* DSM 1731 to produce solvents (Bahl et al., 1982a). In batch cultures (Husemann and Papoutsakis, 1986) or with washed logarithmic growth-phase cells (Ballongue et al., 1985), addition of butyrate (6–20 mM), acetoacetate (12–20 mM), acetate (8–33 mM), and several other C_1 to C_4 straight-chain acids can induce the solventogenic transition in *C. acetobutylicum* ATCC 824. When the culture pH was maintained at 6 and the starting glucose concentration was about 275 mM (about 5%, w/v), solvent production by *C. acetobutylicum* ATCC 824 was observed when 65 mM of butyrate was added early during growth, but it ceased immediately upon glucose depletion (Husemann and Papoutsakis, 1988). The addition of butyrate plus acetate accelerated *C. beijerinckii* strain NRRL B592 (George and Chen, 1983a) and strain NCIMB 8052 (Holt et al., 1984) into the solventogenic transition at neutral pH, but the addition of butyrate or acetate alone was not effective (George and Chen, 1983a). Among the carboxylic acids tested, citric acid (Husemann and Papoutsakis, 1986) and straight-chain carboxylic acids with 5–7 carbon

atoms and branched-chain acids (Ballongue et al., 1985) could not induce the solventogenic transition. The specificity towards the straight-chain C_1 to C_4 carboxylic acids indicates that it is not the acid-base properties of the carboxylic acids that are responsible for their solventogenic triggering effect. Furthermore, specific enzymes or sensors must be involved in transducing the external signal to the transcriptional apparatus.

The concentration of total or undissociated butyric or acetic acid at the onset of solvent production has been the subject of a number of investigations as this parameter is likely a quantitative triggering signal for solventogenesis. Depending on the medium composition and other growth conditions used, the concentration of undissociated butyric acid at the onset of solvent production by *C. acetobutylicum* ranged from about 5 to 20 mM when the culture pH was below 5.5 (Monot et al., 1984; Terracciano and Kashket, 1986; Husemann and Papoutsakis, 1988) and below 5 mM when the culture pH was controlled at 6 (Husemann and Papoutsakis, 1988). A correlation was not found between the onset of solvent production and the external pH, the intracellular pH, the pH difference across the cytoplasmic membrane, and the external and intracellular butyrate and acetate concentrations (Husemann and Papoutsakis, 1988). The correlation between the concentration of undissociated butyric acid and the onset of solvent production is not absolute, and it may reflect a combined effect of the pH and the dissociated butyric acid concentration on solventogenesis (Husemann and Papoutsakis, 1988), as the undissociated butyric acid is a biochemically inert species (Gottwald and Gottschalk, 1985).

Although the time-course of fermentation is similar among different species of solvent-producing clostridia, suggesting a common regulatory mechanism for solventogenesis, there are qualitative or quantitative differences in the response exhibited by different solvent-producing species to the same set of environmental signals. A prominent example is the different pH-dependence for the onset or progress of solvent-production (Johnson and Chen, 1995). In laboratory media, a pH of 5.5 or below is usually considered necessary for *C. acetobutylicum* to show sustained solvent production, and an optimal pH of 4.3 was reported for *C. acetobutylicum* strains DSM 792 and 1731. *C. acetobutylicum* ATCC 824 produced 48 mM butanol and 27 mM acetone when the culture pH was controlled at 6 and the initial glucose concentration was about 550 mM (about 10%, w/v), whereas it produced little butanol or acetone at pH 6 when the initial glucose concentration was about 275 mM (Husemann and Papoutsakis, 1988). In contrast, *C. beijerinckii* NRRL B592 produced these solvents at pH 6.8 with an initial sucrose concentration of 6% (w/v) and without butyrate supplementation (George and Chen, 1983a). *Clostridium saccharobutylicum* NCP262 (formerly *C. acetobutylicum* P262) produced these solvents at pH 5.5–6.5, but not when the pH of the culture was allowed to drop below 4.5 during the early part of the fermentation (Jones and Woods, 1986).

In batch or continuous cultures of *C. acetobutylicum* DSM 1731, phosphate limitation (initial phosphate concentrations between 0.62 and 0.74 mM) has a profound effect on enhancing the consumption of glucose and production of solvents (Bahl et al., 1982b). The onset of solvent production coincided with the exhaustion of phosphate in the medium and with the culture pH falling below 5.

Among the conditions conducive to the onset of solvent production, a high concentration of acids is effective with different species of clostridia. It is interesting to note that the practice of "slopback" (the reuse of the hot stillage [i.e., the fermentation broth after the removal of cells and solvents] to substitute for 10–100% of the volume of water in the fermentor medium) during industrial solvent production resulted in a slightly faster fermentation (McCutchan and Hickey, 1954) and also an increase in yield of solvents (Beesch, 1952; Beesch, 1953; Walton and Martin, 1979). Slopback was practiced to lower water usage, the cost of heating, and the cost of waste disposal. However, it could also be a source of butyric and acetic acids in each batch of fermentor medium. The actual concentrations of butyrate and acetate in the stillage are not clear but could be estimated. When batch cultures of *C. acetobutylicum* DSM 1731 were grown for three days in a medium containing 60 gm/liter of glucose and either a low (0.62 mM) or a high (12.25 mM) concentration of phosphate, the final butyrate and acetate concentrations were 9 and 18 mM, respectively, for the low-phosphate culture, and 68 and 42 mM, respectively, for the high-phosphate culture, whereas the final butanol concentrations were 175 and 47 mM, respectively, for the two cultures (Bahl et al., 1982b). The concentrations of butyrate and acetate in stillage might be within this range.

The onset of solvent production by PJC4BK strains of *C. acetobutylicum* ATCC 824 (these mutant strains have the butyrate kinase, *buk*, gene in the *ptb-buk* locus inactivated) occurs during the exponential phase of growth (Green et al., 1996b; Harris et al., 2000). The total external butyric acid concentration was 1 mM, and the undissociated butyric acid concentration was estimated at 0.4 mM when butanol (about 2 mM) was first detected (Harris et al., 2000). These observations suggest that a high concentration of

butyrate (or its transport) is not involved in triggering the onset of solvent production. Because these mutant strains have a lowered level of butyrate kinase (*C. acetobutylicum* ATCC 824 has a second butyrate kinase gene; see the subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter), it is postulated that the accumulation of butyryl-phosphate or butyryl-CoA might be responsible for triggering solvent production, as previously suggested (Gottwald and Gottschalk, 1985). When the butyrate kinase level was lowered by the antisense RNA technique (see subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter), the peak and final butyrate concentrations were actually higher than that of the control culture (Desai and Papoutsakis, 1999). Because of the high butyrate concentration produced in the presence of anti-*buk*-RNA, it is unclear what triggers the earlier onset of solvent production in the presence of anti-*buk*-RNA.

Relationship to Sporulation Although solvent production can be observed in exponentially growing cells, significant solvent accumulation occurs in cells beyond the logarithmic phase of growth. The duration of solvent production indeed has a close temporal relationship to growth stages, especially sporulation. Weizmann is credited with the discovery of a correlation between a strain's ability to sporulate and its ability to produce solvents as he used repeated pasteurization (100–150 cycles) to select heat-resistant spores for selecting and preserving a productive strain (Prescott and Dunn, 1959a). McCoy and Fred (1941) reported the result of a serial transfer experiment that lasted over two years. When three pure cultures were subjected to 150 cycles of the Weizmann procedure (pasteurization at 100°C for 1–2 min at intervals of 4–5 days to allow spore formation), the initial solvent yield was 26.2% and the final solvent yield was 24.7%, and the ratio of solvents was unchanged. On the other hand, when a strain was subjected to 200–300 transfers using vegetative cells (transfers were made at 12- and 24-h intervals), degeneration was observed by the 10–20th transfer. The solvent yield usually dropped to 0.5–2% before the 50th transfer, and spore formation was almost completely lost.

It is now established that an early step(s) of the switch for solvent production and sporulation is activated by a shared regulatory element. The Spo0A protein has been found to control the switch from acid to solvent production in *C. acetobutylicum* and *C. beijerinckii* (Ravagnani et al., 2000; Harris et al., 2002; Thormann et al., 2002). In *Bacillus subtilis*, the phosphorylated Spo0A protein plays a crucial role both as a negative and as a positive effector of gene expression at early times in the sporulation gene activation program (Baldus et al., 1994). Sequences resembling the Spo0A-binding motif (the 0A box) 5′-TGTCGAA are present in either orientation in the promoter region of the solvent-production operons and are required for transcriptional activation. However, additional upstream sequences are also required for the transcription of the *adhE* gene of *C. acetobutylicum* DSM 792, indicating the involvement of additional transcription effectors for solvent production (Thormann et al., 2002).

Because solvent production and sporulation share a key regulatory element for an early part of the two processes, mature spores are a good source of cells competent for solvent production. However, some asporogenous mutants are able to produce solvents (Meinecke et al., 1984). This is consistent with the interpretation that only an early step(s) of sporulation and solvent production depends on the common regulatory element Spo0A for transcriptional activation, and mutations disabling reactions specific for the late stages of sporulation should not affect solvent production. In fact, even the mutant strain of *C. acetobutylicum* ATCC 824, whose *spo0A* gene was inactivated, expressed the solvent-production genes to a limited extent and produced low levels of acetone and butanol (Harris et al., 2002), indicating that the transcription of the solvent-production genes is greatly enhanced by but not absolutely dependent on Spo0A. Because of the shared regulatory element, cells entering the solvent-producing stage are also entering sporulation, resulting in the formation of resting cells. Therefore, the shared regulatory circuit between solvent production and sporulation effectively sets a practical limit on the duration of active solvent production, regardless of the toxicity of the fermentation products. Because some asporogenous mutants of *C. acetobutylicum* are solvent-producers (see the subsection Strain Degeneration in this Chapter), it may be useful to identify factors that limit the duration of solvent production in these asporogenous mutants. The observation that solvent production by *C. beijerinckii* NRRL B592 is enhanced by high glucose but low yeast extract concentrations (conditions that appear to slow down growth and acid production) and that acid and solvent production can proceed simultaneously (Maddox et al., 2000) suggests that a combination of genetic manipulation and process engineering may significantly prolong solvent production in batch cultures.

Product Pattern As might be predicted from the metabolic pathways shown in Fig. 26, the ratio of

butanol to acetone (or isopropanol) produced by a culture can be expected to vary under different growth conditions. The determining factors could include the level of specific enzymes present, the availability of specific substrates, the supply of reducing power, and the activity of regulatory molecules. The production of acetone depends on the activity of acetoacetate : butyrate/acetate CoA-transferase (see the subsection Metabolic Pathways and Enzymology of Solvent Production in this Chapter) and is hence directly linked to the uptake of butyrate and acetate via CoA-transferase. The uptake of one butyrate or acetate molecule via CoA-transferase results in the production of one acetoacetate molecule, which leads to the production of one acetone molecule. Butyrate taken up via butyrate kinase does not contribute to the production of acetone because it generates butyryl-CoA, whereas acetate taken up via acetate kinase might contribute to acetone production if the resulting acetoacetyl-CoA is metabolized by CoA-transferase with the consumption of additional acids. Addition of acetate, or the practice of slop-back, is thus known to boost acetone production. The production of butanol and isopropanol requires reducing power and is limited to the period when cells metabolize (oxidize) carbohydrates actively.

In industrial solvent fermentation using *C. acetobutylicum*, the product ratio (based on weight) is usually reported as butanol : acetone : ethanol (6 : 3 : 1; Gabriel, 1928; McCutchan and Hickey, 1954). However, the butanol : acetone : ethanol ratio can range from 76.1 : 17.9 : 6.0 or 75.6 : 22.4 : 2.0 to 60 : 38 : 2 with different organisms grown under different conditions (McCutchan and Hickey, 1954). On the basis of sugar consumed, the solvent yield is about 30% (w/w), and it can be nearly quantitative on the mol/mol basis. With slop-back, solvent yields are generally about 34% (w/w; McCutchan and Hickey, 1954). Although butanol is toxic to the solvent-producing clostridia (Moreira et al., 1981; Bowles and Ellefson, 1985; for a discussion of cellular toxicity of alcohols, see "Ethanol" in Organic Acid and Solvent Production, Part II in this Volume), the final concentration of butanol in an industrial fermentation broth is not the highest that could be reached. Instead, it is purposely controlled by the use of a limited amount of carbon substrate so that a batch of fermentation is completed within a desirable period of time and little unused carbon substrate remains in the medium at the end. Whether butanol toxicity affects the progression of sporulation remains to be determined.

Alcohologenic Mode of Growth The production of both acetone and butanol by *C. acetobutylicum* is a characteristic of this species, and the two products are responsible for the organism's continued usefulness during and after World War I. Interestingly, under certain growth conditions, a different mode of fermentation (i.e., production of only butanol and ethanol) by this organism was discovered. Butanol but not acetone is produced when the culture pH is at or above 5.5 (Peguin et al., 1994; Girbal et al., 1995a). The term "alcohologenic" applies to the mode of fermentation that produces only the alcohols butanol and ethanol (Girbal and Soucaille, 1994). Growth conditions leading to alcohologenesis include the addition of methyl or benzyl viologen to the medium (Rao and Mutharasan, 1987; Rao and Mutharasan, 1988; Grupe and Gottschalk, 1992; Peguin et al., 1994; Dürre et al., 1995; Sauer and Dü, 1995), the addition of neutral red to the medium at neutral pH (Girbal et al., 1995b), and the addition of glycerol or glycerol plus pyruvate to a glucose-containing medium (Girbal and Soucaille, 1994; Vasconcelos et al., 1994). During alcohologenesis, the two acetone-producing enzymes, CoA-transferase and acetoacetate decarboxylase, are not detected (Dürre et al., 1995). It is now known that *C. acetobutylicum* has a second aldehyde-alcohol dehydrogenase that is encoded by the *adhE2* gene (CAP0035; see the subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter), and during the alcohologenic mode of growth, butanol and ethanol production is catalyzed by AdhE2 (Fontaine et al., 2002).

Strain Degeneration The solvent-producing clostridia gradually lose their productivity when cells are kept in the vegetative stage for a prolonged period of time (i.e., through serial transfers using growing cells as the inoculum), and this phenomenon is known as "degeneration" (McCoy and Fred, 1941; Kutzenok and Aschner, 1952). Strain degeneration is the result of genetic alterations, and it can be distinguished from the occasional failure of a culture to produce solvents because the growth conditions are unfavorable for sustained solvent production (Maddox et al., 2000). To clearly separate temporary, environmentally caused poor productivity from genetically based strain degeneration, the terms "acid crash" and "acidogenic fermentation" have been proposed (Maddox et al., 2000). Acid crash describes the early cessation of sugar uptake and solvent production when a culture is under a condition (pH below 5 for *C. beijerinckii* NRRL B592) that allows the concentration of undissociated acetic and butyric acids to exceed a threshold level, whereas acidogenic fermentation describes fast growth, sugar utilization, and acid production but slow solvent production when the pH of the culture is kept near neutrality and when yeast extract is present in ample amounts.

To circumvent deterioration of the strains for industrial fermentation, spores were preserved as the stock and repeated pasteurization was performed to select cells competent for solvent production (McCoy and Fred, 1941). The fact that a selection for mature spores also selects cells competent for solvent production indicates that a major mechanism for degeneration is sporulation-related (see the subsection Relationship to Sporulation in this Chapter).

Degeneration occurs more readily in *C. beijerinckii* than in *C. acetobutylicum* (Woolley and Morris, 1990). In continuous cultures, asporogenous mutants of *C. acetobutylicum* DSM 1731 are readily selected (Meinecke et al., 1984). Some asporogenous mutants from the continuous culture reverted to become sporeformers; however, after prolonged growth (35 days) in a continuous culture, a stable asporogenous mutant of *C. acetobutylicum* DSM 1731 became established, but it produced butanol and acetone as the major products. When asporogenous mutants of *C. beijerinckii* NCIMB 8052 were selected in continuous cultures, they degenerated into solvent nonproducing strains (Woolley and Morris, 1990). These mutants spontaneously reverted at a low frequency to the parental phenotype (positive for sporulation, granulose synthesis, and solvent production), indicating that the multiple loss of capacities is the pleiotrophic consequence of a lesion in some global regulatory gene. The discovery of the involvement of the Spo0A protein in the initiation of solvent production could explain the pleiotrophic effect of a single mutation on both solvent production and sporulation (see the subsection Relationship to Sporulation in this Chapter). However, because the molecular change(s) causing degeneration has not been characterized, it is still not known precisely how strain degeneration occurs.

Maintenance of cells in the vegetative state for a prolonged period, either by serial transfers or by continuous cultures, results in cultures of predominantly degenerated cells. During the transition into a culture of degenerated cells, the cell density and the rate of sugar metabolism decrease (Stephens et al., 1985). Interestingly, by maintaining a high cell density in a continuous culture (Stephens et al., 1985) or during serial transfers (Chen and Blaschek, 1999b), the rate of degeneration decreased. The turbidometric technique (Stephens et al., 1985) or the addition of acetate (Chen and Blaschek, 1999b) was used to maintain a high cell density. A mutant strain of *C. beijerinckii* NCIMB 8052 with a truncated peptide deformylase has a slower growth rate and a decreased tendency to undergo degeneration (Evans et al., 1998). The procedures for reducing the rate of degeneration, together with the identification of Spo0A as a key regulator for the initiation of solvent production, should help efforts to elucidate the mechanisms of degeneration and the solventogenic switch.

When compared with the parental strains, the degenerated cells exhibited several different physical and morphological characteristics that may facilitate the detection of degeneration during the preparation of seed cultures for an industrial fermentation. The degenerated cells of *C. beijerinckii* and *C. saccharobutylicum* tend to give larger and translucent colonies with an irregular shape (Adler and Crow, 1987; Woolley and Morris, 1990; Schuster et al., 2001), a longer but thinner cell shape (Adler and Crow, 1987), and a characteristic infrared spectrum (Fourier transform IR) that is distinguishable from the spectra of parental cells at different stages of growth (Schuster et al., 2001).

Metabolic Pathways and Enzymology of Solvent Production The general features of the metabolic pathways (Fig. 26) for solvent production have been known for some time (Rogers, 1986; Jones and Woods, 1986). The enzymology of solvent production was studied mostly with *C. acetobutylicum* and *C. beijerinckii* (Chen, 1993; Bennett and Rudolph, 1995; Dürre and Bahl, 1996), but because of the multiplicity of alcohol and aldehyde dehydrogenases in the solvent-producing clostridia (Chen, 1995), the specific role for each alcohol or aldehyde dehydrogenase in the formation of butanol or ethanol is still a subject of research. The genes encoding the specific solvent-forming enzymes (14–20 in Fig. 26) and the enzymes for the conversion of acetyl-CoA to butyryl-CoA (4–7 in Fig. 26), which are required for both acid and solvent production, have been cloned and sequenced (see below and the subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter). The specific acid-forming reactions (8–11 in Fig. 26) are also important to solvent production because the accumulation of acetate and butyrate during the exponential phase of growth plays a regulatory role in the solventogenic switch (see the subsection Physiology of Solvent Production in this Chapter) and the accumulated acetate and butyrate are reutilized through the activity of the CoA-transferase (16 in Fig. 26) essential to acetone/isopropanol formation.

In the acid- and solvent-producing pathways, three CoA-derivatives (acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA) occupy branch points, and enzymes acting on these branch-point intermediates likely play a pivotal role in regulating the flux of carbon into each specific branch and end product. Three enzymes (thiolase, enzyme 4 in Fig. 26; phosphotransacetylase, enzyme 10; and aldehyde (acetaldehyde) dehydrogenase,

enzyme 19) react with acetyl-CoA, and their relative activities control the amount of acetyl-CoA that is available for the C_2 products (ethanol and acetate) or the C_4 intermediate acetoacetyl-CoA, which is the precursor for acetone/isopropanol, butanol and butyrate. Two enzymes (3-hydroxybutyryl-CoA dehydrogenase, enzyme 5; and acetoacetate:acetate/butyrate CoA-transferase, enzyme 16) react with acetoacetyl-CoA and control the amounts of C_3 (acetone/isopropanol) and C_4 (butanol and butyrate) products formed. Two enzymes (phosphotransbutyrylase, enzyme 8; and aldehyde [butyraldehyde] dehydrogenase, enzyme 14) react with butyryl-CoA and control the amounts of butanol and butyrate produced. The acid- and solvent-producing reactions are linked to ATP formation and $NAD(P)^+$ regeneration. Therefore, the substrate and product concentrations, the energy and redox states, and other cellular conditions can all affect the activities of the branch-point enzymes. Knowledge about the regulatory properties of these enzymes is useful in attempts to control the product ratio of solvent fermentation. The results of in vitro kinetic measurements predict that the activities of these enzymes are subject to regulation by pertinent metabolic intermediates, but in vivo data are difficult to obtain (Chen, 1993; Bennett and Rudolph, 1995; Dürre and Bahl, 1996). The following is a summary of the properties of these enzymes and their structural genes.

Thiolase The condensation of two acetyl-CoA molecules into acetoacetyl-CoA by thiolase (acetyl-CoA:acetyl-CoA *C*-acetyltransferase; enzyme 4 in Fig. 26) is an essential step for the formation of the C_4 precursor for acetone/isopropanol, butanol and butyrate. The reaction is therefore required for both the acid- and the solvent-producing stages of growth. Thiolase has been purified to different degrees of purity from several *Clostridium* species (Chen, 1993; Bennett and Rudolph, 1995). From *C. acetobutylicum* ATCC 824, thiolase has been purified 70-fold to homogeneity, and it has a subunit mol. wt. (MW) of 44 kDa and a native MW indicative of a homotetrameric structure (Wiesenborn et al., 1988). The purified thiolase is sensitive to inhibition by micromolar levels of CoASH in the direction of acetyl-CoA condensation, and it is also inhibited by ATP and butyryl-CoA. The *C. acetobutylicum* thiolase shows a high activity in the pH range of 5.5 to 7.0. It has been suggested that the relative amounts of CoASH and acetyl-CoA regulate the activity of thiolase (Wiesenborn et al., 1988).

The structural gene for the purified thiolase of *C. acetobutylicum* ATCC 824 has been cloned and sequenced (Stim-Herndon et al., 1995), and it encodes a polypeptide of 392 amino acids (calculated MW 41,237). The genome of *C. acetobutylicum* ATCC 824 contains two thiolase genes, with *thlA* located on the chromosome (CAC2873, denoting the gene number on the annotated chromosome sequence) and *thlB* located on the plasmid pSOL1 (CAP0078, denoting the gene number on the annotated plasmid sequence). The *thlA* gene encodes the purified thiolase, and its level of transcription reached a transient minimum 3–7 h after a continuous culture of *C. acetobutylicum* DSM 792 was induced for solvent production (Winzer et al., 2000). The *thlB* gene had a very low level of expression in both acid- and solvent-producing cells, and its physiological function is unknown. The transient decrease in the level of transcription of the *thlA* gene between the acid- and solvent-producing stages of growth may explain the observed variation in the level of thiolase activity in acid- and solvent-producing cells (Chen, 1993).

3-Hydroxybutyryl-CoA Dehydrogenase In the solvent-producing clostridia, 3-hydroxybutyryl-CoA dehydrogenase (β-hydroxybutyryl-CoA dehdyrogenase; enzyme 5) catalyzes the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. The enzyme has been purified from *C. beijerinckii* NRRL B593, and it has subunit and native MWs of 30.8 and 213 kDa, respectively (Colby and Chen, 1992). The enzyme can use either reduced nicotinamide adenine dinucleotide (NADH) or NADPH as a cosubstrate, but NADH gives a 60-fold higher catalytic efficiency and is likely the physiological cosubstrate. The structural gene (*hbd*) for the enzyme of three solvent-producing clostridia has been sequenced. The length (number of amino acid residues) of the deduced polypeptide and its calculated MW are: *C. acetobutylicum* {ATCC824}, 282 and 30,500 (Boynton et al., 1996a); *C. beijerinckii* NRRL B593, 281 and 30,167 (J. Toth and J.-S. Chen, GenBank accession number {AF494018}); and *C. saccharobutylicum* NCP262 (formerly *C. acetobutylicum* P262), 282 and 31,435 (Youngleson et al., 1989). The *C. acetobutylicum hbd* gene is designated CAC2708 on the annotated genome sequence. The amino acid sequence of 3-hydroxybutyryl-CoA dehydrogenase among these three species is highly conserved throughout the length of the polypeptide, with the *C. beijerinckii* enzyme shortened by one amino acid at the C-terminus (J.-S. Chen and J. Toth, unpublished results). The *hbd* gene is part of the BCS (butyryl-CoA synthesis) operon (see the subsection Genes and Operons for Solvent Production and the Genome for Solvent-Producing Bacteria in this Chapter).

Crotonase In the solvent-producing clostridia, crotonase (enoyl-CoA hydratase; enzyme 6 in

Fig. 26) catalyzes the dehydration of 3-hydroxybutyryl-CoA to form crotonyl-CoA. Crotonase has been purified 131-fold (67% yield) to homogeneity from *C. acetobutylicum* (probably strain NRRL B528, as spores were provided by I. Fridovich whose laboratory used this strain; Waterson et al., 1972). The purified enzyme is active only toward C_4 and C_6 substrates, and it has a native MW of 158 kDa and a subunit MW of 40 kDa. The predicted structural gene (*crt*; CAC2712) for *C. acetobutylicum* ATCC824 encodes a polypeptide of 261 amino acids, with a predicted MW of 28.2 kDa (Boynton et al., 1996a). The predicted *crt* gene of *C. beijerinckii* NRRL B593 also encodes a polypeptide of 261 amino acids (predicted MW 28,180; J. Toth and J.-S. Chen, GenBank accession number {AF494018}). The *crt* genes of *C. acetobutylicum* {ATCC824} and *C. beijerinckii* NRRL B593 occupy a similar location in the BCS operon, which consists of five genes for the synthesis of butyryl-CoA (see the subsection Genes and Operons for Solvent Production and the Genome for Solvent-Producing Bacteria in this Chapter).

Butyryl-CoA Dehydrogenase In the solvent-producing clostridia, butyryl-CoA dehydrogenase (enzyme 7 in Fig. 26) catalyzes the reduction of crotonyl-CoA to butyryl-CoA. Butyryl-CoA dehydrogenase has not been purified from the solvent-producing clostridia, and results from studies using cell-free extracts of the clostridia suggest that the immediate electron donor for the enzyme is not NADH or NADPH (Chen, 1993; Boynton et al., 1996a). The structural gene (*bcd*) for the butyryl-CoA dehydrogenase of *C. acetobutylicum* and *C. beijerinckii* has been identified from its conserved amino acid sequence and location in the BCS operon (see the subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter). The predicted *bcd* gene (CAC2711) of *C. acetobutylicum* ATCC824 encodes a polypeptide of 379 amino acids (predicted MW 41,386; Boynton et al., 1996a). The predicted *bcd* gene of *C. beijerinckii* NRRL B593 also encodes a polypeptide of 379 amino acids (predicted MW 41,045; J. Toth and J.-S. Chen, GenBank accession number {AF494018}).

Two open-reading frames (CAC2709 and CAC2710) are present between the *bcd* and *hbd* genes of the BCS operon of *C. acetobutylicum* {ATCC824}, and they were identified as the *etfA* (syn. *fixB*) and *etfB* (syn. *fixA*) genes that code for the subunits of an electron-transferring flavoprotein (ETF; Boynton et al., 1996a). Two corresponding ORFs occur between the *bcd* and *hbd* genes of the BCS operon of *C. beijerinckii* NRRL B593 (J. Toth and J.-S. Chen, GenBank accession number {AF494018}) or preceding the *hbd* gene in *C. saccharobutylicum* NCP262 (formerly *C. acetobutylicum* P262; Youngleson et al., 1995). The ETF of the butyric acid-producing anaerobic bacterium *Megasphaera elsdenii* has been purified and characterized (Whitfield and Mayhew, 1974), and it mediates electron-transfer between NADH and butyryl-CoA dehydrogenase. The *M. elsdenii* ETF is a heterodimer, with the *etfA* and *etfB* genes encoding, respectively, the α- (338 amino acids; MW 36,101) and β- (270 amino acids; MW 29,081) subunits (O'Neill et al., 1998). The *etfA*- and *etfB*-encoded polypeptides of the solvent-producing clostridia are similar in size and amino acid sequence to, respectively, EtfA and EtfB of *M. elsdenii*. It may be postulated that in the solvent-producing clostridia, the *etfA*- and *etfB* genes encode an ETF that is the electron donor for the butyryl-CoA dehydrogenase.

Aldehyde Dehydrogenase Aldehyde dehydrogenase (ALDH; enzymes 14 and 19 in Fig. 26) is responsible for the formation of butyraldehyde from butyryl-CoA and acetaldehyde from acetyl-CoA for the production of, respectively, butanol and ethanol. Different ALDH activities can be measured in a strain of solvent-producing clostridia (Bertram et al., 1990), and the ALDH activity can reside in distinct proteins in different species of solvent-producing clostridia (Toth et al., 1999). A biochemically similar ALDH has been purified from *C. saccharobutylicum* NRRL B643 (formerly *C. acetobutylicum* NRRL B643; Palosaari and Rogers, 1988) and *C. beijerinckii* strains NRRL B592 (Yan and Chen, 1990) and NRRL B593 (Toth et al., 1999). The ALDH from these two species has a native MW of 100–115 kDa and a subunit MW of 55–56 kDa. NADH is a more efficient cosubstrate than NADPH, and butyryl-CoA is a better substrate than acetyl-CoA for these ALDHs. The gene (*ald*) encoding the ALDH has been cloned from two strains of *C. beijerinckii* and sequenced (Toth et al., 1999; Hong, 1999). The *ald* gene of both *C. beijerinckii* strain NRRL B592 and strain NRRL B593 encodes a polypeptide of 468 amino acids, with a predicted mol. wt. of 51,312 (strain NRRL B592) or 51,353 (strain NRRL B593). A probe derived from the *C. beijerinckii ald* gene hybridized to restriction fragments of the genomic DNA of all tested strains of *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* but not of *C. acetobutylicum* (Toth et al., 1999). The *ald* gene is part of the solvent production operon of *C. beijerinckii* (see the subsection Genes and Operons for Solvent Production and the

Genome of Solvent-Producing Bacteria in this Chapter).

Cloning and sequencing of the solvent-production genes of *C. acetobutylicum* strains ATCC 824 and DSM 792 led to the discovery of the *aad*/*adhE* gene (CAP0162) in the solvent-production operon (Fischer et al., 1993; Nair et al., 1994). The *aad*/*adhE* gene is located on the naturally occurring plasmid pSOL1 (see the subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter for the genome structure) and encodes a polypeptide of 862 amino acids (predicted MW 95.2 kDa). The *aad*/*adhE*-encoded polypeptide is related to the AdhE protein of *E. coli* (see "Ethanol" in Organic Acid and Solvent Production, Part II in this Volume) and was therefore proposed as a fused aldehyde-alcohol dehydrogenase (Fischer et al., 1993; Nair et al., 1994), with residue 400 roughly the end of the aldehyde dehydrogenase domain and residue 450 roughly the start of the alcohol dehydrogenase domain. The Aad protein of *C. acetobutylicum* ATCC 824 has been purified to homogeneity (Ismaiel and Chen, 1998), and it has a native MW of 1.5 MDa or above and an observed subunit MW of 97.4 kDa. With NADH as a coenzyme, the purified Aad protein has a 400 times higher aldehyde dehydrogenase activity (with butyryl-CoA) than alcohol dehydrogenase activity (with butyraldehyde) on the basis of the catalytic efficiency or the V_{max}/K_m value (A. I. Ismaiel and J.-S. Chen, unpublished data). A DNA probe encompassing amino acid residues 278 to 556 of the *aad*-encoded polypeptide hybridized to restriction fragments of *C. acetobutylicum* genomic DNA but not to those of three other solvent-producing species of clostridia, suggesting that the Aad/AdhE protein is a source of ALDH activity in *C. acetobutylicum* (Toth et al., 1999).

Clostridium acetobutylicum ATCC 824 contains a second *adhE*-like gene (*adhE*2; CAP0035) on the pSOL1 plasmid, and the *adhE2* gene encodes a polypeptide of 858 amino acids (MW 94.4 kDa), which has a 66.1% identity with the AdhE of *C. acetobutylicum* ATCC 824 (Fontaine et al., 2002). The *adhE*2-encoded protein (AdhE2) is a NADH-dependent aldehyde-alcohol dehydrogenase responsible for butanol production under the alcohologenic condition (see the subsection Physiology of Solvent Production in this Chapter). The AdhE2 protein has been fused with *Strep*-tag II, overexpressed in *E. coli* and purified (Fontaine et al., 2002). Like the purified AdhE, the purified *Strep*-tag II-AdhE2 protein also exhibited higher NADH-dependent butyraldehyde dehydrogenase (0.74 units/mg) than NADH-dependent butanol dehydrogenase (0.18 units/mg) activities.

Alcohol Dehydrogenase Alcohol dehydrogenase (ADH; enzymes 15, 18 and 20 in Fig. 26) is a ubiquitous enzyme, and multiple forms of ADHs, with different molecular structures and physiological roles, are usually found in an organism. Although most ADHs accommodate substrates with different carbon-chain lengths, they generally display a specificity for either primary alcohols (and aldehydes) or secondary alcohols (and ketones), and they show a higher catalytic efficiency (based on the ratio $[k_{cat}]/K_m$) with NAD, NADH, NADP or NADPH as the coenzyme. For the production of butanol, isopropanol and ethanol by the solvent-producing clostridia, ADH catalyzes the final reaction of the respective pathways, and the presence of multiple ADHs may be expected.

The multiplicity of ADH and the relatedness of the ADHs in the solvent-producing clostridia have been reviewed (Chen, 1995). Besides the different primary ADHs, a novel primary-secondary ADH has been purified from two strains of *C. beijerinckii* that produce both butanol and isopropanol (Ismaiel et al., 1993). The primary-secondary ADH of *C. beijerinckii* NRRL B593 is a tetramer of identical subunits (351 amino acids), NADPH-dependent, and equally active in reducing aldehydes and ketones to, respectively, primary and secondary alcohols. Therefore, the primary-secondary ADH is sufficient for the production of both butanol and isopropanol. The gene for the primary-secondary ADH of *C. beijerinckii* NRRL B593 has been cloned and sequenced (Peretz et al., 1997), and the X-ray crystallographic structure of the ADH has been solved (Korkhin et al., 1998).

The solvent-producing clostridia have both NADH-dependent and NADPH-dependent primary ADHs (Dürre et al., 1987; Hiu et al., 1987; Youngleson et al., 1988; Welch, 1991; Yan, 1991). Although NADPH-dependent primary ADHs have been separated from NADH-dependent primary ADHs (Welch, 1991; Yan, 1991), only the NADH-dependent primary ADHs have been purified from *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B592. From *C. acetobutylicum* ATCC 824, four enzymes with primary ADH activities have been purified: the butanol dehydrogenases I and II (BDH I and BDH II), with a native MW slightly higher than 80 kDa and a subunit MW of 42 kDa (Welch et al., 1989; Petersen et al., 1991), and the much larger aldehyde-alcohol dehydrogenases (Aad or AdhE and AdhE2; see the subsection Aldehyde Dehydrogenase for properties of Aad/AdhE and AdhE2 in this Chapter).

BDH I and BDH II of *C. acetobutylicum* ATCC824 are encoded, respectively, by *bdhA* (CAC3299) and *bdhB* (CAC3298), which are contiguous genes but are transcribed separately

(Petersen et al., 1991; Walter et al., 1992). It should be noted that, on the basis of the N-terminal amino acid sequence, the BDH I (NH_2-Met-Leu-Ser-Phe-) described in Walter et al. (1992) and in the annotation of the *C. acetobutylicum* genome sequence was designated "BDH II" (which binds to Blue-Sepharose) by Welch (1991). The *bdhA* and *bdhB* genes code for polypeptides of 389 (MW 43,039) and 390 (MW 43,227) amino acid residues, respectively (Walter et al., 1992). The amino acid sequences of BDH I and BDH II have a 72.9% positional identity. BDH II of Welch (Welch et al., 1989; Welch, 1991) has a 46-fold greater activity with butyraldehyde than acetaldehyde, whereas BDH I (which does not bind to Blue Sepharose) has only a twofold greater activity with the C_4 substrate than with the C_2 substrate.

Clostridium beijerinckii NRRL B592, a strain not producing isopropanol, has three NADH-dependent primary ADHs (Chen, 1995). These three ADHs, designated "ADH-1," "ADH-2" and "ADH-3," are homo- and heterodimers of subunits encoded by the *adhA* gene (388 amino acids; MW 42,617; J. Toth, A. Ismaiel, and J.-S. Chen, GenBank accession number {AF497741}) and the *adhB* gene (388 amino acids; MW 42,715; J. Toth and J.-S. Chen, GenBank accession number {AF497742}). The *adhA*- and *adhB*-encoded polypeptides differ by only 13 amino acids (3%), and except for the C-terminal 40 residues, the *C. beijerinckii* AdhA and AdhB sequences are highly similar to that of Adh-1 of *C. saccharobutylicum* NCP 262 (J. Toth et al., unpublished data). The *adhA*- and *adhB* genes of *C. beijerinckii* NRRL B592 are not contiguous, and they have unrelated flanking sequences. Thus, although the NADH-dependent primary ADHs of the solvent-producing clostridia are structurally related (Chen, 1995), their positions in the respective genomes are different.

Acetoacetate : Acetate/Butyrate CoA-transferase The enzyme (16 in Fig. 26) catalyzes the reversible transfer of the CoA moiety between an acyl-CoA and a carboxylic acid. The physiological reaction of the CoA-transferase, which is synthesized when cells enter the solventogenic phase, is to convert acetoacetyl-CoA into acetoacetate, with acetate or butyrate as the CoA acceptor. Therefore, the activity of the CoA-transferase results in the reutilization of preformed acetate and butyrate, and the CoA-derivatives of the two acids enter the acid- or solvent-producing pathways. When butyrate is the CoA-acceptor, reaction 16 leads to the production of one acetone (or isopropanol) and one butanol or butyrate. When acetate is the CoA-acceptor, the resulting acetyl-CoA will enter the solventogenic or butyrate-forming pathway through the condensation reaction catalyzed by thiolase (enzyme 4 in Fig. 26), and the acetoacetyl-CoA so produced can be used for the production of either acetone/isopropanol (via CoA-transferase) or butanol/butyrate (via 3-hydroxybutyryl-CoA dehydrogenase).

CoA-transferase has been purified from *C. acetobutylicum* ATCC 824 (Wiesenborn et al., 1989a) and *C. beijerinckii* NRRL B593 (Colby, 1993). During purification, the CoA-transferase of these two species requires high concentrations of ammonium sulfate (0.5–0.75 M) and glycerol (15–20%, vol/vol) at pH 7 to preserve activity. The CoA-transferase of *C. acetobutylicum* ATCC 824 has unusually high K_m values for acetate (1.2 M) and butyrate (0.66 M); whereas the enzyme from *C. beijerinckii* NRRL B593 has K_m values of 0.5 M for acetate and 10 mM for butyrate. The K_m values for acetoacetyl-CoA range between 21 and 56 μM with acetate or butyrate as the cosubstrate for the CoA-transferases from both species (Chen, 1993).

The CoA-transferase of *C. acetobutylicum* and *C. beijerinckii* has a native MW of 85–93 kDa and appears to be a tetramer composed of two types of subunits. The structural genes (*ctfA* and *ctfB*) for the subunits of CoA-transferase have been cloned from *C. acetobutylicum* strains ATCC 824 (Petersen et al., 1993) and DSM 792 (Fischer et al., 1993) and from *C. beijerinckii* strains NRRL B592 (Hong, 1999) and NRRL B593 (J. Toth et al., GenBank accession number {AF157306}). The *ctfA* and *ctfB* genes are conserved in the two species, and they are flanked by similar solvent-production genes in the two species. However, the actual flanking gene or the orientation of the flanking gene differs in the two species (see the subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter). The *C. acetobutylicum* ATCC 824 *ctfA* gene (CAP 0163) encodes a polypeptide of 218 amino acids (MW 23,643), and the *ctfB* gene (CAP 0164) encodes a polypeptide of 221 amino acids (MW 23,626). The *ctfA* and *ctfB* genes of *C. beijerinckii* strain NRRL B593 encode polypeptides of, respectively, 217 amino acids (MW 23,231) and 221 amino acids (MW 23,710). The *ctfA* and *ctfB* genes of *C. beijerinckii* strains NRRL B592 are very similar to the respective genes of *C. beijerinckii* strain NRRL B593 (Hong, 1999).

Acetoacetate Decarboxylase Acetoacetate decarboxylase (AADC; enzyme 17 in Fig. 26) catalyzes the irreversible conversion of acetoacetate to acetone and CO_2. AADC is thus a crucial enzyme for solvent production because of its consumption of acetoacetate to allow the CoA-transferase to continue to channel preformed

butyrate and acetate into solvent production. AADC of *C. acetobutylicum* strains BY (the Weizmann strain) and NRRL B528 is a stable protein and has been extensively studied, and the literature has been briefly reviewed (Chen, 1993).

AADC of *C. acetobutylicum* is an oligomeric protein (native MW about 300–330 kDa; Hamilton and Westheimer, 1959; Gerischer and Dü, 1990). Its structural gene (*adc*; CAP 0165) encodes a polypeptide of 244 amino acids (calculated MW 27,519; Gerischer and Dü, 1990; Petersen et al., 1993). The *adc* gene of *C. beijerinckii* strains NRRL B592 and NRRL B593 encodes a polypeptide of 246 amino acids (calculated MWs 27,392 and 27,353, respectively) for the two strains (Hong, 1999; J. Toth and J.-S. Chen, GenBank accession number {AF157305}).

Phosphotransbutyrylase and Butyrate Kinase

Phosphotransbutyrylase (PTB; enzyme 8 in Fig. 26) catalyzes the conversion between butyryl-CoA and butyryl phosphate, whereas butyrate kinase (BK; enzyme 9 in Fig. 26) catalyzes the conversion between butyryl phosphate and butyrate and ATP (Chen, 1993). PTB and BK are thus key enzymes for the formation of butyric acid. Theoretically, PTB and BK can catalyze the ATP-dependent reutilization of preformed butyrate for solvent production (Husemann and Papoutsakis, 1989), but direct evidence for any significant role played by PTB and BK in this respect is still lacking.

PTB has been purified from *C. acetobutylicum* ATCC 824 (Wiesenborn et al., 1989b) and *C. beijerinckii* NRRL B593 (Thompson and Chen, 1990), and the two purified PTBs are similar in native MW (264 versus 205 kDa), subunit MW (31 versus 33 kDa), and in the K_m for butyryl-CoA (0.11 versus 0.04 mM) and phosphate (14 versus 6.5 mM). The PTB of *C. beijerinckii* NRRL B593 also reacts with acetoacetyl-CoA (with a K_m of 1.1 mM) in the presence of phosphate, and coenzyme A-SH (CoASH) is a product of the reaction (Thompson and Chen, 1990).

PTB is encoded by the *ptb* gene. The *ptb* gene of *C. acetobutylicum* ATCC 824 (CAC 3076) encodes a polypeptide of 296 amino acids (calculated MW 31,546; Walter et al., 1993), whereas the *ptb* gene of *C. beijerinckii* NCIMB 8052 encodes a polypeptide of 302 amino acids (calculated MW 32,445; Oultram et al., 1993). PTB appears to be a hexamer or an octamer of identical subunits. The *ptb* gene follows a *buk* gene (for BK) to form an operon in these two organisms. In *C. acetobutylicum* ATCC 824, a second *buk* gene (CAC 1860) is present (Huang et al., 2000); the gene is expressed but its physiological significance is unknown.

Both butyrate kinases of *C. acetobutylicum* ATCC 824 have been purified. The first butyrate kinase (BK-I) was purified from *C. acetobutylicum* ATCC 824 (Hartmanis, 1987), whereas the second was purified from a recombinant *E. coli* expressing BK-II (Huang et al., 2000). The kinetic properties of BK-I (native MW 85 kDa) were measured in the acyl phosphate-forming or reverse direction. The *buk* gene for BK-I (CAC 3075) of *C. acetobutylicum* ATCC 824 encodes a polypeptide of 355 amino acids (calculated MW 39,002; Walter et al., 1993). The *buk* gene of *C. beijerinckii* NCIMB 8052 also encodes a polypeptide of 355 amino acids (calculated MW 38,438; Oultram et al., 1993).

It has been speculated that a decrease in the flux of butyryl-CoA going into butyrate formation may favor the production of butanol. Use of the antisense RNA technique to decrease the expression of either the BK or the PTB gene in *C. acetobutylicum* caused a lowering of both PTB and BK activities (but to a different degree), yet it did not have any significant impact on butyrate formation, suggesting that butyrate formation is not controlled by the levels of PTB and BK (Desai and Papoutsakis, 1999). However, the antisense RNA against the PTB gene caused an increase in solvent production, whereas the antisense RNA against the BK gene caused a decrease in solvent production, although both caused decreases in PTB and BK activities. A *C. acetobutylicum* mutant (PJC4BK) with the gene for BK inactivated showed superior solvent productivity between pH 5.0 and 5.5 (Harris et al., 2000), whereas it exhibited good solvent productivity at pH greater than or equal to 5.5 (Green et al., 1996b). Therefore, a decrease in the BK activity by any of these three manipulations had very different effects on the solvent productivity, which perhaps illustrates the complexity of the mechanisms regulating solvent production.

Phosphotransacetylase and Acetate Kinase

Phosphotransacetylase (PTA; enzyme 10 in Fig. 26) and acetate kinase (AK; enzyme 11 in Fig. 26) catalyze consecutive reactions analogous to those catalyzed, respectively, by phosphotransbutyrylase (PTB) and butyrate kinase (BK). Thus, PTA catalyzes the conversion between acetyl-CoA and acetyl phosphate, and AK catalyzes the conversion between acetyl phosphate and acetate and ATP (Chen, 1993). The two enzymes PTA and AK are potentially involved in ATP-dependent reutilization of preformed acetate during solvent production; however, like the situation involving PTB and BK, direct evidence in support of such a role is lacking.

PTA and AK activities have been measured in cell-free extracts of solvent-producing clostridia, and these activities can be separated from those

attributable to PTB and BK, respectively (Chen, 1993). PTA has only been partially purified from *C. beijerinckii* (Chen, 1993), whereas AK has been purified from *C. acetobutylicum* DSM 1731 (Winzer et al., 1997). AK of *C. acetobutylicum* DSM 1731 has a native MW of 87–94 kDa and a measured subunit MW of 43 kDa. The K_m values for acetyl phosphate, Mg-ADP, acetate, and Mg-ATP are, respectively, 0.58, 0.71, 73, and 0.37 mM. Results of Northern blot analysis show that there is no significant difference in the transcription of the acetate kinase gene (*ack*) in cells of *C. acetobutylicum* DSM 1731 under acid- and solvent-producing conditions (Winzer et al., 1997).

The *C. acetobutylicum* ATCC824 genes for PTA (*pta*; CAC 1742) and AK (*ack*; CAC 1743) have been cloned and sequenced, and the *pta* gene precedes the *ack* gene in forming an operon (Boynton et al., 1996b). The *pta* gene encodes a polypeptide of 333 amino acids (calculated MW 36.2 kDa), whereas the *ack* gene encodes a polypeptide of 401 amino acids (calculated MW 44.3 kDa).

Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria As shown in Fig. 26, the enzymes catalyzing reactions 16–18 (between acetoacetyl-CoA and acetone or isopropanol) and the enzymes catalyzing reactions 14 and 15 (between butyryl-CoA and butanol) are specifically required for solvent production. The genes encoding these enzymes (*ctfA* and *ctfB* for acetoacetate:butyrate/acetate CoA-transferase, *adc* for acetoacetate decarboxylase, and *ald* or *aad*/*adhE* for aldehyde and alcohol dehydrogenase) form monocistronic or polycistronic operons. All characterized strains of solvent-producing clostridia have a gene cluster, which contains the acetone-forming genes and most of the butanol-forming genes.

In *C. acetobutylicum* strains ATCC 824 and DSM 792, the main gene cluster for solvent production consists of two converging operons (Dürre et al., 2002) of the following organization (arrowheads indicating the direction of translation):

aad/*adhE* ►-R *ctfA* ►-R *ctfB* ►-R ◄-l *adc*

The solvent-production or "*sol*" operon contains the *aad*/*adhE* and the *ctf* genes, whereas the *adc* operon is monocistronic. The *aad*/*adhE* gene and the separately transcribed *adc* operon are characteristics of this species (Hong, 1999; Toth et al., 1999). Transcriptional regulation of the solvent-production genes of *C. acetobutylicum* has been reviewed (Dürre et al., 2002), and the Spo0A protein is a significant component of the regulatory networks for solventogenesis (Harris et al., 2002; Thormann et al., 2002).

In *C. beijerinckii* strains NRRL B592 and NRRL B593, the *ald* gene which encodes an aldehyde dehydrogenase precedes the *ctfA* gene to give a solvent-production operon of the following organization (Hong, 1999; Toth et al., 1999; Toth, J. and J.-S. Chen, GenBank accession numbers {AF157305} and {AF157306}):

ald ►-R *ctfA* ►-R *ctfB* ►-R *adc* ►-R

Both *C. acetobutylicum* and *C. beijerinckii* have genes located elsewhere for the multiple alcohol dehydrogenases. Whereas the *sol* and *adc* operons of *C. acetobutylicum* are located on the pSOL1 plasmid, the contiguous but monocistronic *bdhA* and *bdhB* genes are located on the chromosome (see subsection Metabolic Pathways and Enzymology of Solvent Production in this Chapter). *Clostridium beijerinckii* NRRL B592 has *adhA* and *adhB* genes, which encode the subunits of three dimeric isozymes of primary alcohol dehydrogenases, and the *adhA* and *adhB* genes are not contiguous genes (GenBank accession numbers {AF497741} and {AF497742}). The isopropanol-producing *C. beijerinckii* NRRL B593 has an *adh* gene encoding the primary-secondary alcohol dehydrogenase (Peretz et al., 1997). Although the *adhA* gene of *C. beijerinckii* NRRL B592 and the *adh* gene of *C. beijerinckii* NRRL B593 are structurally unrelated, both of them are preceded by an *stc* gene (Peretz et al., 1997; Hong, 1999; GenBank accession numbers {AF157307} and {AF497741}), which could encode an enhancer-binding protein belonging to the NtrC family (Osuna et al., 1997), and are followed by a *hydG* gene (Pedroni et al., 1995; Rakhley et al., 1999), which could encode an electron-transfer subunit of a redox enzyme. The conserved location of the *stc* gene relative to the *adh* or *adhA* gene in the two strains of *C. beijerinckii* suggests a regulatory role for the *stc* gene in the expression of the *adh* or *adhA* gene, but this relationship remains to be established.

The enzymes catalyzing reactions 5–7 (between acetoacetyl-CoA and butyryl-CoA) are required for the formation of both butyric acid and butanol (Chen, 1993). The five genes encoding these enzymes (*hbd* for 3-hydroxybutyryl-CoA dehydrogenase, *crt* for crotonase, and *bcd* for butyryl-CoA dehydrogenase) and an electron-transfer protein (*etfA* and *etfB*) form the "*bcs*" operon (Boynton et al., 1996a; Toth, J. and J.-S. Chen, GenBank accession number {AF494018}). The genes of the *bcs* operon are conserved in *C. acetobutylicum* and *C. beijerinckii* and have the following organization:

crt ►-R *bcd* ►-R *etfB* ►-R *etfA* ►-R *hbd* ►-R

Because the proteins encoded by these genes are needed for both primary and secondary metabolism, the promoter region of the *bcs* operon must differ from that for the *sol* operon.

In *C. saccharobutylicum* NCP 262, the following arrangement of solvent production-related genes has been described (Youngleson et al., 1995):

etfB ►-R *etfA* ►-R *hbd* ►-R *adh-1* ►-R

The *adh-1* gene encodes an NADPH-linked alcohol dehydrogenase, and its transcription peaks shortly before the onset of solvent production, suggesting the functioning of other *adh* genes later in the growth cycle (Youngleson et al., 1995). The *etfA* and *hbd* genes (the DNA sequence preceding the *etfB* gene has not been reported) are transcribed during both acid- and solvent-producing phases of growth.

Among the alcohol dehydrogenases of the solvent-producing clostridia, the enzymes encoded by the *bdhA* and *bdhB* genes of *C. acetobutylicum* ATCC 824, the *adhA* and *adhB* genes of *C. beijerinckii* NRRL B592, and the *adh-1* gene of *C. saccharobutylicum* NCP 262 are related (Walter et al., 1992; Chen, 1995; Toth, J. et al., unpublished results; GenBank accession numbers {AF497741} and AF497742}). The roles of these *adh* genes in butanol formation will need further clarification.

The Genome of Clostridium acetobutylicum *ATCC824* The genome of *C. acetobutylicum* consists of a chromosome of about 4 Mb and a megaplasmid of about 200 kb (Cornillot and Soucaille, 1996; Cornillot et al., 1997a; Cornillot et al., 1997b). The genome sequence of *C. acetobutylicum* ATCC 824 has been determined (Nolling et al., 2001). The chromosome is 3,940,880 bp in length (GenBank accession number {AE001437}), with a total of 3,740 polypeptide-encoding ORFs and 107 stable RNA genes having been identified and accounting for 88% of the chromosomal DNA. The average length of the intergenic regions is about 121 bp. The megaplasmid, pSOL1, is 192,000 bp in length (GenBank accession number {AE001438}) and appears to encode 178 polypeptides. There appear to be two unrelated cryptic prophages in the chromosome. The first spans about 90 kb and includes about 85 genes (CAC1113 to CAC1197), whereas the second spans about 60 kb and includes about 79 genes (CAC1878 to CAC1957). Genes for three distinct insertion sequence-related proteins are present on the chromosome, but only one of these is intact. It is believed that no active insertion sequence elements are present in the *C. acetobutylicum* genome.

Four genes (*aad*/*adhE*, *ctfA*, *ctfB* and *adc*; CAP0162 to CAP0165), which are required for butanol- and acetone-production under acidic growth conditions, occur as two converging operons on the pSOL1 plasmid. The pSOL1 plasmid also contains the second copy of the aldehyde-alcohol dehydrogenase gene (*adhE2*; CAP0035), which is required for butanol production under the neutral or alcohologenic growth conditions (Fontaine et al., 2002). In addition, the pSOL1 plasmid contains genes for a pyruvate decarboxylase (CAP0025) and an alcohol dehydrogenase (CAP0059), whose physiological roles have not been determined. The genes for two characterized alcohol dehydrogenases (CAC3298 and CAC3299) are present on the chromosome, and one or both of them may function during butanol production (Dürre et al., 2002).

On the basis of the genome sequence, the number of recognizable sporulation genes is smaller in *C. acetobutylicum* than in *Bacillus subtilus*. Some of the Spo0 and SpoV genes have not been detected in *C. acetobutylicum*, but the gene encoding an apparent ortholog of the Spo0A protein (CAC2071) has been identified. The number of identified genes for spore coat biosynthesis, spore germination, and septum formation is also smaller in *C. acetobutylicum* than in *B. subtilis* (Nolling et al., 2001), but it is not known how much of the difference can be attributed to mechanistic differences in the sporulation-germination processes between the two organisms.

A large number of genes, some of which are organized in apparent operons, have been identified for substrate utilization and transport, and they occur on both the chromosome and the megaplasmid (Nolling et al., 2001). For example, putative genes for cellulose degradation are found on the chromosome (CAC0910–CAC0919, CAC0561, and CAC3469), whereas putative genes for xylan degradation are found on both the chromosome (CAC0617 and CAC0706) and the megaplasmid (CAP0071, CAP0114, CAP0115, and CAP0117–CAP0120). Knowledge of genes for substrate utilization and their regulation could be useful in attempts to expand the substrate range of *C. acetobutylicum* or other solvent-producing species.

The Genome of C. beijerinckii *NCIMB 8052 Clostridium beijerinckii* NCIMB 8052 has a circular, 6.7-Mb chromosome (Wilkinson and Young, 1995). A combined physical and genetic map shows that the genes for acetoacetate decarboxylase (*adc*) and CoA-transferase (*ctfA* and *ctfB*) are located at some distance from either an alcohol dehydrogenase gene (*bdh*) or the genes for butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb*). The "*deg*" gene, whose integrity is related to the strain's tendency to lose solvent productivity (degeneration), was later identified as the fms gene encoding the peptide deformylase (Evans et al., 1998).

The Genome of C. saccharobutylicum *NCP 262 Clostridium saccharobutylicum* NCP 262 has a circular, 5.3-Mb chromosome (Keis et al., 2001b).

The positions of genes required for the formation of butyric acid as well as of two genes for alcohol dehydrogenases have been located on the map. The position of the genes for acetone formation (*ctfA*, *ctfB* and *adc*) has not been reported.

Tools for the Genetic Manipulation of Solvent-Producing Clostridia Since the 1980s, much progress has been made in the development of tools for the genetic manipulation of solvent-producing clostridia (Young et al., 1989; Mitchell, 1998). The basic tools are now available for carrying out a variety of genetic and molecular biological experiments with the solvent-producing clostridia.

Mutagenesis experiments with solvent-producing clostridia have been performed in a number of laboratories. Irradiation with ultraviolet (UV) light is not effective (Bowring and Morris, 1985), whereas several alkylating agents have been used successfully (Allcock et al., 1981b; Bowring and Morris, 1985; Hermann et al., 1985; Lemmel, 1985; Junelles et al., 1987; Rogers and Palosaari, 1987; Clark et al., 1989; Cueto and Mendez, 1990; Annous and Blaschek, 1991; Gutierrez and Maddox, 1992). Mutagenesis with transposons has been used with good results (Woolley et al., 1989; Bertram et al., 1990; Babb et al., 1993; Sass et al., 1993; Mattsson and Rogers, 1994; Kashket and Cao, 1995). Homologous recombination for directed mutagenesis has been demonstrated in *C. acetobutylicum* (Green and Bennett, 1996a) and in *C. beijerinckii* (Wilkinson and Young, 1994) using nonreplicative plasmids and in *C. acetobutylicum* using a replicative plasmid (Harris et al., 2002). The antisense RNA technique (Desai and Papoutsakis, 1999) provides a strategy for altering the phenotype without modifying the targeted gene itself.

A dominant selective marker is an essential element of a gene-transfer system. The erythromycin resistance gene of the streptococcal plasmid pAMβ1 has been widely used (Young et al., 1989). When selection for erythromycin resistance needs to be performed under acidic culture conditions over an extended period of time, the more acid-stable clarithromycin is used in place of erythromycin (Mermelstein and Papoutsakis, 1993a). The *Bacillus* plasmid pIM13 has provided the replicon for several shuttle vectors that can be maintained in *E. coli* and *C. acetobutylicum* (Lee et al., 1992), *C. beijerinckii* (Li, 1998), *C. saccharobutylicum* (Azeddoug et al., 1992), and *C. saccharoperbutylacetonicum* (Truffaut et al., 1989). Plasmids isolated from clostridia also provided replicons for the construction of shuttle vectors (Yoshino et al., 1990; Yoon et al., 1991; Lee et al., 1992). *Clostridium acetobutylicum* ATCC 824 contains a restriction endonuclease *Cac*824I whose recognition sequence 5′-GCNGC occurs frequently in *E. coli* plasmids. To protect the *E. coli-Clostridium* shuttle vector from restriction by *Cac*824I, in vivo methylation of the plasmid in *E. coli* by the *Bacillus subtilis* phage ϕ 3T I methyltransferase has been successfully used (Mermelstein and Papoutsakis, 1993b).

Different methods, including conjugative and protoplast-mediated transfers, have been developed for the introduction of vectors carrying specific genes into the solvent-producing clostridia (Mitchell, 1998). Much effort was put into the development of conjugative transfer systems (Oultram and Young, 1985; Reysset and Sebald, 1985; Yu and Pearce, 1986; Oultram et al., 1987; Bertram and Dü, 1989; Woolley et al., 1989; Williams et al., 1990) and the protoplast-mediated transfers (Reid et al., 1983; Lin and Blaschek, 1984; Jones et al., 1985). However, it is the electroporation technique that has made it easier to transform different strains of solvent-producing clostridia. Conditions of electroporation for the following strains are available: *C. acetobutylicum* ATCC 824 (Mermelstein et al., 1992), *C. acetobutylicum* DSM 792 (Nakotte et al., 1998), *C. beijerinckii* NCIMB 8052 (Oultram et al., 1988), *C. beijerinckii* NRRL B592 (Birrer et al., 1994; Li, 1998), and *C. beijerinckii* NRRL B593 (Li, 1998).

A good reporter gene is required for the study of promoters. The commonly used reporter systems, such as the *E. coli* β-galactosidase or the green fluorescent protein (GFP), are not suitable for the solvent-producing clostridia because of the biased codon usage pattern (AT-rich), an acidic environment, and the absence of molecular oxygen (O_2) in the clostridia. A β-galactosidase encoded by the *lacZ* gene of *Thermoanaerobacterium thermosulfurogenes* EM1 has been used as a reporter in *C. acetobutylicum* ATCC 824 (Tummala et al., 1999). However, a β-galactosidase is present in *C. acetobutylicum* NCIMB 2951 (Hancock et al., 1991). The use of β-galactosidase as a reporter in the solvent-producing clostridia may be limited if other strains also contain this enzyme. The *gusA*-encoded β-glucuronidase activity was successfully used as a reporter in *C. beijerinckii* NCIMB 8052 (Ravagnani et al., 2000). The secondary alcohol dehydrogenase activity (reduction of acetone to isopropanol) of the primary-secondary ADH of *C. beijerinckii* NRRL B593 has been used as a reporter in non-isopropanol-producing strains, such as *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B592 (Li, 1998). Further development of efficient reporter systems for the solvent-producing clostridia should facilitate the characterization of the promoters for solvent-production operons.

Commercial Applications

COMMERCIAL PRODUCTION Acetone, butanol and isopropanol are now produced by synthetic processes using petrochemicals as the starting material. However, the fermentation process is in operation in China (Jones and Keis, 1995; R.-X. Shen, personal communication). Use of the fermentation process in other countries has been reported (Billig, 1992), but the scale of the operation and other details have not. In Austria, *C. beijerinckii* NRRL B592 is being tested on the pilot-plant scale (50 liters for the first stage and approximately 150 liters for the second stage) to convert low-grade potatoes into acetone and butanol, and on a demonstration scale, tests are planned (Gapes, 2000b). commercial production of acetone, butanol, and isopropanol is briefly described below.

Acetone Most of the world's acetone is now obtained as a coproduct of phenol production by the cumene process. In the cumene-to-phenol process, benzene is alkylated to cumene, which is oxidized to cumene hydroperoxide, and the latter is cleaved to acetone and phenol. Dehydrogenation of isopropanol accounts for most of the acetone that is produced to meet the demand in excess of that supplied by the phenol process. The economics of acetone production are unusual in that the supply depends on the production of phenol, whereas the demand is controlled by the uses of acetone. When the consumption of acetone grows at a slower rate than the growth of demand for phenol, an excess in the supply of acetone occurs. More than 75% of the world's and about 95% of the United States' acetone production now comes from the cumene-to-phenol process. World production of acetone in 1990 was about three million metric tons per year, of which about one third was made in the United States. It has been predicted that acetone as a coproduct from the cumene-to-phenol process will continue to dominate supply, and production of "on-purpose" acetone will probably decline as supplies of by-product acetone increase (Howard, 1991), which effectively eliminates the need to use a fermentation process to produce acetone, except where the local economic situations may favor the fermentation process.

Butanol The principal commercial source of *n*-butanol is through hydrogenation of *n*-butyraldehyde, which is obtained from the Oxo reaction of propylene. The Oxo process produces *n*-butyraldehyde and isobutyraldehyde. The mixture may be separated before hydrogenation of *n*-butyraldehyde to *n*-butanol, or the mixture is hydrogenated and the products, *n*-butanol and isobutanol, are separated afterwards (Billig, 1992). As of January 1, 1990, the total United States capacity to produce *n*-butanol (591,000 tons or 1,182 million lbs per year) was roughly equal to the combined capacity of Western Europe and Southeast Asia (Billig, 1992). Between 1985 and 1995, the United States production of *n*-butanol increased from 716 to 1,493 million lbs (Anonymous, 1996b).

Isopropanol Isopropanol is generally known as the first petrochemical (Logsdon and Loke, 1999). It is manufactured from propylene by either an indirect hydration process (the sulfuric acid process) or direct hydration. Isopropanol is also produced by the hydrogenation of acetone, which is a coproduct of the cumene-to-phenol process (Logsdon and Loke, 1996). In addition, isopropanol is produced from crude acetone, which is generated as a by-product of propylene oxide manufacture (Anonymous, 2001b). Between 1986 and 1996, the United States annual production of isopropanol remained in the range 1,272–1,474 million lbs (Anonymous, 1997). The demand for 1999 and 2000 was, respectively, 1,180 and 1,200 million lbs, whereas the production capacity of the United States plants was 1,875 million lbs per year (Anonymous, 2001b).

USES *Acetone* Acetone is an excellent solvent for a wide range of industrial materials including gums, waxes, resins, fats, greases, oils, dyestuffs and cellulosics. Thus, solvent applications remain the largest uses for acetone worldwide, where both acetone and chemicals derived from acetone (the aldol chemicals) are used as solvents. However, because acetone is classified as a volatile organic compound, little or no growth in the solvents market is expected, although acetone is a normal constituent of the environment and is readily biodegradable (Howard, 1991).

Acetone is used as a carrier for acetylene, in the manufacturing of a variety of coatings and plastics, and as a raw material for the synthesis of a wide range of products including ketene, methyl methacrylate, and bisphenol A. Following solvent applications, the second largest use for acetone is for the manufacturing of acetone cyanohydrin, which is converted to methacrylates, and this use for acetone has been growing steadily. However, the fastest growing outlet for acetone is for the manufacturing of bisphenol A, which is used in the manufacturing of polycarbonate. It is interesting to note that although a mole of acetone is consumed in the production of a mole of bisphenol A, the process actually causes the net production of a mole of acetone because two moles of acetone are formed for every two moles of phenol formed (Howard, 1991).

Acetone is potentially useful in blends with gasoline as it can increase the oxygen content, the octane rating, and the water tolerance of the fuel (Bolt, 1980; Noon, 1982). Acetone can also be blended with butanol, ethanol and water to serve as an automobile fuel (see the next subsection Butanol in this Chapter).

Butanol *n*-Butanol is used as a direct solvent in paints and other surface coatings. Other direct solvent applications are in the formulation of pharmaceuticals, waxes and resins. Additional commercial markets for *n*-butanol include plasticizer esters, butylated melamine-formaldehyde resins, and mono-, di- and tributylamines. Butyl acetate is one of the more important derivatives of *n*-butanol and is employed as a solvent in rapid drying paints and coatings. *n*-Butanol and butyl acetate act synergistically to serve as a latent solvent system for nitrocellulose lacquers and thinners to give a solvent system stronger than either solvent alone.

Butyl glycol ethers such as 2-butoxyethanol are the largest-volume derivatives of *n*-butanol used in solvent applications. They are used in vinyl and acrylic paints, lacquers, varnishes, and aqueous cleaners to solubilize organic surfactants.

The largest-volume commercial derivatives of *n*-butanol, however, are *n*-butyl acrylate and methacrylate. These are used principally in emulsion polymers for latex paints, in textile applications, and in impact modifiers for polyvinyl chloride (Billig, 1992).

n-Butanol has been proposed as a blending agent with gasoline for the internal combustion engines (Noon, 1982; Bata et al., 1991; Ladisch, 1991) and with diesel (Ladisch, 1991). Besides being a component in the alcohol-gasoline blends, *n*-butanol can be blended with acetone, ethanol and water in a ratio of 51 : 25 : 6 : 18 (by weight) and the blend performed well in a spark-ignited, internal combustion engine (Noon, 1982).

n-Butanol can increase the water tolerance of gasoline-ethanol blends (Bolt, 1980; Noon, 1982). The water tolerance of the gasoline-ethanol blends increases with the ethanol concentration and temperature. At room temperature, a 25% ethanol blend with gasoline can tolerate about 1% of water. If 2% of water is present in the blend, most of the ethanol will separate from gasoline in a few seconds and settle to the bottom, which is known as phase separation. Phase separation renders the ethanol-gasoline blend unfit as an automobile fuel (Bolt, 1980). Addition of butanol to the blend increases the water tolerance to prevent phase separation (Bolt, 1980; Noon, 1982).

Isopropanol Isopropanol is used as chemical intermediates, solvents, and a component of many medical products. Estimated United States uses in 1993 were as chemical intermediates, 34%; personal care and household products, 24%; coating and ink solvent, 15%; processing solvent, 12%; pharmaceuticals, 10%; and miscellaneous uses, 5% (Logsdon and Loke, 1996). A more recent report (Anonymous, 2001b) gave the following breakdown: direct solvent uses, 46%; chemical derivatives, 36%; household and personal care products, 12%; pharmaceuticals, 4%; and acetone, 2%.

Isopropanol is used in the production of other chemicals such as derivative ketones, isopropylamines, and isopropyl esters. The use of isopropanol in the production of monoisopropylamine for herbicides (primarily glyphosate) continues to be the fastest growing segment (Anonymous, 2001b). A minor use for isopropanol is to serve as a feedstock for the production of acetone to meet the demand in excess of the coproduct acetone from phenol production. However, isopropanol is also produced from crude acetone, which is generated as a by-product of propylene oxide manufacture (Anonymous, 2001b).

Because of its balance between alcohol, water, and hydrocarbon-like characteristics, isopropanol is an excellent, low cost solvent free from government regulations and taxes that apply to ethanol. The lower toxicity of isopropanol favors its use over methanol. Consequently, isopropanol is used as a solvent in many consumer products and industrial products. It is used widely as a solvent for cosmetics, and many aerosol products contain isopropanol as a solvent. Because it is a good solvent for a variety of oils, gums, waxes, resins and alkaloids, isopropanol is used for preparing cements, primers, varnishes, paints, and printing inks (Logsdon and Loke, 1996).

Isopropanol is used as an antiseptic and disinfectant for home, hospital and industry. Rubbing alcohol, an aqueous solution of 70% (vol/vol) isopropanol, exemplifies the use of isopropanol in healthcare products. Other examples include 30% (vol/vol) isopropanol solutions for medicinal liniments, tinctures of green soap, scalp tonics, and tincture of mercurophen. It is also contained in pharmaceuticals such as local anesthetics, tincture of iodine, and bathing solutions for surgical sutures and dressings. Over 200 medical uses of isopropanol have been tabulated (Logsdon and Loke, 1996).

The use of diisopropyl ether as a fuel additive may become a significant outlet for isopropanol (Logsdon and Loke, 1996). Isopropanol itself is a useful blending component for gasoline because of its heating value, oxygen content, and octane rating (Wagner et al., 1980; Schoutens

and Groot, 1985a; Owen and Coley, 1990; Houben, 1995).

Areas of Research and Development

MICROBIOLOGY OF SOLVENT PRODUCTION The patents issued between the 1910s and 1940s contained many names of solvent-producing bacteria. Studies of industrial strains that are available from culture collections identified four species of *Clostridium* (Johnson et al., 1997; Keis et al., 2001a). Among these four species, *C. beijerinckii* is represented by many characterized strains, whereas the other three have few available strains. Considering the success of earlier investigators in isolating solvent-producing bacteria from nature, it is reasonable to expect that more solvent-producing organisms are yet to be isolated. There are new efforts in this area, such as the isolation of new solvent-producing clostridia from Columbian soil (Montoya et al., 2001). The new microbiological study may help provide novel solvent-producing organisms, from which more genetic determinants and phenotypic traits may be evaluated for the further development of industrial solvent fermentation.

ENZYMOLOGY, GENETICS, AND REGULATION OF SOLVENT PRODUCTION Efforts to define the enzymology of solvent production by the clostridia continue. Although the enzymes for most of the solvent-forming reactions have been purified from at least one strain of clostridia and characterized, the specific roles of the multiple aldehyde and alcohol dehydrogenases (Chen, 1993; Chen, 1995) are yet to be defined. The enzyme butyryl-CoA dehydrogenase also has not been characterized. Therefore, the enzymology for the three consecutive reactions leading to the formation of butanol is a subject of research. The characterization of the second aldehyde-alcohol dehydrogenase (AdhE2) from *C. acetobutylicum* ATCC 824 (Fontaine et al., 2002) and the cloning of the two genes (*adhA* and *adhB*) encoding the three primary ADH isozymes of *C. beijerinckii* NRRL B592 (GenBank accession numbers {AF497741} and {AF497742}) represent efforts in this area.

The regulation of the expression of the solvent-production operons is yet to be understood. The promoters for the solvent-production operons and the transcriptional factors for these promoters are the subjects of research. The completion of the genome sequence of *C. acetobutylicum* ATCC 824 and the sequence of the solvent-production operons from different species will facilitate the study of the components of the regulatory circuit. The identification of the Spo0A-binding sequence in the promoter region of solvent-production operons of *C. acetobutylicum* (Thormann et al., 2002) and *C. beijerinckii* (Ravagnani et al., 2000) as well as the role of Spo0A in solvent production by *C. acetobutylicum* (Harris et al., 2002) should accelerate the pace of research in this area. The development of better reporter systems for the study of promoter function in different solvent-producing species will be crucial to progress in this area.

Among the properties of solvent-producing clostridia, strain degeneration can have a serious impact on a commercial operation. Strain degeneration is the result of genetic alterations, but besides a decrease in solvent production, it is accompanied by other phenotypic changes (e.g., change in colony morphology or cell size [Adler and Crow, 1987] and Fourier transform infrared [FT-IR] spectra of cells [Schuster et al., 2001]), which may be useful indicators for the early detection of strain degeneration.

It has been observed that a truncated but functional peptide deformylase, which reduces the growth rate of the mutant (Evans et al., 1998), or an elevated acetate concentration in the growth medium (Chen and Blaschek, 1999a) will lower the frequency of degeneration of *C. beijerinckii* NCIMB 8052. At present, it is not known how many different classes of genetic changes may result in strain degeneration and how the proportion of degenerated cells in a population may change under different culture conditions. Research in this area will help define the molecular basis for strain degeneration and lead to procedures for the prevention and early detection of degeneration.

METABOLIC ENGINEERING With the genes for the acid- and solvent-forming pathways cloned from *C. acetobutylicum* ATCC 824, it becomes possible to manipulate the metabolic pathways in this and other species for the purpose of improving solvent production. Both gene inactivation and gene overexpression have been used. Nonreplicative integrational plasmids containing internal *buk* or *pta* gene fragments were used to inactivate genes encoding butyrate kinase (*buk*) or phosphotransacetylase (*pta*), respectively (Green et al., 1996b). Inactivation of the *buk* gene reduced butyrate kinase activity and increased butanol production (Green et al., 1996b; Harris et al., 2000). Interestingly, the onset of solvent production occurred earlier (in the exponential phase of growth) in the BK^- mutant (PJC4BK) and the final solvent concentration was significantly higher when the growth pH was lowered from 5.5 to 5.0 (Harris et al., 2000). When anti-*buk*-RNA was used to lower the level of butyrate kinase (Desai and Papoutsakis, 1999), the onset of solvent production also occurred earlier than in the control

culture. An earlier onset of solvent production allows a longer production period. The levels of butanol, acetone, and ethanol reached 225 mM (16.7 gm/liter), 76 mM (4.4 gm/liter), and 57 mM (2.6 gm/liter), respectively, when the BK^- mutant (PJC4BK) was grown at pH 5.0, indicating that the benchmark butanol concentration of 180 mM in the industrial fermentation broth is not due to butanol toxicity.

When a plasmid carrying the *aad* gene (encoding the aldehyde-alcohol dehydrogenase) was introduced into the butyrate kinase mutant (BK^-) to increase the level of the aldehyde-alcohol dehydrogenase, the resultant strain PJC4BK (pTAAD) produced similar amounts of butanol and acetone as PJC4BK, but 98 mM (4.5 gm/liter) of ethanol. Work with PJC4BK (pTAAD) indicated that the *aad*-encoded aldehyde-alcohol dehydrogenase did not limit butanol production under the fermentation conditions used. The level of ethanol produced by PJC4BK (pTAAD) approached the level (5 gm/liter) produced by several species of ethanol-producing clostridia (Rogers and Gottschalk, 1993).

A synthetic operon (the *ace* operon) for acetone production was constructed by placing the *C. acetobutylicum adc*, *ctfA* and *ctfB* genes (see subsection Genes and Operons for Solvent Production and the Genome of Solvent-Producing Bacteria in this Chapter) under the control of the *adc* promoter. Plasmid pFNK6 carrying the *ace* operon was introduced into *C. acetobutylicum* ATCC 824, and the resultant strain ATCC 824 (pFNK6) had an earlier onset of solvent production at pH 5.5 and 6.5 and produced higher levels of acetone and butanol than the parent strain (Mermelstein et al., 1993). Interestingly, a plasmid-control strain containing a vector without the *ace* operon also produced higher levels of solvents, but levels were lower than that produced by ATCC 824 (pFNK6).

A synthetic operon (the *ace4* operon) composed of four *C. acetobutylicum* ATCC 824 genes (*adc*, *ctfA*, *ctfB* and *thl*) under the control of the *thl* promoter was constructed to allow *E. coli* to produce acetone (Bermejo et al., 1998). The thiolase gene (*thl*) is included for the conversion of acetyl-CoA to acetoacetyl-CoA (see the subsection Metabolic Pathways and Enzymology of Solvent Production in this Chapter). One of the transformed *E. coli* strains, ATCC 11303 (pACT), produced 125–154 mM of acetone when sodium acetate was added to glucose-fed cultures. Besides their potential usefulness for acetone production, it was suggested that the recombinant strains may be useful hosts for recombinant protein production in that detrimental acetate accumulation can be avoided (Bermejo et al., 1998).

Because isopropanol is potentially a more desirable by-product than acetone for industrial butanol fermentation, a secondary alcohol dehydrogenase can be introduced into acetone- and butanol-producing strains that possess other superior properties, such as a broad substrate range or an early onset of solvent production. The *adh* gene encoding a primary-secondary alcohol dehydrogenase from *C. beijerinckii* NRRL B593 has been successfully expressed in *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B592 and has enabled the transformants to produce isopropanol (instead of acetone) and butanol as major products (Li, 1998). Expression of the *C. beijerinckii* NRRL B593 primary-secondary alcohol dehydrogenase in *E. coli* allows the transformant to produce isopropanol from added acetone (Peretz et al., 1997).

Continuous Solvent Production A continuous fermentation is superior to a batch process because of more effective uses of the fermentation facility. Since the early 1980s, much effort has been devoted to the characterization of the parameters and the development of a continuous culture system for solvent production (see also patents listed in Table 34). On the basis of the results of these studies, it was concluded that no single growth-limiting factor specifically induces solvent production in a chemostat. In glucose-, nitrogen-, or magnesium-limited chemostats, steady-state solvent production was low or difficult to maintain, and an application of these kinds of limitations to an industrial process seems unlikely (Dürre and Bahl, 1996). However, phosphate- or sulfate-limitation promoted solvent production (Bahl et al., 1982b; Dürre and Bahl, 1996).

A two-stage phosphate-limited culture system allowed *C. acetobutylicum* DSM 1731 to produce solvents continuously (Bahl et al., 1982b). At the first stage, the cells are growing under conditions that induce the onset of solvent production. At the second stage, growth is not possible because the limiting nutrient is exhausted, and this stage is thus devoted to the conversion of remaining sugar to solvents. The dilution rate and temperature were 0.125 h^{-1} and 37°C for the first stage and 0.04 h^{-1} and 33°C for the second stage. In a one-stage continuous culture, butanol and acetone concentrations as well as the rate of glucose consumption increased when the dilution rate was decreased from 0.13 to 0.025 h^{-1}. Although a low dilution rate favors a high product concentration, the productivity (gm of butanol $\cdot$ liter^{-1} $\cdot$ h^{-1}) decreased with decreasing dilution rate, and at dilution rates below 0.025 h^{-1}, stable culture conditions could no longer be obtained. A two-stage continuous culture of *C. acetobutylicum* ATCC 824 was operated at dilution rates of

0.08 and 0.04 h^{-1} for the first and second stages and yielded similar results (Godin and Engasser, 1990).

A two-stage continuous culture, with on-line solvent removal by membrane evaporation, was carried out with *C. beijerinckii* NRRL B592 (Gapes et al., 1996). In this study, porous LECA (Lightweight Expanded Clay Aggregates) particles (particle size 5–10 mm) were included at both stages. The internal (accessible to cells) volume of the LECA particles represented 12 and 15% of the total volume (consisting of the working volume of the fermentor, the internal volume of the LECA particles, and the volume of the recirculation loop) of the first and second stages, respectively. The use of the LECA particles at both stages resulted in a significant increase in the final solvent concentration and solvent productivity at the second stage. The true dilution rate for each stage of the culture is difficult to determine because the medium inside the LECA particles must have a lower dilution rate than the medium outside the particles. Accordingly, the cells inside and outside of the LECA particles are likely at different physiological states. Continuous cultures of solvent-producing clostridia show periodic fluctuations of metabolic activities (known as metabolic oscillation). The inclusion of a membrane evaporation module in the recirculation loop of the second cultivation stage seems to reduce the magnitude of metabolic oscillations (Gapes et al., 1996). It should be informative to compare cells collected at different oscillatory cycles to see whether they represent different genotypic populations of cells, which differ in their capacity for substrate utilization, solvent production, and sporulation.

Active solvent production by *C. acetobutylicum* or *C. beijerinckii* at the second stage of a two-stage continuous culture may be analogous to solvent production in the batch culture, where solvent-producing cells are growing very slowly or not growing as they proceed to complete sporulation. Results suggest that the Spo0A regulatory protein is involved in the initiation of solvent production, besides its role in the initiation of sporulation (see the subsection Physiology of Solvent Production in this Chapter). If active solvent production by the wildtype cell is limited to a period during which the cell does not commence new division (although the optical density of the culture continues to increase owing to an increase in cell mass), it becomes apparent that a true chemostat or continuous culture of the wildtype solvent-producing cells should be difficult to maintain. For the same reason, cell immobilization or recycling will not be practical for continuous solvent production by the wildtype cells. However, appropriate asporogenous mutants (Meinecke et al., 1984; Lemme and Frankiewicz, 1985), whose lesion is at a step beyond the onset of solvent production and sporulation, could allow the use of immobilized cells for continuous solvent production (Largier et al., 1985). New research in this area will probably use asporogenous or sporulation-deficient mutants that are selected for prolonged active solvent production.

Product Recovery Solvent fermentation produces a dilute solution of butanol, acetone or isopropanol. The recovery of these products by distillation is an energy-intensive process. Unless the final product concentrations are elevated, the development of alternative methods for product recovery may improve the economics of this fermentation. Techniques for the alternative methods could include the use of membranes (reverse osmosis, perstraction, pervaporation and membrane evaporation), adsorbents, liquid-liquid extraction, gas striping, and chemical recovery methods (Dürre and Bahl, 1996; Dürre, 1998). Each of these techniques has its advantages and shortcomings, but all of them can be designed to allow on-line product recovery, which is desirable for a continuous solvent-production system (Gapes et al., 1996; Dürre, 1998).

Pilot Plant Studies Since the 1980s, two studies of solvent fermentation at the pilot-plant scale have been reported. A pilot project was started in Soustons, France, to evaluate the commercial potential of producing solvents for gasoline-substitute fuels. It was designed to use mainly hydrolysates of cereal straw and corn stover as the substrates. The raw materials were pretreated by steam explosion and then hydrolyzed by *Trichoderma* cellulase complex to yield fermentable substrates, and the aim was to produce one ton of solvents from 6–7 tons of raw materials (Nativel et al., 1992; Nimcevic and Gapes, 2000). Batch fermentations using strains of *C. acetobutylicum* were performed at 2 m^3 and 50 m^3 scales. Yields of one ton of solvents from 7.7 tons of corn cobs were reported. Economic evaluation of the process revealed that the costs of acetone-butanol fermentation from lignocellulosic materials are strongly dependent on the market value of the by-product lignin (Marchal et al., 1992).

Another pilot-plant study was performed in Austria, using potatoes as the raw materials (Nimcevic and Gapes, 2000). The pilot plant was designed for different modes of operation, including batch, fed-batch, and continuous. For continuous fermentation, potatoes were mashed and then liquified with a relatively small amount of amylase to prevent blockage of the equipment. The study used *C. beijerinckii* NRRL 592 and a two-stage setup (Gapes et al., 1996), with

the longest continuous fermentation lasting about four weeks. The first-stage fermentor was a gas-lift bioreactor with a working volume of 50 liters, and the second stage fermentor had a working volume up to 300 liters and was equipped with an on-line gas-stripping unit. For product removal and recovery, different on-line and off-line techniques were tested, and they included distillation, vacuum distillation, rectification, gas-stripping, and reverse osmosis. Membrane systems were not tested for product recovery at the pilot plant because it was assumed that the relatively low solvent flux through the membrane would require too large a membrane area for commercial operation. The results of the pilot-plant study have not been published yet. However, the solvent fermentation is attractive to certain localities because it provides an alternative to ethanolic fermentation: when there is a glut of ethanol, those regions which traditionally produce ethanol from surplus and waste starchy materials can now produce butanol and acetone or isopropanol instead (Gapes, 2000b).

Economic Evaluation Following the oil embargo in 1973 and the drastic rise in the price of crude oil, a renewed interest in the butanol-acetone fermentation has required periodic evaluation of the economic feasibility of this fermentation utilizing different substrates, engineering designs, and financing (Solomons, 1976; Lenz and Moreira, 1980; Gibbs, 1983; Schoutens and Groot, 1985a). These studies considered molasses, milk whey, and lignocellulosic materials as the source of the carbohydrates for the fermentation, as the cost of the carbon substrate is invariably the largest single cost item. The question "where is the sugar to come from" (Solomons, 1976) remains valid today. Evaluations utilizing corn or low-grade potatoes as the raw material yielded encouraging results (Gapes, 2000a; Qureshi and Blaschek, 2001a).

The analysis by Gapes (2000a) shows that the process can be run economically in niche markets on a relatively small industrial scale, processing low-grade agricultural products. The niche market is usually a rural region where cheap, low-grade substrates are available and the conversion of the raw material into bulk chemicals meets the specific needs of the locality, as exemplified by the many small ethanol distilleries that have been operated around Europe for many decades. On the basis of the average world market price of butanol (0.61 EUR/kg) and acetone (0.45 EUR/kg) for the five years preceding 2000, it was calculated that the break-even price of substrate lies between 0.05 and 0.09 EUR/kg for a grass-root plant or between 0.09 and 0.13 EUR/kg if an existing plant can be modified at low cost. The price of agricultural products, such as grains (primarily produced for food and feed), is thus too high under the conditions assumed in this analysis. Utilization of low-grade substrates, such as frozen potatoes, mycotoxin-contaminated corn, and surplus sugar beet, makes the fermentation economical. As suggested by several authors, butanol-acetone production by a fermentative process probably has higher capital costs but lower production costs than production by the petrochemical industry process. Gapes also suggests that from the viewpoint of investment costs alone, it is unlikely that a continuous operation is of great advantage because of the increased equipment cost for maintaining sterility during the fermentation.

The evaluation of a process using corn and the hyper-butanol-producing strain of *C. beijerinckii* BA101 in batch reactors at a plant with a capacity of 153,000 metric tons per year of butanol, acetone and ethanol gave the following results. At a corn price of US$79.23 per ton (US$2.01 per bushel) and a solvent yield of 0.42 gm per gm of glucose, the price for butanol is projected to be US$0.34 per kg. If the price of corn is at US$197.10 per ton, the price of butanol rises to US$0.47 per kg. Production from a grass-root plant would result in a butanol price of US$0.73 per kg, when the price of corn is US$79.23 per ton, or US$0.88 per kg, when the price of corn is US$197.10 per ton, or US$1.07 per kg, when the price of corn is US$197.10 per ton and no credit for gases is taken. In this evaluation, acetone and ethanol are treated as by-products, and a by-product credit is included in the calculation of the price of butanol. The price of petrochemical-derived butanol was US$1.21 per kg as reported in the Oct. 16, 2000, issue of *Chemical Market Reporter* (Qureshi and Blaschek, 2001a).

Patents and Regulatory Issues

There are two periods during which the majority of patents for bacterial solvent production were issued. The first period is between 1910 and 1950, and the second period started in 1982. The first period is characterized by the use of newly isolated bacteria (Beesch, 1952; Prescott and Dunn, 1959a), whereas the second period is characterized by the use of mutant strains derived from organisms in the culture collections (Table 34). Many of these patents have expired, but they continue to provide useful information for the correct identification and classification of industrial strains preserved in the culture collections (Jones and Keis, 1995; Keis et al., 1995).

Table 34. United States patents issued after 1981 for butanol production by fermentation.

Patent no.	Date	Inventors	Title
4,326,032	1982	Grove, L. H.	Process for the production of organic fuel
4,368,056	1983	Pierce, S. M., Wayman, M.	Diesel fuel by fermentation of wastes
4,424,275	1984	Levy, S.	Continuous process for producing *n*-butanol employing anaerobic fermentation
4,443,542	1984	Hayashida, S., Ogata, S., Yoshino, S.	Process for the production of butanol and novel microorganism composition used therein
4,520,104	1985	Heady, R. E., Frankiewicz, J. R.	Production of butanol by a contiuous fermentation process
4,521,516	1985	Lemme, C. J., Frankiewicz, J. R.	Strain of *Clostridium acetobutylicum* and process for its preparation
4,539,293	1985	Bergstrom, S. L., Foutch, G. L.	Production of butanol by fermentation in the presence of cocultures of *Clostridium*
4,560,658	1985	Datta, R., Zeikus, J. G.	Production of butanol by fermentation in the presence of carbon monoxide
4,568,643	1986	Levy, S.	Continuous process for producing *n*-butanol employing anaerobic fermentation
4,628,116	1986	Cenedella, R. J.	Vinyl bromide extraction of butyric acid and butanol from microbial fermentation broth
4,690,897	1987	Squires, C. H., Heefner, D. L., Evans, R. J., Kopp, B. J., Yarus, M. J.	Method for transformation of anaerobic microorganisms
4,757,010	1988	Hermann, M., Fayolle, F., Marchal, R.	Production of *Clostridium acetobutylicum* mutants of high butanol and acetone productivity, the resultant mutants, and the use of these mutants in the joint production of butanol and acetone
4,777,135	1988	Husted, G. R., Santangelo, J. D., Bostwick, D. W.	Method for producing butanol by fermentation
4,905,761	1990	Bryant, R. S.	Microbial enhanced oil recovery and compositions
5,063,156	1991	Glassner, D. A., Jain, M. K., Datta, R.	Process for the fermentative production of acetone, butanol and ethanol
5,192,673	1993	Jain, M. K., Beacom, D., Datta, R.	Mutant strain of *C. acetobutylicum* and process for making butanol
5,210,032	1993	Kashket, E. R.	Degeneration-resistant solventogenic clostridia
6,358,717	2002	Blaschek, H., Annous, B., Formanek, J., Chen, C.-K.	Method of producing butanol using a mutant strain of *Clostridium beijerinckii*

The new wave of patent applications coincided with the rise in interest in using fermentation as an alternative to the petrochemical-dependent synthetic routes for the production of butanol and acetone. For reasons of economics and environment protection, the use of industrial waste or lignocellulosic residues, instead of primary food products, as the substrates for the fermentation is desired (Pierce and Wayman, 1983). There are propositions to use butanol, acetone or isopropanol as components of fuels (Grove, 1982; Pierce and Wayman, 1983) or to use solvents produced in situ for enhanced oil recovery (Bryant, 1990). Another reason for making solvent fermentation important pertains to the desired use of fermentation-derived butanol as an extractant in the preparation of foods, flavors and pharmaceuticals to reduce the potential of carcinogen carryover from the petroleum-based synthetic butanol (Blaschek et al., 2002).

Prospects

The demise of solvent fermentation in North America and East Asia between the late 1950s and the early 1960s resulted from both the competitive uses for molasses, which drove up the cost of raw materials, and the rise of the petrochemical industry, which drove down the price of the chemically synthesized butanol and acetone. However, this industrial fermentation did not totally disappear after the 1960s. The continued operation of the fermentation in South Africa between 1936 and 1982 illustrates the importance of the local conditions in determining the cost effectiveness and the necessity of the fermentation. The use of butanol as an extractant by the food and pharmaceutical industries may also create a demand for the fermentation product as it does not contain the carcinogens that may be present in butanol produced from petrochemicals. The continued use of the fermentation

process to produce butanol in China may partly be due to this consideration.

With an increasing world population and an increased pressure to preserve the productivity of the land and the aquatic system, the use of petrochemicals and the further deterioration of the environment by pollution must be curtailed. Therefore, in addition to conserving the petroleum reserve, the production of valuable chemicals such as butanol, acetone and isopropanol from fermentable wastes or low-grade agricultural products can also help to improve the quality of the environment as well as the economy of localities where such raw materials are generated. Results of economic evaluations indicate that butanol production by fermentation can be profitable in niche markets where the cost of raw material is kept low (Gapes, 2000a; Qureshi and Blaschek, 2001a). The cost for raw material will probably remain the most significant item in the economics of solvent fermentation. The genome of *C. acetobutylicum* contains several sets of genes for the utilization of abundant and inexpensive polysaccharides. If solvents can be produced from lignocellulosic substrates, the fermentation will be even more attractive.

The economics of solvent fermentation can be further improved by increasing the final concentration of solvents in the fermentation broth so that the cost for product recovery is lowered. Although butanol is toxic to the cell, results of genetic manipulations show that significantly higher concentrations of butanol and acetone can be achieved when the metabolic machinery is altered, indicating that butanol toxicity is not the limiting factor for the final solvent concentration. Our understanding of the enzymology and regulation of solvent fermentation suggests that because of the shared regulatory element (Spo0A) for the onset of both solvent production and sporulation and because of the cell's progression toward sporulation, active solvent production has a limited duration and hence productivity within the life cycle of a *Clostridium* cell. It is thus tempting to postulate that if the regulation for the onset of solvent production can be disconnected from the regulation for the onset of sporulation (or if the sporulation process can be delayed or interrupted), the period of active solvent production may be significantly prolonged to increase the final concentration of solvents. At present, it appears that isopropanol is a more desirable by-product than acetone for butanol fermentation, because an adequate supply of acetone is available through phenol production. If there should be an additional demand for acetone or the route for acetone production changes, the product pattern can be adjusted through metabolic engineering so that the best combination is obtained.

Section 9: 1,3- and 1,2-Propanediol Production

Introduction

Three-carbon diols (1,3- and 1,2-propanediol) are of significant commercial interest. The first (1,3 propanediol; PDO; also known as "trimethylene glycol") can be produced by glycerol fermentation by several different microorganisms, including *Clostridium*, *Enterobacter*, *Klebsiella* and *Lactobacillus* (Rayner, 1926; Mickelson and Werkman, 1940). The fermentative production was first described in 1881 (Biebl et al., 1999). The reader is referred to several reviews on microbial production of 1,3-propanediol (Deckwer, 1995; Zeng et al., 1997; Cameron et al., 1998; Biebl et al., 1999). The second (1,2 propanediol; 1,2-PD; also known as "propylene glycol") is produced naturally by a few organisms such as *Thermoanaerobacterium thermosaccharolyticum* and various *Clostridium* spp. (Tran-Din and Gottschalk, 1985; Cameron and Cooney, 1986; Sanchez-Riera et al., 1987). *Escherichia coli* has been engineered to produce 1,2-propanediol, and this development may provide a competitive biotechnological route of production (Altaras and Cameron, 2000). The microbial production of 1,2-propanediol has been summarized in reviews (Cameron et al., 1998; Bennett and San, 2001).

Large quantities of 1,2-propanediol are made by a chemical process using propylene oxide as raw material. Consumption in the United States is estimated to be in the range of 850 million pounds per year (Ouellette, 2000). Propylene glycol is a viscous liquid that is used primarily in unsaturated polyester resins. Pricing of propylene glycol, as listed in the January 1, 2001, issue of *Chemical Marketing Reporter*, is about US$0.59 per pound. The relatively low cost of chemically produced propylene glycol makes it difficult for a biological process to compete economically.

On the other hand, 1,3-propanediol has been more difficult to produce via chemical synthesis, and for many years its high price limited its application. Sold as a specialty chemical, 1,3-propanediol was priced in the range of US$30/kg (Biebl et al., 1999). This high cost sparked interest in exploring the economics of a biological route, especially as PDO is a desirable monomer for polymer synthesis. Deckwer (1995) estimated that the cost of 1,3-propanediol produced by fermentation was highly dependent on glycerol, the primary raw material. He estimated that more than 2/3 of the production cost of 1,3-propanediol was due to glycerol, which sells for about US$0.65–0.80 per pound (Anonymous, 2001a).

The prospects for 1,3-propanediol changed significantly when Shell Chemical announced the commercialization of a new polyester in 1995 called "Corterra." This new polyester is a combination of terephthalic acid and 1,3-propanediol. The potential for widespread use of this polyester (polytrimethylene terephthalate [PTT]) lies in fiber applications, as it has excellent properties (Welling, 1998). Shell developed a lower cost chemical route to the monomer, but industrial interest in biotechnological routes remained.

As mentioned earlier, only a few microorganisms possess the ability to ferment glycerol anaerobically to 1,3-propanediol. Glycerol is converted to pyruvate through dihydroxyacetone phosphate. In *Klebsiella*, the pyruvate is cleaved to acetyl-CoA and formate, with ethanol, acetate, CO_2, and H_2 being generated. A balance of reducing equivalents is required, resulting in the conversion of glycerol to 3-hydroxypropionaldehyde via a vitamin B12-dependent glycerol dehydratase, and then subsequent reduction to 1,3-propanediol (Streekstra et al., 1987). DuPont and Genencor undertook a new biotechnological approach (adding genes to propanediol-producing hosts that allowed for the usage of dextrose as the substrate; Chotani et al., 2000). With the number of tools in molecular biology increasing and the potential benefits of constructing new pathways, the metabolic engineering approach towards production of 1,3-propanediol has become an important one (Cameron et al., 1998).

The anaerobe *Thermoanaerobacterium thermosaccharolyticum* can produce 1,2-propanediol from dextrose, whereas other microorganisms use costly 6-deoxysugars. Propylene glycol produced by chemical means is a mixture of R and S isomers. The pure R enantiomer, which may have added value as a chiral molecule, is produced by microbial processes. This organism can produce 1,2-propanediol from a variety of sugars, which may help lower the cost of production (Altaras et al., 2001). The naturally occurring strains do not produce 1,2-propanediol at high amounts, so an alternate approach involving metabolically engineered *E. coli* was followed. *Escherichia coli* typically makes 1,2-propanediol from costly 6-deoxyhexose sugars; however, expression of either glycerol dehydrogenase or methylglyoxal synthase resulted in anaerobic production from glucose (Altaras and Cameron, 1999). Many challenges still remain in the development of a bioprocess that can be competitive with the chemical route in the production of 1,2-propanediol.

To become competitive with a chemical process, one that is microbially based must overcome hurdles: 1) the cost of agricultural raw material versus the relative low cost of petrochemical feedstocks, and 2) purification, including both water removal and separation from a complex mixture. The approaches described in subsequent sections focus on using lower cost raw material and achieving gains in yield and titer.

Scientific Background

1,3-Propanediol The study of microbial production of 1,3-propanediol has an interesting history (reviewed by Biebl et al., 1999). It is one of the oldest fermentation products known and has been studied for over 100 years. For a number of years, interest in the fermentation was due to its potential as an outlet for surplus glycerol. Glycerol can be made via a chemical process, or it can be derived from various agricultural fats during the production of fatty acids and soaps. Increased availability of low-cost glycerol might be expected in the future, as it is a by-product of such processes as transesterification of fats for biodiesel production as well as the process for ethanol production by yeast.

Glycerol can be used aerobically for growth by a large number of microorganisms. Very few organisms are able to form PDO, which is typically formed as an anaerobic product of glycerol. Only bacteria in the genera *Klebsiella*, *Enterobacter*, *Citrobacter*, *Lactobacillus*, *Clostridium* (Biebl et al., 1999) and *Ilyobacter* (Stieb and Schink, 1984) have been shown to make PDO. Most of the work has been done utilizing *Klebsiella*, *Enterobacter* and *Clostridium* (Heyndrickx et al., 1991; Bouvet et al., 1994; Barbirato et al., 1995; Petitdemange et al., 1995; Solomon et al., 1995; Barbirato et al., 1996a; Zeng, 1996; Ahrens et al., 1998; Biebl et al., 1998).

Glycerol is converted via two pathways: an oxidative one to pyruvate and a reductive pathway to PDO. The oxidative pathway involves a NAD^+-linked enzyme, glycerol dehydrogenase, which converts glycerol to dihydroxyacetone. Interestingly, dihydroxyacetone has uses in the cosmetics industry (Barbirato et al., 1998). Dihydroxyacetone is then phosphorylated by DHA kinase and is then further metabolized to pyruvate through glycolytic reactions. From pyruvate, a mixture of end products is formed that varies among the different anaerobic glycerol utilizers (Forage and Lin, 1982b). The oxidative and reductive pathways are shown in Fig. 28.

In the reductive pathway, 3-hydroxypropionaldehyde is formed by the action of a vitamin B12-dependent glycerol dehydratase. The 3-hydroxypropionaldehyde is then reduced by the enzyme 1,3-PD dehydrogenase. The oxidative and reductive pathways of glycerol dissimilation form a balance, as the role of the PDO pathway is to regenerate reducing equivalents in the form

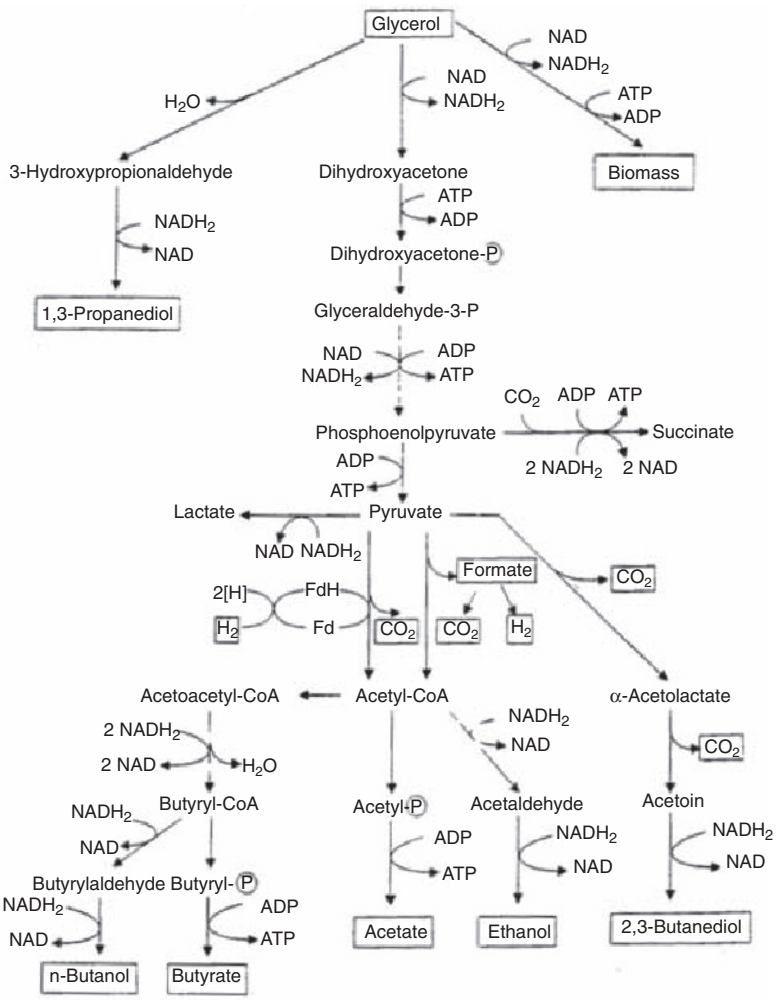

Fig. 28. Biochemical pathways of glycerol fermentation of representative microorganisms. From Biebl et al. (1999), with permission.

of reduced nicotinamide dinucleotide ($NADH_2$) produced from the dihydroxyacetone pathway (Deckwer, 1995; Zeng et al., 1997; Cameron et al., 1998).

The genes for the anaerobic dissimilation of glycerol are termed "the *dha* system." Dihydroxyacetone and glycerol are inducers of glycerol dehydrogenase, dihydroxyacetone kinase, glycerol dehydratase and 1,3-PD dehydrogenase (Forage and Foster, 1982a). Each of these genes and the resulting gene products have been studied in some detail and will be discussed below.

The first gene of the oxidative pathway is a NAD^+-linked glycerol dehydrogenase. The enzyme differs from other glycerol dehydrogenases such as the one found in *E. coli*. *Escherichia coli* does not contain the *dha* genes, but rather uses glycerol aerobically via an ATP-dependent kinase and an *sn*-glycerol 3-phosphate dehydrogenase (St. Martin et al., 1977). However, a NAD^+-glycerol dehydrogenase was isolated from a mutant *E. coli* strain with defects in two genes for aerobic glycerol dissimilation, which provided a forced selection for growth on glycerol. This glycerol dehydrogenase was purified and studied and was found to have a broad substrate specificity, including glycerol as well as a number of substituted diols (Tang et al., 1979). This *E. coli* enzyme was studied and found to be immunochemically similar to a glycerol dehydrogenase in *Klebsiella pneumoniae*, but not to the glycerol dehydrogenase of the *dha* system (Tang et al., 1982). This gene was later identified as *gldA* and is identical to D-1-amino-2-propanol oxidoreductase of *E. coli*. It maps near the *glpFKX* operon, and hydroxyacetone was shown to induce its expression (Trunger and Boos, 1994).

Glycerol dehydrogenase (E.C. 1.1.1.6) and dihydroxyacetone kinase (EC 2.7.1.29) were cloned and purified from *Citrobacter freundii*. Glycerol dehydrogenase is a hexamer of a polypeptide of 43 kDa. Like the *E. coli* glycerol dehydrogenase, the *Citrobacter* enzyme also has a broad substrate specificity. The DHA kinase is a dimer of a 57-kDa polypeptide. This enzyme is

very specific for its substrates dihydroxyacetone and ATP. DHA kinase shows some structural similarities to the glycerol kinase of *E. coli*. The glycerol dehydrogenase gene and the DHA kinase gene were denoted "*dhaD*" and "*dhaK*," respectively (Daniel et al., 1995b).

The *Citrobacter freundii* gene for 1,3-propanediol dehydrogenase (*dhaT*) has been cloned and overexpressed in *E. coli*. The enzyme is made up of eight identical subunits of 43.4 kDa and is able to catalyze a number of oxidation and reduction reactions. The enzyme oxidized a number of alcohols and was most active with diols containing two primary alcohol groups separated by one or two carbon atoms. This dehydrogenase reduced several aldehydes, with its greatest activity toward 3-hydroxypropionaldehyde. The authors compared the sequence of the *C. freundii* enzyme with alcohol dehydrogenases and concluded that it belonged to a novel family of type III alcohol dehydrogenases (Daniel et al., 1995a). The *dhaT* gene was also cloned from *C. pasteurianum*, and it showed an 89.8% similarity to the *C. freundii* gene (Luers et al., 1997).

The enzyme 1,3-propanediol dehydrogenase has also been purified from *E. agglomerans* and showed a pH optimum of 7.8. The enzyme was inhibited by NAD^+ and PDO, which may help to explain the finding that *E. agglomerans* accumulates 3-hydroxypropionaldehyde (Barbirato et al., 1997b).

Glycerol dehydratase is a key enzyme in the production of PDO from glycerol. It has been proposed that it is the rate-limiting step in PDO formation for *C. butyricum* (Abbad-Andaloussi et al., 1996), *K. pneumoniae* (Ahrens et al., 1998), and *C. freundii* (Boenigk et al., 1993). The enzyme has been purified and it requires coenzyme B12 (adenosylcobalamin) for activity (Schneider, et al., 1970). The genes from *Citrobacter freundii* (Daniel and Gottschalk, 1992; Seyfried et al., 1996), *Clostridium pasteurianum* (Macis et al., 1998), *Klebsiella pneumoniae* (Tobimatsu et al., 1996), and *Klebsiella oxytoca* (Tobimatsu et al., 1995) have been cloned and studied. The enzymes have sequence homology and similar substrate specificities and catalyze the conversion of 1,2-diols to deoxy aldehydes. The enzymes are similar in molecular weight and are made up of three subunits. In *Citrobacter freundii* the enzyme is $\alpha 2\beta 2\gamma 2$ and has a molecular weight of approximately 196 kDa (Seyfried et al., 1996).

The *dha* genes from *Klebsiella pneumoniae* were imported by conjugation into *E. coli*. The *E. coli* was then able to grow anaerobically on glycerol, but it did not produce PDO. All enzymes of the *dha* regulon were detected except for glycerol dehydratase. The growth yield of the *E. coli* was reduced and may be explained by the lack of glycerol dehydratase and difficulties in achieving redox balance (Sprenger et al., 1989). The presence of adenosylcobalamin was shown to be important for functioning of glycerol dehydratase. Addition of this cofactor and reduction of growth temperature from 37 to 28°C restored glycerol dehydratase activity and allowed *E. coli* to produce PDO from introduced *C. freundii dha* genes (Daniel and Gottschalk, 1992).

In a different study, PDO production from glycerol was seen using *E. coli* containing cosmid vector-introduced *K. pneumoniae* genes. The yield of PDO from glycerol in complex media was much higher than in defined media. The yield of 0.46 moles/mole was comparable to that reported for the *Klebsiella*, which was the source of the *dha* genes. In this case, apparently the glycerol dehydratase functioned in *E. coli*, and the authors speculate that this could be due to the large size of the DNA insert (Tong et al., 1991).

Glycerol dehydratase is inactivated by its substrate, glycerol. In *Citrobacter freundii*, *dhaF* and *dhaG* were identified as being responsible for reactivation of glycerol dehydratase. Coenzyme B12, ATP, and Mg^{+2} were necessary for this reactivation. By transcriptional analysis these two genes were shown to be expressed in glycerol but not glucose grown cells. The *dhaF* and *dhaG* subunits were purified and shown to form a tightly bound complex with a molecular mass of 150 kDa. The purified complex was tested for its ability to reactivate glycerol dehydratases of *C. freundii*, *K. pneumoniae*, *C. pasteurianum*, and the diol dehydratases of *K. oxytoca*, *Salmonella typhimurium* and *Propionibacterium freudenreichii*. The *C. freundii* and *K. pneumoniae* dehydratases were the only enzymes reactivated, which indicated that the *dhaF-dhaG* complex is specific for glycerol dehydratases of closely related organisms and does not function to reactivate diol dehydratases (Seifert et al., 2001). Similar reactivation gene products were found in *Klebsiella oxytoca* and were named "*ddrA*" and "*ddrB*" (Tobimatsu et al., 1999).

The genes encoding for glycerol dehydratase in *C. freundii* are the *dhaBCE* genes, which form part of the *dha* regulon. As mentioned earlier, the other key enzymes (*dhaD*, *dhaK* and *dhaT*) form part of the pathway. There is also a transcriptional activator protein *dhaR* (Daniel et al., 1999). The genes encoding for glycerol dehydratase in *Klebsiella oxytoca* are termed "*pddA*," "*pddB*," and "*pddC*" (Tobimatsu et al., 1995).

The mix of products derived from the dihydroxyacetone to pyruvate pathway varies depending on the microorganism. *Klebsiella* can produce ethanol, formate, acetic acid, and 2,3-

butanediol, while *Clostridium* can also produce butyric acid. Some strains of *C. pasteurianum* also form butanol (Heyndrickx et al., 1991). The pathways of glycerol fermentation and subsequent pyruvate use as drawn by Biebl et al. (1999) are shown in Fig. 28. The mix of products varies depending on the organism and the growth conditions, including pH and glycerol concentration.

Effort in calculating the theoretical yields of PDO and the mix of products formed has been considerable. To achieve economic production and maximize PDO yield, it is desirable to understand and eventually optimize by-product formation. The story is a complex one, as many different growth conditions have been examined and a variety of responses from different microorganisms can be seen.

The formation of PDO and its by-products under different conditions has been measured for *Klebsiella* and *Clostridium*, and some examples are given in the following paragraph. Using *Klebsiella aerogenes* NCTC 418, various growth-limited conditions were measured in an anaerobic chemostat. Under glycerol-limited conditions, energy generation was lower than expected owing to PDO formation. Under other growth limiting conditions such as phosphate, ammonia, or sulfate limitation, it was found that the products acetate, ethanol, succinate and PDO were formed but in differing amounts depending on the limitation. When glycerol was given in a pulse to glycerol-limited cultures, the glycerol was consumed quickly with most of the glycerol being converted to PDO and acetate (Streekstra et al., 1987).

A calculation of theoretical yield was done for *Klebsiella pneumoniae*. Two separate cases for ATP generation were assumed, one for acetic acid production and the other for ethanol. The acetic acid pathway was calculated to have a five times higher PDO yield than the ethanol pathway, while the ethanol pathway gave higher biomass and ATP yields. The theoretical maximum yield when acetate and not ethanol is produced is 0.64 moles of PDO/mole of glycerol (Zeng et al., 1993).

The theoretical yields for various conditions were calculated for *C. butyricum* and tested in chemostat culture. The products butyrate and/or acetate are necessary for generating ATP for biosynthesis. Butyrate production is more efficient for biomass synthesis as it yields a higher amount of ATP. The maximum PDO production occurs when butyrate and hydrogen are not produced. The theoretical maximum PDO yield is 0.72 moles of PDO/mole of glycerol in the case of no butyrate and no hydrogen formation.

The theoretical results were compared with experimental data of *C. butyricum* grown under glycerol limitation and excess in continuous culture. Significant differences were seen with the highest yield value of PDO seen under conditions of glycerol excess. The effects of dilution rate in continuous culture were also measured on *K. pneumoniae* and *C. butyricum*. Both reached about 80% of the theoretical maximum under high dilution rate, while *Klebsiella* reached a much higher yield and productivity under low dilution rate (Zeng, 1996).

Zeng et al. (1997) summarized this work and wrote equations for the biomass yield and the formation of various products by *C. butyricum*. The equation for biomass formation is

$$C_3H_8O_3 + 3NH_3 + 35ATP \rightarrow 3C_4H_7O_2N + 4NADH_2 + 6H_2O \quad (16)$$

For acetate formation:

$$C_3H_8O_3 \rightarrow C_2H_4O_2 + 2NADH_2 + 2ATP + CO_2 + H_2 + H_2O \quad (17)$$

For butyrate formation:

$$2C_3H_8O_3 \rightarrow C_4H_8O_2 + 2NADH_2 + 3ATP + 2CO_2 + 2H_2 \quad (18)$$

For 1,3-propanediol formation:

$$C_3H_8O_3 + NADH_2 \rightarrow C_3H_8O_2 + 2H_2O \quad (19)$$

The equations represent reactions in the glycerol fermentation that the cell needs to regulate to achieve an overall balance in energy generation, reducing equivalents, and biomass. The various reactants are balanced in the overall cell metabolism. The authors calculated cases where either one or the other pathway is the only one used for energy generation and with maximum or no H_2 formation. The acetic acid pathway gave a PDO yield about 30% higher than butyric acid formation, with a theoretical maximum PDO yield of 0.65 moles of PDO/mole of glycerol with H_2 formation. Thus, the acetate pathway is more attractive for PDO production (Zeng et al., 1997).

When *Enterobacter agglomerans* is grown anaerobically in a chemostat with glycerol, PDO is the major product, and acetate, ethanol, and formate are also produced in significant amounts, while lactate and succinate are minor products. When *E. agglomerans* is grown on glucose, PDO is not made and the enzymes of the *dha* regulon are decreased by 30- to 120-fold. The effects of different growth rates were also measured. When the dilution rate increased from

0.05–0.31 h^{-1}, the activities of the enzymes of the oxidative pathway (glycerol dehydrogenase and DHA kinase) decreased 2.5-fold. With the higher dilution rate, glycerol dehydratase increased 34-fold and the 1,3-propanediol dehydrogenase level remained stable. Enzymes of central metabolism, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and pyruvate formate lyase all increased slightly, except for pyruvate dehydrogenase, which was reduced slightly. Pyruvate dehydrogenase, which is normally expressed aerobically, does appear to be responsible for a portion of the carbon flow in *E. agglomerans* under anaerobic glycerol-utilizing conditions. The flow of pyruvate through pyruvate formate lyase was reduced at higher dilution rate (Barbirato et al., 1997a).

Hydrogen is generated during glycerol fermentation and it should not be considered as merely a waste product. When anaerobic growth under low residual glycerol was compared for *C. butyricum* and *K. pneumoniae*, it was *K. pneumoniae* that was able to incorporate more of the glycerol electrons into hydrogen. When various media components such as phosphate, ammonia, and sulfate are limited, hydrogen but not PDO formation is also limited (Solomon et al., 1994).

The balance and recovery of reducing equivalents in glycerol fermentations are important to the final yield. Data from various authors were compared for *K. pneumoniae* fermentations run under different glycerol concentrations. Under glycerol limitation, a portion of the $NADH_2$ released from the dehydrogenases is oxidized through pathways other than those leading to PDO, ethanol, lactate, and 2,3-butanediol. These reducing equivalents are discharged by the cell as molecular hydrogen. In cultures with glycerol excess, the majority of reducing equivalents appear in PDO and only a small part is released as hydrogen. The authors suggest that the interconversion of $NADH_2$ and H_2 is mediated by pyruvate : ferredoxin reductase and hydrogenase (Zeng et al., 1993). This mechanism is also likely operating in *Clostridium*, as with some strains of *Clostridium* the theoretical yield of PDO was exceeded and it was attributed to some portion of ferredoxin-bound molecular hydrogen transferring reducing equivalents to NAD, which was used to reduce additional glycerol to PDO (Biebl et al., 1992).

Much of the work on PDO production has been done with only a few strains. Various laboratories have gone to various environments to isolate new PDO producers to look for microorganisms with potentially superior properties. For example, several strains were isolated from sewage sludge, and the PDO yield and mix of products were compared. These isolates were identified as *Klebsiella* and *Citrobacter*. The *Citrobacter* strains yielded primarily PDO and acetic acid, with only small quantities of lactic acid and ethanol formed, while the *Klebsiella* strains produced more ethanol and lactic acid. *Citrobacter* had a higher product yield than *Klebsiella*, yielding 0.65 moles/mole of glycerol compared to the *Klebsiella* yield of 0.53 moles/mole of glycerol. *Citrobacter* had an overall productivity lower than that of one of the *Klebsiella* strains, as *K. pneumoniae* DSM 2026 had the highest titer and the best productivity (Homann et al., 1990).

The ability to use glycerol is not widely distributed among the *Clostridium*. A number of clostridal strains from culture collections were tested which did not grow on glycerol. Using a selection of growth on glycerol, four strains were isolated from soil and mud samples. All were identified to be *C. butyricum*. The most active strain, designated "SH1" and deposited as DSM 5431, was able to produce 56 g/liter of PDO in 29 hours from 110 g/liter of glycerol in a batch fermentation (Biebl et al., 1992).

Additional PDO-producing *Clostridia* have been isolated and their by-products have been characterized. Strains of *C. butyricum* and *C. pasteurianum* were examined for both solvent and hydrogen production from glycerol. In batch fermentations, *C. butyricum* LMG 1212t3 converted glycerol to PDO, and the addition of acetate increased glycerol utilization fourfold with more hydrogen and butyrate and less PDO formed. The other strain examined, *C. pasteurianum* LMG 3285, used most of the glycerol in the presence or absence of acetate and produced mainly butanol and hydrogen, along with butyrate, ethanol, acetate, formate and PDO (Heyndrickx et al., 1991).

Several enterobacteria were compared for their ability to produce PDO. *Escherichia coli* lacks the *dha* regulon and is able to grow on glycerol using only the genes of the *glp* regulon, which require the presence of molecular oxygen or nitrate as an exogenous electron acceptor. *Klebsiella pneumoniae* is known to utilize glycerol and produce PDO whereas *Klebsiella oxytoca* made much less PDO from glycerol. *Klebsiella planticola*, *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis* and *Hafnia alvei* did not produce PDO from glycerol. Some of the *Klebsiella* did not possess a *dha* regulon, while others demonstrated activity of some of the enzymes in the pathway. *Klebsiella oxytoca* had 1,3-propanediol dehydrogenase activity and no glycerol dehydratase activity. *Klebsiella ozaenae* has only glycerol dehydrogenase activity (Bouvet et al., 1994). Like among the clostridia, the ability to grow anaerobically on glycerol and form PDO does not appear to be widespread among species of *Klebsiella*.

A microorganism isolated from the anoxic mud of a distillery wastewater digestor, *Enterobacter agglomerans*, was also shown to ferment glycerol to PDO. Acetate is the primary by-product, but formate, ethanol, succinate and lactate are also formed. The initial concentration of glycerol affects the fermentation profile of products. At the high glycerol concentration of 100 g/liter, the PDO yield is higher, lactate concentration is reduced, and ethanol is not produced. Acetate was the primary by-product formed that generated ATP. At high glycerol concentrations, the substrate was not completely consumed, which suggested the accumulation of an inhibitory factor (Barbirato et al., 1995).

The inhibitory compound (3-hydroxypropionaldehyde) accumulates when glycerol is added at high initial concentrations. The highest levels are seen in *E. agglomerans*, but 3-hydroxypropionaldehyde is also observed in cultures of *C. freundii* and *K. pneumoniae* grown in high glycerol concentrations. Both natural and synthesized 3-hydroxypropionaldehyde were shown to have a bacteriostatic effect (Barbirato et al., 1996a). Culture pH affected 3-hydroxypropionaldehyde formation, as it accumulated much earlier at pH 6 compared to pH 8. As 3-hydroxypropionaldehyde accumulated, the NAD/NADH ratio increased. The authors propose that the PDO dehydrogenase is inhibited by NAD, and this increased nucleotide ratio could be responsible for the accumulation of 3-hydroxypropionaldehyde. The effects of 3-hydroxypropionaldehyde were measured on the activity of the enzymes involved in the anaerobic dissimilation of glycerol, and the enzyme glycerol dehydrogenase demonstrated the greatest sensitivity (Barbirato et al., 1996b).

The fermentation of glycerol to PDO is also sensitive to the various products produced. By use of a pH auxostat, growth of *Clostridium butyricum* was shown to be inhibited by concentrations of 60 g/liter of PDO, 27 g/liter of acetic acid, and 19 g/liter of butyric acid. Since acetic and butyric acid would be produced at lower levels in a typical fermentation, the inhibition by PDO is expected to be the most important consideration. Butyric acid appears to be more toxic than acetic acid. The undissociated forms of the acids are responsible for the inhibitory effects in fermentation (Biebl, 1991).

Externally added acids appear to have a two to three-fold lower inhibition than those produced by the microorganism, which could be attributed to transport through membrane lipids (Zeng et al., 1994b). The time of addition of PDO also affects its inhibition, as an initial PDO concentration of 65 g/liter stops glycerol fermentation, whereas with an initial concentration of PDO (50 g/liter) and glycerol (70 g/liter), the final PDO concentration reaches 83.7 g/liter with complete consumption of glycerol (Colin et al., 2000). This suggests that high PDO concentrations inhibit growth, but not the actual PDO formation.

1,2-Propanediol The chemical synthesis of 1,2-propanediol (1,2-PD) results in the mixture of the R and S isomer. Direct fermentation processes yield either the R or the S isomer, which may provide an advantage as a chiral synthon. Two main biochemical processes for the synthesis of 1,2-PD are known. The first pathway involves the metabolism of deoxyhexoses through the intermediate lactaldehyde. In the second pathway, methylglyoxal (Inoue and Kumura, 1995) is derived from dihydroxyacetone phosphate, and methylglyoxal is reduced to 1,2-propanediol. The microbial formation is covered in reviews (Cameron et al., 1998; Bennett and San, 2001).

The formation of 1,2-propanediol by catabolism of 6-deoxyhexose sugars such as fucose and rhamnose by bacteria and yeast has been known for many years (Suzuki and Onishi, 1968; Ghazvinizadeh et al., 1972; Cocks et al., 1974; Turner and Robertson, 1979; Weimer, 1984b). This pathway forms S-1,2-propanediol through cleavage of the sugar into dihydroxyacetone phosphate and S-lactaldehyde, which are then reduced to S-1,2-propanediol. A low cost source of these 6-deoxysugars is not readily available, making this fermentation route impractical. A diagram of the pathway as it is understood is below and taken from the review by Bennett and San (Bennett and San, 2001; Fig. 29).

Clostridium sphenoides DSM 614 was shown to form both D(–)-1,2-PD and D(–)-lactate from glucose but only under phosphate limiting conditions. Ethanol and acetate were the major products at a phosphate concentration of 0.4 mM and above. The 1,2-PD formation began at phosphate concentrations below 80 μM. Lactate also increased under low phosphate conditions. Besides glucose, 1,2-PD was formed from D-fructose, cellobiose, L-rhamnose and L-fucose. The activities of the enzymes methylglyoxal synthase, methylglyoxal reductase, and 1,2-PD dehydrogenase were all detected in the cell. Methylglyoxal synthase, the first enzyme of the methylglyoxal bypass, is strongly inhibited by phosphate (Tran-Din and Gottschalk, 1985). The possible routes to 1,2-propanediol are summarized by Bennett and San (Bennett and San, 2001; Fig. 30).

Besides *Clostridium sphenoides*, the organism *Clostridium thermosaccharolyticum* (now called "*Thermoanaerobacterium thermosaccharolyticum*") also produces R-1,2-PD and acetol from

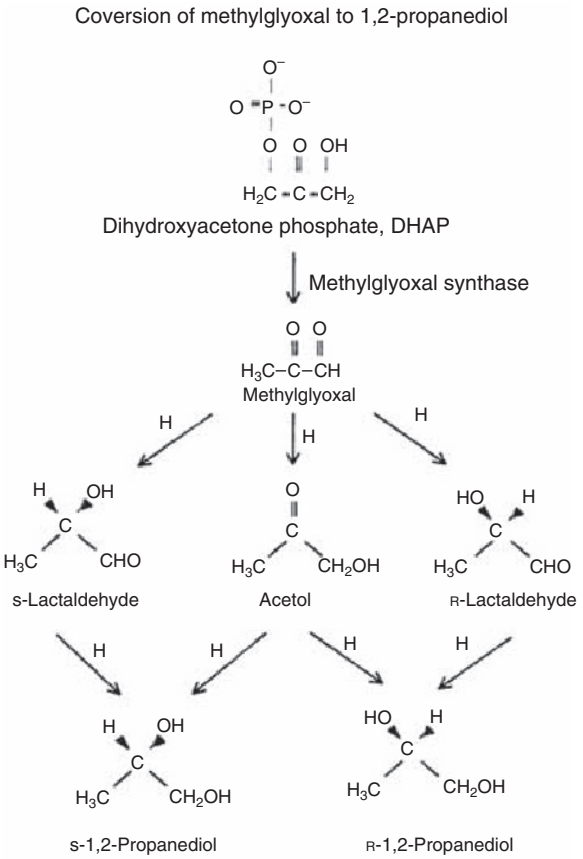

Fig. 29. Conversion of deoxy sugars to 1,2-propanediol. From Bennett and San (2001), with permission.

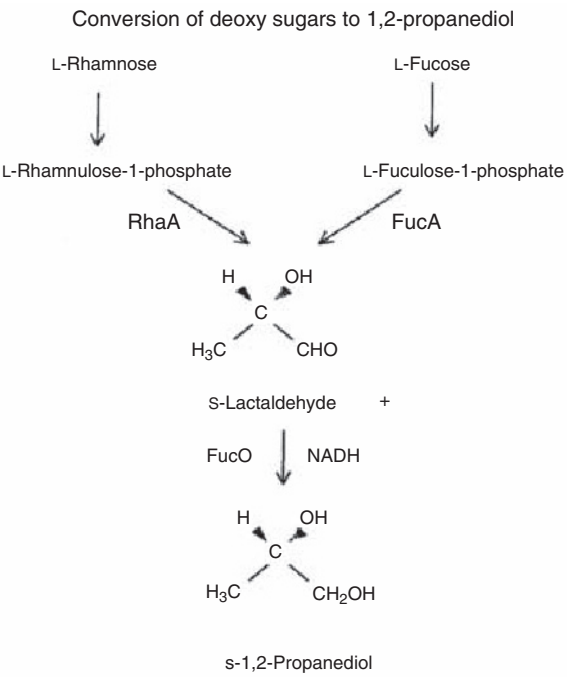

Fig. 30. Routes to 1,2-propanediol synthesis through methyglyoxal. From Bennett and San (Bennett and San, 2001), with permission.

glucose. Other products made include acetate, ethanol, and lactate. In a fed-batch fermentation, the yield of 1,2-PD on glucose was 0.27g/g at 30.7 hours. The maximum titer reached was 7.9 g/liter. In contrast to *C. sphenoides*, the *C. thermosaccharolyticum* production of 1,2-PD was not enhanced by phosphate limitation. A maximum theoretical yield of 0.42 grams of 1,2-PD per gram of glucose was calculated (Cameron and Cooney, 1986).

A novel route to 1,2-PD by the anaerobic conversion of lactic acid by *Lactobacillus buchneri* was described. Lactic acid had been shown to disappear in silage inoculated with *L. buchneri*. This organism anoxically converts 2 moles of lactic acid to 1 mole of acetic acid and 1 mole of 1,2-propanediol. This conversion does not occur unless the organism is at a pH below 5.8. In the proposed pathway, 1 mole of lactic acid is converted to 1,2-PD through lactaldehyde, while the other mole of lactic acid is converted through pyruvate to acetate (Oude Elferink et al., 2001).

The enzyme methylglyoxal synthase, which catalyzes the conversion of dihydroxyacetone phosphate to methylglyoxal, has been cloned from *Clostridium acetobutylicum* and expressed in *E. coli*. This enzyme has been found in number of bacteria. The clostridial enzyme has a pH optimum of 7.5, and when dihydroxyacetone phosphate, glyceraldehyde, and glyceraldehyde-3-phosphate were examined as substrates, only dihydroxyacetone phosphate could be converted to methylglyoxal (Huang et al., 1999). Other work with this pathway has included improvement of the *Thermoanaerobacterium thermosaccharolyticum* fermentation as well as the metabolic engineering of *E. coli* to produce 1,2-PD. This work will be discussed in the subsection Research and Development in this Chapter.

Commercial Applications

The product 1,3-propanediol and the properties of polytrimethylene terephthalate (PTT) were discovered in the early 1940s, but the high cost of PDO manufacture inhibited widespread commercial use (Anonymous, 1999). In the mid-1990s, Shell Chemical Company announced that it had developed a new chemical route to the production of PDO. It called its new PTT-based fiber "Corterra." Shell's process uses a hydroformylation of ethylene oxide and recovers the intermediate 3-hydroxypropionaldehyde which is then hydrogenated to PDO. Water and impurities are removed to produce a fiber grade PDO

(Shelley and D'Aquino, 1999). Shell first constructed a small development plant that had a nameplate capacity of 3,200 metric tons per year. It has constructed a world-scale PDO plant at Geismar, Lousiana, with a capacity of 75,000 metric tons per year that was scheduled to be online in late 1999. This is enough PDO to make just under 300,000 metric tons of PTT. The true potential of this new polymer may be in replacing large parts or all of the polyethylene terephthalate market (PET), which worldwide is around 20 million metric tons (Greenberg, 1999).

Degussa developed a chemical synthesis process for PDO in which propylene is catalytically converted to acrolein, which is then hydrated to 3-hydroxypropionaldehyde. PDO is formed by hydrogenation, and fiber-grade material is produced by distillation. This technology was licensed by DuPont, and Degussa and DuPont have been working together to produce PDO at a 20-million-lb/yr facility in Germany that came online in 1998. DuPont has named their polyester "Sonora." The polypropylene route is not believed to be as economical as the ethylene oxide route, primarily owing to the cost of the process plant (Shelley and D'Aquino, 1999).

DuPont is pursuing a third route to PDO, with a bioprocess that uses glucose as a starting material. This work has been done in collaboration with Genencor (Potera, 1997). The anaerobic conversion of glycerol to 1,3-propanediol was well known and has been documented in the scientific literature over many years. The DuPont and Genencor team of scientists created a microorganism that was able to convert carbohydrate to PDO, or "3G" as it is referred to by DuPont (Shelley and D'Aquino, 1999). With this new technology, low-cost renewable resources can be used as a raw material source. A glycerol dehydratase or diol dehydratase enzyme is expressed in a microorganism such as *E. coli*, which is able to utilize sugars such as glucose. Initial results showed that the level of PDO produced by recombinant microorganisms was very low (Laffend et al., 1997).

DuPont and Genencor have reported a 500-fold improvement in productivity (Moore, 1999). They announced that they would extend their research and development (R&D) collaboration through the end of 2001. DuPont is scaling up the bioprocess by an announced joint venture with Tate and Lyle. At their Decatur, Illinois, corn-wet mill, A.E. Staley, a subsidiary of Tate and Lyle, produces raw materials from renewable resources such as corn. PDO production is being tested by DuPont at a 200,000-lb/yr pilot plant there, and DuPont has said that it expects commercialization of the bio-based PDO in 2003 (Wood, 2001). Though the product can in part be called a "green" chemical since the PDO is derived from renewable resources, the terephthalate part of the polymer is derived from petrochemicals.

In large, commodity scale fermentations such as this one, the price of raw materials is significant and can account for 70% or more of total cost (Wilke, 1999). The variability in supply and raw material cost can affect the cost of production. Since most of the scientific effort on PDO in the past was conversion of glycerol, the DuPont/Genencor process should represent a potential cost advantage since it is based on glucose. Glycerol prices range between US$1 and $2/kg. At the yield of around 50% on glycerol, the cost of PDO would be high compared to production on glucose, which sells for below US$0.20 per pound (Wilke, 1999).

The long-term prospects for PDO appear excellent, as the fibers made from PTT are expected to compete with nylon and spandex and are touted to have superior properties. According to Shell, PTT fibers are chemical and stain resistant but have elasticity and good colorfastness. The fibers also dye easily and will be priced in the range of $US0.90–1 per pound. The Shell Corterra fiber won the R&D magazine award as one of the most technologically significant new products in 1998 (Welling, 1998). Besides apparel, the fibers can be used in carpet, engineering thermoplastics, and non-wovens. Other potential commercial applications include paints, adhesives, laminates and coatings (Anonymous, 1999).

While PDO use is just beginning to grow and become a large volume commodity chemical, propylene glycol is already made in vast quantities. Total production in the United States is estimated at over one billion pounds, with unsaturated polyester resins accounting for about 25% of the demand (Ouellette, 2000). One of the major uses of unsaturated polyester resins (UPR) is in construction, and demand for UPRs and hence propylene glycol is driven by the strength of the economy. As propylene glycol is safer than ethylene glycol, its use in antifreeze has climbed so that almost one fifth of the demand for propylene glycol goes to antifreeze. Other uses for propylene glycol include liquid laundry detergent, pharmaceuticals and cosmetics, and other miscellaneous uses (Anonymous, 1996a). The fact that it is clear and also has good emollient properties makes it desirable for these uses. Propylene glycol is "generally regarded as safe" (GRAS) by the United States Food and Drug Administration and has use in human foods and certain animal foods.

The demand for propylene glycol is expected to grow about 3% per year in the United States. The manufacture is by a chemical process using

hydration of propylene oxide. Propylene oxide is made from propylene, and prices of propylene have increased, tied to oil price increases. Propylene oxide also enjoys a high demand due to the growth of its biggest end use, polyurethanes (Ouellette, 2000). With propylene oxide costs being between $US0.20 and 0.30 per pound, it is difficult to envision a biological route to propylene glycol that can compete economically. However, as mentioned previously, the chemical route makes a mixture of isomers whereas the biological route makes a pure isomer. If chirality turns out to be important in an end use, then a bio-based process becomes much more attractive. Although there has been work on bio-based processes in academic labs, in contrast to PDO, no commercial activity is evident for a fermentation process for propylene glycol.

Research and Development

1,3-Propanediol With the increasing potential of use of PDO in polymers, there has been an impetus to improve the yield and rates of the various PDO producing strains. Various types of reactors and feeding strategies, the use of cosubstrates, and isolation of new organisms have all been approaches towards improvement of this fermentation based on glycerol. Also, metabolic engineering approaches have been used to widen the substrate range and to combine pathways of various microorganisms. The different approaches will be discussed in greater detail below.

A great deal of the focus has been on *Clostridium butyricum*, and the production of PDO from glycerol has been demonstrated at a scale of 2 m^3 using the strain *C. butyricum* DSM 5431. In this work, air-lift and stirred tank reactors were compared, and no difference in product formation was seen. The effect of pH and temperature was also studied and a temperature of 35°C and a pH of 7.0 were selected as optimal. PDO (46–58 g/liter) and productivities (2.3–2.9 g/liter/h) were seen at both small and large volume (Gunzel et al., 1991).

A fed-batch strategy was used to avoid substrate inhibition. This system measured CO_2 produced, and the glycerol feed volume was calculated on the basis of a constant stoichiometry. Using strain VPI 3266 of *Clostridium butyricum*, batch and fed-batch fermentation processes were compared. Generally, the fed-batch results were about twofold better, with 65 g/liter of PDO produced at a rate of 1.2 g/liter/h and a PDO yield of 0.57. The fed-batch approach helps circumvent the organism's sensitivity to high initial concentrations of glycerol (Saint-Amans et al., 1994). Another important process feature is the composition of the medium. A low nutrient, or minimal, medium was devised for *C. butyricum*. Biotin was shown to be the sole growth factor required by this organism, and when biotin was present, the organism was able to produce 67 g/liter PDO from 129 g/liter of glycerol, with a mixture of acetate and butyrate as by-products. Use of this media also provided evidence that nitrogen could be a limiting factor, especially when the carbon-to-nitrogen ratio was less than 81 : 1 (Himmi et al., 1999).

Continuous fermentations have also been used to improve PDO productivity. In a continuous *Klebsiella pneumoniae* fermentation, when glycerol was added in excess at an inlet substrate feed of approximately 74 g/liter, PDO and various by-product acids were the major products. Under glycerol limitation (an inlet concentration of approximately 15 g/liter), ethanol and hydrogen were the major products. The effect could be monitored by the evolution rates of hydrogen and CO_2. In glycerol excess, CO_2 evolution becomes higher than H_2 evolution, while it is the reverse in glycerol limitation (Solomon et al., 1994). Understanding the effects of various dilution rates on product distribution will help maximize the output of a PDO fermentation process.

Dilution rate was also shown to have an effect on PDO production by *K. pneumoniae*, with the highest PDO concentration achieved with a low dilution rate. This result differs from others mentioned below, and the authors state that in previous studies, the continuous cultures were run in a different fashion, with the substrate concentration in the feed constant, while the dilution rate was varied. In this study, the maximum experimental values seen were a PDO concentration of 35–48 g/liter and a productivity of 4.9–8.8 g/liter/h (Menzel et al., 1997).

In contrast, in a *Citrobacter freundii* fermentation, the highest PDO productivity was obtained under conditions of glycerol limitation at a high dilution rate. The highest values reported were 41.5 g/liter of PDO produced from 80.5 g/liter of glycerol, with an overall productivity of 1.38 g/liter/h (Boenigk et al., 1993). Streekstra et al. (1987) also showed that a higher dilution rate improved productivity with *Klebsiella*.

There has also been some work on new reactor types in the production of PDO. Pflugmacher and Gottschalk (1994) used cells of *Citrobacter freundii* DSM 30040 immobilized on polyurethane carrier particles. In this case, the productivity increased with the dilution rate, and the maximum productivity was very high, at 8.2 g/liter/h. A cell recycling system was tried with *Clostridium butyricum* using hollow fiber modules made from polysulfone. In this system, a productivity increase was seen; however, it could

be achieved only in a narrow section of the theoretical range (Reimann et al., 1998b). The potential of new types of reactors and fermentation configurations still needs additional exploration before an optimal system is found for PDO production from glycerol.

Identifying new microorganisms with various desirable features for PDO production is another avenue that has been explored. Some desirable features for PDO production that would help to reduce fermentation operating costs include the ability to grow at higher temperature, the ability to grow on crude glycerol stocks, tolerance to high substrate and product concentrations, and the ability to produce PDO at a higher rate.

Various mud and soil samples were examined and several glycerol-fermenting clostridia were isolated. The most active strain was able to convert up to 110 g/liter of glycerol to 5 g/liter of PDO in 29 h. In this strain, H_2 production was lower than expected, and reducing potential was transferred from ferredoxin to NAD, which increased PDO yields (Biebl et al., 1992). This strain, SH1, was renamed "DSM 5431" and was the parent strain used in further work to isolate more mutants with even more desirable characteristics.

Product tolerant mutants of *C. butyricum* DSM 5431 were isolated by Abbad-Andaloussi et al. (1995). Besides showing product tolerance, these mutants also yielded a higher biomass in fermentation. To further explore the use of these mutants, a fed-batch strategy was used which coupled the feeding of glycerol and ammonium to alkali consumption. A mixture of glycerol, ammonium chloride, and sodium phosphate was fed when initial glycerol was nearly three quarters depleted. Using this feeding strategy, one of the mutants was able to reach a concentration of 70 g/liter of PDO. The greatest increase, however, appeared to be in volumetric productivity, which more than doubled from 0.62–0.83 g/liter/h to 1.4–2.4 g/liter/h (Reimann and Biebl, 1996).

The mutants of DSM 5431 showed other interesting properties. Under conditions of glycerol excess, the parent strain showed a significant decrease in substrate conversion, while the product tolerant mutants continued to consume glycerol and form product at a constant level. The key enzymes of PDO formation are higher in the mutant strains. With increased carbon flow, the wildtype, but not the mutants, showed increased levels of NADH and NAD^+ and acetyl-CoA. The wildtype generated more reducing equivalents by producing more acetate and less butyrate (Reimann et al., 1998a).

One way by which cost can be addressed is by the use of lower cost, cruder raw materials in fermentation. Ten new environmental *Clostridium* strains were isolated and identified as *C. butyricum*. Of these ten, four were able to ferment industrial glycerol, which was derived from transesterification of rapeseed oil, while *Clostridium* sp. obtained from culture collections could not. The best new isolate, named "E5," was also more resistant to high levels of PDO. The new isolate was able to attain PDO yields on crude glycerol comparable to those of DSM 5431 on purified glycerol, reaching 58 g/liter of PDO from 109 g/liter of glycerol (Petitdemange et al., 1995). Another *Clostridium butyricum* isolate was also tested on industrial glycerol under a variety of conditions, including single-stage and two-stage fermentation. The maximum volumetric productivity was shown to be 5.5 g/liter/h. The strain was also shown to be very tolerant of high PDO concentration, up to 80 g/liter (Papanikolaou et al., 2000).

Another means of achieving process efficiency is by the use of thermophilic bacteria. With bacteria able to withstand high temperatures, hot effluents from fat cleavage plants can be used without cooling. It may also be possible to easily remove volatile by-products such as ethanol from the broth. A number of thermophilic producers of PDO from glycerol were isolated and the most active strain, called "AT1," was investigated further. Its pH optimum was 5.8–6.0 and temperature optimum was 58°C. In batch fermentations, its productivity was much lower than that of mesophilic *Clostridium* and it appeared to be inhibited strongly by PDO and various by-products. This initial work showed promise, but further work needs to be done in improving this new strain or continuing to screen additional thermophilic strains (Wittlich et al., 2001).

The work described above has focused on the use of glycerol as the fermentation substrate. Several laboratories have also examined the use of cosubstrates by adding another hydrogen donor substrate to glycerol. Tong and Cameron (1992) used an *E. coli* with the *K. pneumoniae dha* genes and glycerol co-fermented with either glucose or xylose. The best cell and PDO yield were obtained on glucose and glycerol. With glycerol alone, the PDO yield was 0.46 moles/mole compared to 0.63 moles/mole with glucose and glycerol. Lactate, formate and acetate were the primary by-products observed in most of the *E. coli* fermentations.

Three fermentable cosubstrates (glucose, 1,2-ethanediol and 1,2-propanediol) were used in *C. butyricum* and *C. freundii* fermentations. Glucose was shown to enhance PDO yield in *C. butyricum* but not in *C. freundii*. On 1,3-ethanediol, the products were acetate and ethanol, and on 1,2-propanediol, the primary product was 2-propanol. The diols used electrons from

glycerol that were oxidized to acids for an even hydrogen balance. Because approximately two times more glucose than glycerol is required to enhance yields, addition of glucose as a cosubstrate does not appear promising as an economic advantage in *C. butyricum* (Biebl and Martin, 1995).

In yet another approach, cofermentations were carried out using mixed cultures of glycerol producers such as *Saccharomyces cerevisiae* grown together with glycerol utilizers such *C. freundii*, *K. pneumoniae* and recombinant *E. coli*. PDO levels varied depending on the organisms used and the ratio of the organisms. PDO production was observed when various fermentable sugars were used, as well as during fed-batch production (Haynie and Wagner, 1997).

Another very promising approach for improvement of biological PDO production is the use of metabolic engineering to create organisms with new capabilities. A number of different approaches have been tried with varying degrees of success. The genes of the *Klebsiella dha* regulon were introduced into *E. coli* by conjugation. *Escherichia coli* was able to then grow anaerobically on glycerol, but it did not produce PDO. This was most likely due to the lack of glycerol dehydratase (Sprenger et al., 1989). When the appropriate cofactor for glycerol dehydratase was supplied to *E. coli* containing the *Citrobacter freundii dha* regulon, it was able to produce PDO at relatively high amounts (Daniel and Gottschalk, 1992).

Tong et al. (1991) detected production of PDO after transfer of the *dha* genes of *K. pneumoniae* into *E. coli*. The introduction of a new pathway into a microorganism is termed "metabolic engineering." Taking this concept further, Skraly et al. (1998) constructed an operon of PDO genes that could be used in a number of hosts and where the regulation could be manipulated. The *dhaB* gene and the *dhaT* gene, which code for glycerol dehydratase and 1,3-propanediol oxidoreductase, respectively, were put under the control of a single promoter. The constructed operon also contained certain restriction sites that allow new promoters to be easily inserted. With the artificial operon, *E. coli* did produce PDO; however, the mix of products was different than previously reported.

The use of metabolic pathway engineering in *E. coli* may provide advantages over the natural producers. The tools to modify *E. coli* are readily available, the substrate range may be broadened, and genes can be added and subtracted to obtain optimal genes for the pathway (Cameron et al., 1993). This approach was also demonstrated in industrial laboratories and descriptions appear in the patent literature.

Raw materials, especially the carbon source, are a large portion of the cost of the PDO fermentation. As mentioned previously, the cost of glycerol can be fairly high and a lower cost substrate such as glucose or even biomass-derived sugar is more attractive. This is the approach taken by DuPont and Genencor as evidenced by several patents. Glycerol or diol dehydratase was placed into microorganisms able to use various sugars, and the production of PDO from one microorganism using a sugar such as glucose was demonstrated (Laffend et al., 1997; Laffend et al., 2000). The diol dehydratase enzyme that is responsible for the degradation of 1,2-propanediol and is part of the *K. pneumoniae pdu* operon was isolated and used. *Escherichia coli* transformants were able to use this enzyme to produce PDO from glycerol (Nagarajan and Nakamura, 1998). A further improvement cited is to use a "protein X" gene, which consists of an open reading frame (ORF) coding for a protein responsible for in vivo activation of dehydratase activity. "Protein X" codes for a 51 KD polypeptide that was originally thought to be a subunit of the diol dehydratase activity. Host cells with this "protein X" and with the three known genes which encode subunits of diol dehydratase show increased PDO production (Diaz-Torres et al., 2000).

Another aspect of the DuPont and Genencor development was to transfer genes responsible for glycerol production into a host that did not naturally produce glycerol. The genes coding for the enzymes glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase were transformed into *E. coli*, and glycerol production was seen (Bulthuis et al., 1998).

The various approaches were combined into recombinant organisms that contained all the following genes: glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, glycerol dehydratase, and 1,3-propanediol oxidoreductase. Microorganisms including *E. coli*, various *Klebsiella* species, and *Saccharomyces* were shown to produce PDO from glucose (Nakamura et al., 2000). The DuPont and Genencor effort has focused on *E. coli* because of the number of genetic tools available and since *E. coli* does not naturally produce PDO, no natural regulation needs to be overcome. The constructed *E. coli* strain equals or exceeds glycerol based natural organisms, with approximately 140 g/liter of PDO produced from glucose in 45 hours (Chotani et al., 2000).

1,2-Propanediol The industrial focus on 1,3-propanediol has sparked interest in the microbial production of 1,2-propanediol. Some work has focused on the fermentation process of 1,2-propanediol as well as the metabolic engineering

of pathways for its production. In early work with *C. thermosaccharolyticum*, various process conditions were examined such as temperature, pH, gas phase composition, and substrate concentration. This work was conducted in a volume of 2 liters. The maximum cell concentration achieved was in the range of 1.0 to 1.3 g/liter. The temperature range examined was 50–65°C and the optimal temperature for production was 60°C. At higher temperatures, lactate decreased and ethanol increased. The pH range studied was from 6.0 to 7.2. At the optimal pH of 6.0, a concentration of 5.6 g/liter of 1,2-PD was obtained. Other fermentation conditions were examined such as the use of H_2 in the headspace as well as the use of yeast extract, which was shown to have an important effect on 1,2-PD production. The optimal fermentation conditions were defined as 45 g/liter of glucose, 4.5 g/liter of yeast extract, a pH of 6.0, a temperature of 60°C, and the use of N_2 gas. Under these conditions, the final 1,2-PD concentration achieved was 9.05 g/liter with a yield of 0.20 g/g of glucose (Sanchez-Riera et al., 1987).

The cost of production of 1,2-PD could be lowered by the use of low cost, renewable sugars. D-Glucose, L-arabinose, and D-xylose are the major sugars from cellulosic biomass. Their use for 1,2-PD production was examined using *T. thermosaccharolyticum*. Also tested were L-galactose and lactose; lactose is of interest because it can be obtained at low cost from cheese whey (Altaras et al., 2001).

In earlier work, *T. thermosaccharolyticum* did not grow on lactose and D-galactose (Cameron and Cooney, 1986). This organism was selected to grow on these sugars by multiple serial transfers. When tested in fermentations, these adapted organisms produced primarily ethanol and lactate, and no 1,2-PD was detected. The five-carbon sugars arabinose and xylose showed more promise, and both yielded 1,2-PD during fermentation. Arabinose was fermented at a rate and yield similar to glucose, while xylose fermentation was slower and the yield was approximately half. Whey permeate was shown to be a useful substrate for 1,2-PD production but only after hydrolysis of the lactose and supplementation with yeast extract (Altaras et al., 2001).

Another approach towards the improvement of 1,2-PD production is the use of metabolic engineering. *Escherichia coli* was chosen as the host organism because many tools are available for genetic modification. Although it does not produce 1,2-PD from glucose, it is able to produce the intermediate methylglyoxal (Cameron et al., 1998). *Escherichia coli* strains expressing rat lens aldolase produced 1,2-PD and acetol from glucose (Altaras and Cameron, 1999).

Instead of rat lens aldolase, glycerol dehydrogenase from either *E. coli* or *K. pneumoniae* was overexpressed in *E. coli*. In this case *E. coli* produced R-1,2-PD from glucose. The metabolic pathway probably involves the reduction of methylglyoxal to R-lactaldehyde by the glycerol dehydrogenase, and the R-1,2-PD is produced by reduction of R-lactaldehyde. The overexpression of *E. coli* methylygloxal synthase alone causes *E. coli* to produce about the same amount of 1,2-PD as produced by the strains with overexpressed glycerol dehydrogenase. When both genes are overexpressed, the improvement in 1,2-PD production is even greater. Although the metabolic engineering of *E. coli* is promising, the maximum titer of 0.7 g/liter of 1,2-PD is low (Altaras and Cameron, 1999).

Further improvements were made by a series of metabolic engineering strategies. Activities of enzymes leading to the production of the byproduct, lactate, were abolished. A complete 1,2-PD pathway comprising glycerol dehydrogenase (*gldA*), methylglyoxal synthase (*mgs*), and 1,2-PD oxidoreductase (*fucO*) was put in the lactate minus background. The use of alcohol dehydrogenase in place of 1,2-PD oxidoreductase was also evaluated, but the use of 1,2-PD oxidoreductase ultimately gave better results. Using a fed-batch fermentation, *E. coli* lacking lactate dehydrogenase and containing *gldA*, *mgs*, and *fucO* genes gave the highest titers of 4.5 g/liter with a yield of 0.19 g of 1,2-PD per gram of glucose consumed (Altaras and Cameron, 2000). As *E. coli* is tolerant to over 100 g/liter of 1,2-PD (Cameron et al., 2000) and can be improved significantly by metabolic engineering, the potential for improving a process employing recombinant *E. coli* strains is significant.

Patents and Regulatory Issues

In the production of monomers such as PDO and 1,2-PD, there are many patents that cover the chemistry and process of synthesizing these chemicals. The separation of these chemicals from the synthetic mix is another important area of technology (Malinowski, 1999; Malinowski, 2000). There is patent activity, especially on the various new technologies for the chemical synthesis of PDO that have lowered the cost of the monomer and opened it for more widespread application. There are also undoubtedly numerous patents on the formulation of the various polymers that contain PDO and 1,2-PD and the applications and end use of these chemicals. It is beyond the scope of this review to cover the chemical process and application patents for PDO and 1,2-PD, inasmuch as a search of the United States Patent Database using the key

Table 35. Patents/patent applications for 1,3-propanediol and 1,2-propanediol production in the bioprocess area.

Assignee	Patent number	Year	Technology
Unilever	U.S. patent 5,164,309	1992	Production of PDO from glycerol by *Citrobacter*
Henkel Kommanditgesellschaft auf Aktien, Gesellschaft für Biotechnologische Forschung mbH	U.S. patent 5,254,467	1993	Production of PDO from glycerol
DuPont	U.S. patent 5,686276	1997	Production of PDO from a fermentable carbon source using a single microorganism
DuPont	U.S. patent 5,599,689	1997	Use of mixed microbial cultures to produce PDO
DuPont	U.S. Patent 5,821,092	1998	Production of PDO from glycerol with a recombinant bacteria expressing diol dehydratase
DuPont and Genencor	U.S. Patent 6,025,184	2000	Production of PDO from a fermentable carbon source by a microorganism containing a glycerol or diol dehydratase
DuPont and Genencor	U.S. patent 6,013,494	2000	Recombinant organisms for PDO production containing a variety of genes
Genencor	U.S. patent 6,136,576	2000	Use of recombinant microorganisms for PDO production
Genencor	PCT WO 00/70057	2000	Mutant 1,3-propanediol dehydrogenase
DuPont	PCT WO 01/12833	2001	Production of PDO from a fermentable carbon source in a single organism
Institut National de la Recherche Agronomique, Institut National des Sciences Appliquees de Toulouse, Centre National de la Recherche Scientifique	PCT WO 01/04324	2001	Production of PDO in the absence of coenzyme B12 or its precursors
DuPont	PCT WO 01/11070	2001	Isotopic fingerprinting of PDO produced from a fermentable carbon source
Wisconsin Alumni Research Foundation	U.S. patent 6,087,140	2000	Microbial production of 1,2-propanediol from sugar

Abbreviations: U.S., United States; PDO, 1,3 propanediol; PCT, Patent Cooperation Treaty; and PCT WO, international patent.

word "propylene glycol" turns up more than one thousand patents!

In PDO production by biological processes, the number of patents is fairly small, with the greatest patent activity occurring in the past five years. The patent activity for biological production of 1,2-PD is even less and will be summarized with the PDO patents. Although patents have been referenced where appropriate in the text, a summary of some of the patents on biological production is listed (Table 35).

Prospects for Production of 1,3-Propanediol and 1,2-Propanediol by a Bioprocess

There are many issues to be addressed in the production of any intermediate for the chemical industry with the use of a microbial process. Often the raw material is a chief cost component, so the microorganism must be able to use low cost substrates such as glucose derived from corn processing or other sugars that may be derived from the processing of low cost biomass. The yield of the product on the substrate as well as the ability to reach a fairly high product concentration is also important. Also, the by-products produced by the microorganism are important as they both impact yield as well as the design of the separation process.

Activity in designing new PDO processes using both a chemical route and a biological one has been considerable. Quite often, large volume chemicals are made by more than one chemical process, so it is possible that in the future, PDO may be made by more than one process, with one being a competitive, economic biological one. The advances in recombinant DNA technology

and the ability to create microorganisms with new pathways will help drive the production of industrial chemicals by biological means.

The possibility of future production of PDO by a biological process appears bright. By combining pathways of more than one organism into *E. coli*, DuPont and Genencor report a microorganism that can produce around 140 g/liter of PDO from glucose in less than 50 hours (Chotani et al., 2000). The availability of tools for genetically manipulating *E. coli* suggests that further improvements are likely to be made. Other challenges that remain will be the separation of PDO from broth and the purification of PDO such that it can be used in fiber production.

The near-term prospects for production of 1,2-PD via a biological process do not seem likely. A biological process must compete with well-known large-scale chemical processes using low-cost raw materials. The titers of 1,2-PD are still low; however, the use of host organisms such as *E. coli* may provide some opportunity to increase levels. An interest in bioprocess development may be sparked if a market or product opportunity can be identified for the racemically pure product produced by microorganisms (Cameron et al., 1998).

In the future, the economics of production of chemicals by biological processes may become more attractive with dwindling supplies and higher prices of oil. A bioprocess can also provide opportunities for the agricultural sector in the use of renewable resources such as corn. At some point, public interest in the use of renewable resources may also drive further development of these diols as well as other chemical products.

Section 10: 2,3-Butanediol Production

Introduction

A colorless odorless liquid, 2,3-butanediol (2,3-BD) is also called "2,3-butylene glycol," "dimethylethylene glycol," or "2,3-dihydroxybutane." It can be produced by a number of microorganisms in the genera *Serratia*, *Pseudomonas*, *Bacillus* and *Klebsiella*, but most investigators have focused on *Klebsiella pneumoniae* (also known as "*Klebsiella oxytoca*") and *Bacillus polymyxa*, which is now known as "*Paenibacillus polymyxa*" (Nakashimada et al., 2000). Reviews have covered the history, uses, and technical developments in this field (Afschar et al., 1993; Garg and Jain, 1995; Syu, 2001). The fermentation was described in the early part of the 20th century, but the height of interest came during wartime, as 2,3-BD can be converted to 1,3-butadiene, which is used in synthetic rubber (Syu, 2001).

Production of 2,3-BD is via a mixed acid fermentation pathway that also leads to a mix of acetate, lactate, formate, succinate and ethanol. Juni and Heym (1956) proposed that 2,3-BD is produced in bacteria from pyruvate through the intermediates α-acetolactate and acetoin. Also, microorganisms can degrade 2,3-BD (i.e., it is biodegradable), a feature that may add to its attractiveness as an industrial chemical.

2,3-BD has three stereoisomers: *dextro-* and *levo-* forms that are optically active and an optically inactive *meso-* form. Different microorganisms produce different stereoisomers, but generally a mixture of two is formed (Syu, 2001). Although stereoisomer formation has been studied and a new mechanism proposed (Ui et al., 1998), in much of the 2,3-BD-related work, stereoisomer formation is not measured.

Much of the focus of 2,3-BD work has been on the fermentation, and high productivities and high 2,3-BD concentration in fermentors have been achieved. Concentrations of 102.9 g/liter of butanediol and acetoin were achieved in 32 hours with *Enterobacter aerogenes* (Zeng et al., 1994a) and 113 g/liter of these combined products were achieved from *Klebsiella pneumoniae* (Yu and Saddler, 1983). Bioconversion yields of 0.45 g/g of glucose were also observed (Sablayrolles and Goma, 1984). The application of molecular biology and strain improvement may help to improve the fermentation process, although surprisingly little has been reported. The genes of the 2,3-BD operons were cloned from *Klebsiella terrigana* and *Enterobacter aerogenes* and characterized (Blomqvist et al., 1993). Reports describe the cloning of the gene responsible for the formation of *meso*-2,3-BD, which opens up the avenue of a strain producing a pure isomer (Ui et al., 1996; Ui et al., 1998).

The economics of the fermentation may be helped by the fact that the best 2,3-BD producers are able to grow and make product on a variety of sugars. The use of various low cost materials such as whey and hydrolyzed biomass has been investigated and shows promise for future use and will be discussed in more detail later (Yu et al., 1982b; Laube et al., 1984; Lee and Maddox, 1986; Champluvier et al., 1989).

The primary economic barrier to commercialization may not be the fermentation, but rather the separation of 2,3-BD from the media. It does not separate well by distillation, and chemical conversion of 2,3-BD in the broth and subsequent distillation are costly (Afschar et al., 1993). Other separation techniques that have been examined include salting out (Afschar et al., 1993) and countercurrent steam stripping (Garg and Jain, 1995). Continued fermentation and downstream improvements and a change in the economics of petrochemicals may at some future

date make 2,3-BD production from a bioprocess economically attractive.

Scientific Background

2,3-Butanediol is produced via a mixed acid pathway with a variety of end products. By this pathway the organism can make a less inhibitory neutral compound such as 2,3-BD in place of acid production (Johansen et al., 1975). *Bacillus polymyxa* catalyzes a number of biosynthetic reactions which the cell balances for energy and growth. The following reactions as described by de Mas et al. (1988) are included to illustrate the variety of end-products in parallel pathways:

$$\text{Glucose} \rightarrow 6CO_2 + 4ATP + 10NADH \quad (20)$$

$$\text{Glucose} \rightarrow 2\ \text{acetate} + 2CO_2 + 4ATP + 4NADH \quad (21)$$

$$\text{Glucose} \rightarrow \text{acetoin} + 2CO_2 + 2ATP + 2NADH \quad (22)$$

$$\text{Glucose} \rightarrow \text{butanediol} + 2CO_2 + 2ATP + NADH \quad (23)$$

$$\text{Glucose} \rightarrow 2\ \text{ethanol} + 2CO_2 + 2ATP \quad (24)$$

$$\text{Glucose} \rightarrow 2\ \text{lactate} + 2ATP \quad (25)$$

Reduced nicotinamide adenine dinucleotide (NADH) can be used to generate ATP through the electron transport system. The balance of the reactions and end products in the cell is affected by the oxygen availability and the oxygen uptake rate (deMas et al., 1988). Considerable effort has been spent on oxygen transfer and oxygen uptake in improving the fermentation. This topic will be discussed in more detail in the subsection "Research and Development" in this Chapter.

Juni and Heym (1956) described a cyclic pathway for the dissimilation of 2,3-butanediol. A number of microorganisms oxidize 2,3-BD to acetic acid. The pathway from Juni and Heym is shown in the following figure modified by Syu (2001). The three stereoisomeric forms of 2,3-BD are: 1) D(–) or R,R, 2) L(+) or S,S, and 3) *meso* or R,S.

Three enzymes are involved in the synthesis of 2,3-BD: α-acetolactate synthase (EC 4.1.3.18), α-acetolactate decarboxylase (EC 4.1.1.5) and butanediol dehydrogenase (also known as diacetyl [acetoin] reductase; Larsen and Stormer, 1973; Johansen et al., 1975; Stormer, 1975). Two different enzymes form acetolactate from pyruvate. The first, termed "catabolic α–acetolactate synthase," has a pH optimum of 5.8 in acetate and is part of the butanediol pathway. The other enzyme, termed "anabolic α–acetolactate synthase" or "acetohydroxyacid synthetase," has been well studied and characterized and will not be discussed here. This enzyme is part of the biosynthetic pathway for isoleucine, leucine, and valine and is coded for by the *ilvBN*, *ilvGM*, and *ilvH* genes in *E. coli* and *Salmonella typhimurium* (Bryn and Stormer, 1976).

The second enzyme in the butanediol pathway is acetolactate decarboxylase, which has a pH optimum of about 6.3 and which catalyzes the decarboxylation of acetolactate to acetoin. The third enzyme, diacetyl (acetoin) reductase, catalyzes a reversible reduction of acetoin to 2,3-BD and an irreversible reduction of diacetyl to acetoin. It is a tetrameric enzyme and requires NADH (Larsen and Stormer, 1973).

In *Klebsiella terrigena* and *Bacillus subtilis*, this enzyme is found within the acetoin cluster of genes. Interestingly, in *Lactobacillus lactis*, this gene is found within the gene cluster that encodes the enzymes of branched chain amino acid synthesis. This enzyme has different kinetic properties than the enzymes of *K. terrigena* and *B. subtilis* and may play a key role in acetolactate flux in *L. lactis* (Goupil-Feuillerat et al., 1997).

Acetate induces the three enzymes and activates acetolactate synthase. In a study comparing wildtype *Enterobacter aerogenes* and mutants deficient in 2,3-BD and acetoin production, during the highest phase of 2,3-BD production, the three enzymes of the pathway constituted approximately 2.5% of the protein in the cell. Butanediol production also appears to play a role in regulating the NADH/NAD ratio (Johansen et al., 1975).

Different mechanisms have been proposed for the formation of the various stereoisomers of 2,3-BD. Some of the initial difficulty was in obtaining a pure stereoisomer for study. A fermentation route to synthesis of pure *meso*-isomer was demonstrated in *Serratia*. *Bacillus polymyxa* was used to prepare the D(–)-isomer, and *Bacillus cereus* was used to make L(+)-isomer. Conditions such as pH, temperature, and shaking were modified to optimize production of the various isomers (Ui et al., 1983).

One of initial models for stereoisomer formation was postulated by Taylor and Juni (1960) for *K. pneumoniae*. They proposed the existence of an acetoin racemase, L(+) 2,3-BD dehydrogenase and D(–) 2,3-BD dehydrogenase. The L(+) 2,3-BD dehydrogenase would convert L(+) acetoin to L(+) 2,3-BD and *meso* 2,3-BD, whereas the D(–) 2,3-BD dehydrogenase would reduce D(–) acetoin to D(–) 2,3-BD and *meso*-2,3-BD.

A newer model for *K. pneumoniae* was similar to the earlier one in that it included an acetoin racemase. However, in this newer model, D(–) acetoin is converted to *meso*-2,3-BD, and L(+) acetoin is converted to L(+) 2,3-BD. This model

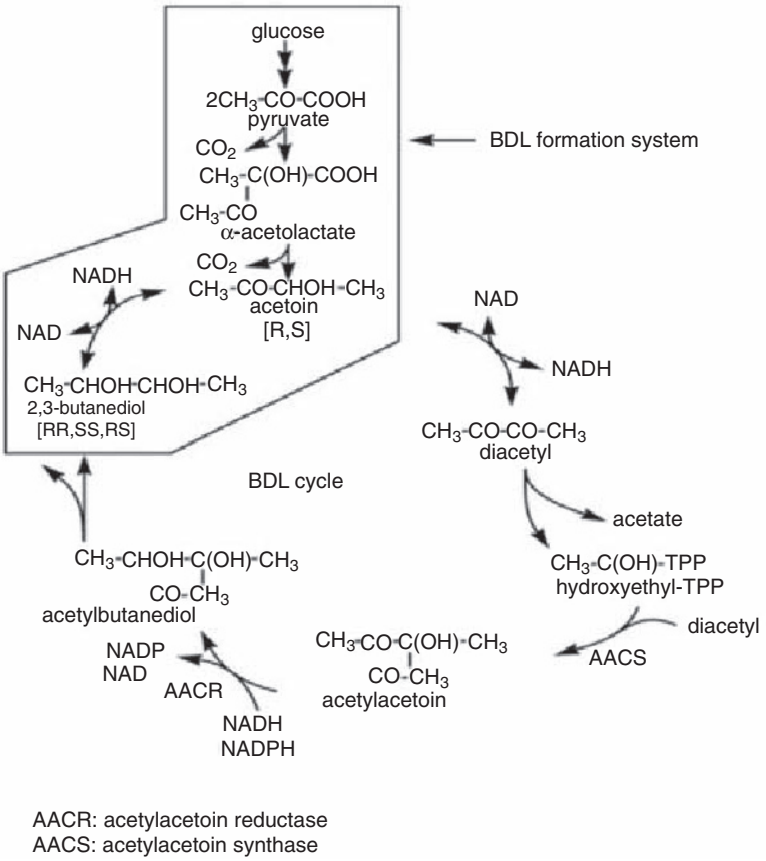

Fig. 31. The 2,3-butanediol cycle in bacteria, as proposed by Juni and Heym (1956). The boxed area represents 2,3-BD formation in bacteria. BDL, butanediol; TPP, thiamine pyrophosphate; AACS, acetylacetoin synthase; AACR, acetylacetoin reductase; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate. Redrawn from Syu (2001), with permission.

is based on the purification and separation of the two acetoin reductases and the determination of their stereospecificity (Voloch et al., 1983).

A novel mechanism for stereoisomer formation was described for *Bacillus polymyxa*. The RR-acetoin formed from pyruvate is converted into RR-butanediol by diacetyl (acetoin) reductase. The same enzyme reduces diacetyl to RR-acetoin. An S-acetoin-forming diacetyl reductase converts diacetyl to SS-acetoin. The racemic acetoin molecules are acted upon by a butanediol dehydrogenase, which generates either RR-butanediol or *meso*-butanediol (Ui et al., 1986).

Bacillus cereus was shown to have the enzymes of the butanediol cycle. Acetylacetoin was reduced to two new stereoisomers of acetylbutanediol by two separate acetylacetoin reductase enzymes. These isomers were subsequently converted by acetylbutanediol hydrolase to RR- and *meso*-2,3-BD (Ui et al., 1998). Clearly the production of the various stereoisomers is a complex issue that will require additional study and the purification of the various enzymes.

The production of 2,3-BD by microorganisms requires the balancing of a number of fermentation reactions in the cell. Mathematical models can be very useful to explain the interrelation of equations, and these models need to be verified by experimental data. Various models have been described and the reader is referred to Papoutsakis and Meyer (1985).

The acid end products of fermentation can inhibit growth and butanediol formation. Large quantities of butanediol, up to 130 g/liter, were not strongly inhibitory, while 0.45 g/liter of acetic acid can completely inhibit growth of *K. oxytoca*. Butanediol inhibition is believed to be due to the reduction of water activity, while acetic acid and not its salt is the inhibitory metabolite (Fond et al., 1985). In *Enterobacter aerogenes*, ethanol was also shown to be an inhibitory metabolite for growth (Zeng and Deckwer, 1991).

Klebsiella pneumoniae and other 2,3-BD producing microorganisms are able to use a variety of five and six carbon sugars to produce 2,3-BD. Pentoses are metabolized through a common intermediate, D-xylulose-5-phosphate. In *K. pneumoniae*, 3 moles of pentose are converted to 5 moles of D-glyceraldehyde-3-phosphate, which is an equivalent yield of D-glucose through glycolysis (Jansen and Tsao, 1983). The conversion

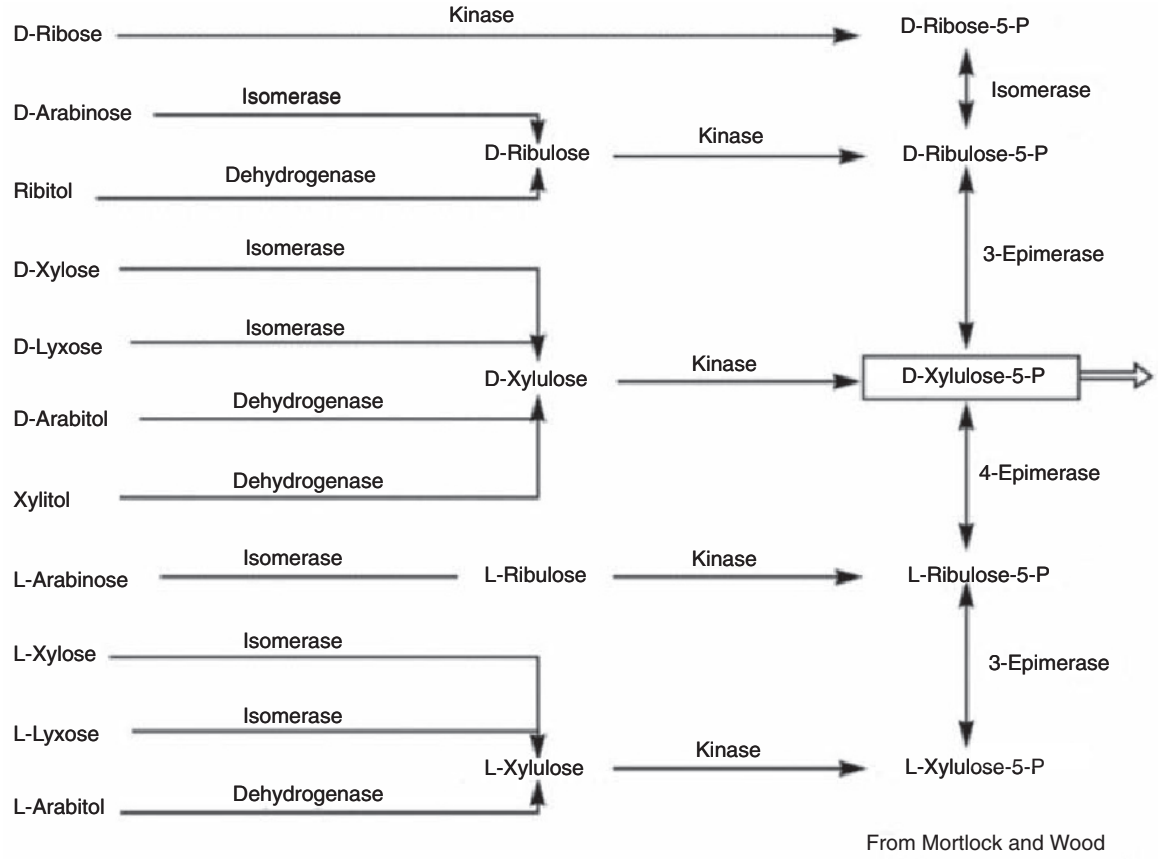

Fig. 32. Conversion of pentoses and pentitols. From Jansen and Tsao (1983), with permission.

of pentoses and pentitols is shown in Fig. 32, as described by Jansen and Tsao (1983).

There have been a number of fermentation studies on 2,3-BD formation. One of the most crucial factors identified is the amount and timing of oxygen supplied. A higher oxygen transfer coefficient favors the production of biomass while inhibiting butanediol production in *Aerobacter aerogenes*. Oxygen suppresses the production of ethanol, but some butanediol can be produced in the presence of oxygen. The maximum oxygen transfer coefficient (Kla) was found to be in the range of 50–100 h^{-1} (Sablayrolles and Goma, 1984).

Various control strategies for oxygen have been tried to maximize butanediol production. In one study, oxygen transfer rate (OTR) was used to maintain growth rate and specific oxygen uptake rate. When a set oxygen transfer rate was maintained that kept the culture at a constant level of oxygen limitation, the final butanediol concentration was similar to an experiment where OTR was uncontrolled. The improvement in the OTR-controlled fermentation was in the rate of 2,3-BD formation, as the productivity or rate of formation of 2,3-BD was 18% higher (Beronio and Tsao, 1993).

Control of microaerobic fermentations by oxygen transfer rate has limitations, as the distribution of oxygen may differ in various types and sizes of reactors. In a somewhat different approach, control by respiratory quotient was attempted. Optimum 2,3-BD production was seen at a respiratory quotient between 4.0 and 4.5, and product concentration (butanediol and acetoin) greater than 100 g/liter was measured. This was compared to a control fermentation where an oxygen transfer rate of 35 mmol/liter/h was maintained. In this case, the respiratory quotient eventually decreased to about 1.5 and product formation was only about 85 g/liter. Biomass in the fermentation with respiratory quotient control was higher than in the OTR-controlled fermentation (Zeng et al., 1994a).

Continuous culture can also be used to determine the optimum oxygen supply. In one system using *K. pneumoniae*, with an inlet feed glucose concentration of 100 g/liter, the optimum

oxygen transfer rate (defined as when maximum product was formed) was determined to be 25.0–35.0 mmol/liter/h. Increasing the oxygen input and thus the OTR reduced the product yield (Ramachandran and Goma, 1988).

In another continuous culture study, maximum oxygen uptake rates (OUR) were determined and they differed depending on the dilution rate. By varying the speed of the impeller with constant aeration, different OURs could be obtained. As dilution rate increased, the yield and product concentration decreased. Product formation was not dependent on growth rate, and even at low or no growth rates, higher specific productivity can be expected. At low OURs, the cells use fermentation for growth and maintenance and continue to convert substrate to 2,3-BD. The authors suggest that a cell recycling or cell immobilization system is a strategy used to take advantage of the potential productivity of nongrowing cells (Zeng et al., 1990).

There is some evidence that the availability of oxygen can also affect the optical purity of 2,3-BD isomers produced. In a continuous culture system with *Paenibacillus polymyxa*, when airflow was increased, the optical purity of 2,3-BD decreased (Nakashimada et al., 1998). Certainly control of oxygen is a critical parameter in the 2,3-BD fermentation process.

Commercial Applications

At present, 2,3-BD is sold as a commercial chemical intermediate. Interest in its production by fermentation peaked during World War II because 1,3-butanediene needed for synthetic rubber production could be made from 2,3-BD. With the rise of the petroleum industry and increase in the availability of cheap raw materials, industrial fermentations for 2,3-BD were no longer of commercial interest (Rosenberg, 1980).

In one method of production via petrochemical means, *n*-butenes can be separated by distillation from hydrocarbons. Treatment with hydrogen peroxide converts butanes such as 1-butene or 2-butene to their epoxide. A glycol such as 2,3-BD is then derived from the epoxide of 2-butene (Szmant, 1989).

The boiling and freezing points of 2,3-BD are 180–184°C and –60°C, respectively. It is either a colorless liquid or in crystalline form. It can be dehydrogenated to diacetyl, a highly valued flavoring agent in food products (Syu, 2001). It can also be dehydrated to methyl ethyl ketone, which has uses as a liquid fuel additive and a solvent. The levo-form of 2,3-BD has potential use in antifreeze. Butanediol can be esterified and in this form could be used as a precursor for drugs and cosmetics. Other potential industrial applications include inks, plasticizers and moistening agents (Garg and Jain, 1995). One of the attractive features of butanediol is its biodegradability. Numerous microorganisms are able to consume it at high rates. This makes it an environmentally friendly product (Afschar et al., 1993).

Butadiene appears to be the key intermediate for further conversion and other potential new uses of 2,3-BD. Butadiene can be dimerized to styrene, a large volume chemical intermediate for the polymer industry (Jansen and Tsao, 1983). Styrene is an eight-carbon molecule with an aromatic ring. Conversion of 2,3-BD to butadiene is not economically attractive. The butadiene market demand has dropped significantly, as its major styrene polymer end markets, automobile parts and construction, are also down sharply. Although the price of butadiene is presently in the range of $US0.20 per pound, future improvement in the economy is expected to increase demand for butadiene and possibly the price (Hoffman, 2001).

At the low selling price of butadiene, it is difficult to see how biologically produced material could be made cost competitive. Perhaps the greatest potential for 2,3-BD may lie in higher-value specialty product uses in such industries as chemical, pharmaceutical, and personal care. Better understanding of the market opportunity is necessary as well as further development of the various 2,3-BD chemical conversion processes.

Research and Development

There have been a number of fermentation studies for the production of 2,3-butanediol. From a process standpoint, it is not easy to make a direct comparison between the various studies because, often, types of fermentors and combinations of process conditions were different. One major focus has been the use of biomass and biomass-derived sugars for the production of 2,3-BD. There is good indication that with the proper conditions, large quantities of 2,3-BD can be made from low-cost or waste sugars.

It is possible to use high concentrations of glucose in *E. aerogenes* NRRL B199 fermentations and achieve good yields. At a glucose concentration of 195 g/liter, the yield of 2,3-BD was 0.45 g/g. However, there was some growth inhibition with such initial high substrate concentrations. At the high substrate concentration, only 2.4 g/liter of ethanol was produced. The productivity was over 1 g/liter/h, making this an attractive process (Sablayrolles and Goma, 1984). When a fed-batch approach was used with *K. pneumoniae* to overcome initial inhibitory sugar concentrations, up to 88 and 113 g of butanediol and acetyl methyl carbinol could be produced

from 190 g of xylose and 226 g of glucose, respectively (Yu and Saddler, 1983).

It is not surprising that various process conditions such as pH will also have an effect on fermentation. Biomass concentrations of *E. aerogenes* were shown to increase between pH of 5–7, while 2,3-BD production was optimum between pH 5.5 and 6.5 and dropped off at a higher pH (Zeng et al., 1990). *Paenibacillus polymxya* also showed a pH optimum for production of 2,3-BD between 5.7 and 6.3 in a chemostat system (Nakashimada et al., 1998).

The effect of added acetic acid also changed with pH. At pH of 5.5, less than 1 g/liter of acetic acid inhibited product formation, whereas at a culture pH of 6.7, it took roughly ten times more acetic acid to show the same effect. This inhibitory effect is due to the undissociated form of acetic acid (Zeng et al., 1990).

With *K. pneumoniae*, acetic acid was shown to stimulate 2.3-BD production up to two- to threefold when added at concentrations less than 1%. This media was at a pH of 6.5 and contained glucose, xylose and various nutrients. The stimulation by acetic acid may be due to activation of the 2,3-BD pathway enzymes. Yeast extract, urea, ammonium sulfate and trace elements were also shown to improve yields in this system (Yu and Saddler, 1982a). A variety of acids were found to enhance 2,3-BD production in *P. polymyxa*: acetate, propionate, pyruvate and succinate showed an effect, while *n*- and *iso*-valerate, *n*- and *iso*-butyrate, formate, malate and lactate had no effect. Acetic acid was the best and also did not reduce the optical purity of the 2,3-BD; thus a feeding strategy with acetic acid may show promise for this organism (Nakashimada et al., 2000).

The use of low cost biomass sources as a substrate for 2,3-BD production has received considerable attention as a way to improve the economics of the process. The main components of woody biomass are lignin, cellulose and hemicellulose. Cellulose is a polymer of D-glucose, while hemicellulose is a polymer containing mostly the five carbon sugars, D-xylose, L-arabinose and D-ribose (Rosenberg, 1980).

Klebsiella is a versatile organism and is able to utilize many sugars for growth and 2,3-BD production. In one study where xylose was evaluated, maximum cell growth occurred at a sugar concentration of 20 g/liter (Jansen and Tsao, 1983). The stoichiometry of the conversion of pentoses is equivalent to that of the conversion of hexoses. As seen with glucose fermentations, there is a growth phase in fermentors with xylose, and when oxygen becomes limiting, then 2,3-BD production is seen. In media with 100 g/liter of xylose, the pH optimum for growth is 5.2, but growth drops off sharply at pH lower than 5.2. A xylose concentration of 20 g/liter is optimum for growth, as higher sugar concentrations lower the water activity. Oxygen supply is critical and variations in oxygen supply can change the yields of the various products produced by *Klebsiella*. The best 2,3-BD production rate was shown to occur at an oxygen transfer rate of 0.027 moles/liter/h. With an initial xylose concentration of 100 g/liter, the average butanediol production rate was 1.35 g/liter/h (Jansen et al., 1984). Added succinate at 10 g/liter was shown to improve 2,3-BD productivity from xylose, while higher concentrations of succinate were inhibitory (Eiteman and Miller, 1995).

Pre-treated cellulosic materials have been tested with *Klebsiella* for butanediol production with some success. *Klebsiella* was grown on acid-hydrolyzed wood hemicellulose, and 2,3-BD yields of 0.4–0.5 g/g were obtained. The authors proposed that such high yields were partially due to the ability of *Klebsiella* to simultaneously ferment uronic acids, such as D-glucuronic and D-galacturonic acid, present in the wood samples. Other compounds in wood (such as furfural and lignin derivatives) are inhibitory to the bacteria if the wood hydrolysate is added at too high a concentration (Yu et al., 1982b). A study comparing the effects of a number of inhibitors on *Klebsiella* was reported. Sulfate and furfural up to concentrations of 0.2% w/v reduced 2,3-BD yield, and phenolic compounds inhibited bacterial growth (Frazer and McCaskey, 1991).

A more efficient approach to use of woody biomass was simultaneous saccharification and fermentation (SSF) for 2,3-BD production. Culture filtrates of *Trichoderma harzianum* were added as a source of hydrolytic enzymes. SSF can shorten overall process times and relieve end product inhibition. The mixture of sugars derived from both cellulose and hemicellulose can be used for 2,3-BD production, thus eliminating the costs of separating the sugars (Yu and Saddler, 1985a).

Klebsiella was grown on woody biomass in coculture with *T. harzianum*. The fungal growth medium was found to inhibit the growth of *Klebsiella*, therefore, resting cells of *Klebsiella* were used. Both the hemicellulose and cellulose sugar streams were used for 2,3-BD production, and yields of approximately 30% of theoretical were obtained (Yu et al., 1985b).

Another potential low cost substrate is cheese whey. Using *K. pneumoniae* cells immobilized in calcium alginate, a productivity of 2.3 g/liter/h on whey permeate was demonstrated. The system was stable during seven weeks of continuous operation (Lee and Maddox, 1986). In another study, *K. oxytoca* was shown to grow poorly on lactose, but it grew very well on the component sugars, glucose and galactose. The aeration rates

affected growth on lactose; low aeration results in a lower cell growth rate. One possible factor for the lower growth rate on lactose may have been inefficient transport into the cell (Champluvier et al., 1989).

Molasses appears to have potential value as a substrate for 2,3-BD production. *Klebsiella oxytoca* can ferment molasses at high concentrations. In one batch experiment, as much as 280 g/liter of molasses was converted to 118 g/liter of 2,3-BD and 2.3 g/liter of acetoin. These high concentrations are needed inasmuch as molasses is a relatively expensive substrate, and the separation costs of the final product may be difficult from such a complex carbohydrate source (Afschar et al., 1991).

Various new process concepts have been proposed for 2,3-BD production. The prospect of making both 1,3-propanediol and 2,3-BD in the same fermentation was proposed using *K. pneumoniae* and glycerol as a substrate. In continuous culture, if the pH was lowered stepwise, 2,3-BD formation started at pH 6.6. This fermentation has the potential to be economically attractive as few by-products are formed (Biebl et al., 1998).

New process methods have been investigated for 2,3-BD production. An improvement of productivity over a continuous or batch system was observed in a cell recycle system using *Klebsiella*. One drawback occurred when biomass built up past a certain level; at this point, the coefficient of mass transfer for oxygen decreased and the viscosity increased owing to microbial polysaccharide production (Ramachandran and Goma, 1988). A novel process technology that improved the use of lactose was employed using coimmobilized cells of *Kluyveromyces lactis* with *Klebsiella oxytoca*. The yeast cells were permeabilized with solvent and used as a source of lactase. The rate of production on lactose was similar to that of immobilized cells on glucose (Champluvier et al., 1989).

Other microorganisms have been studied for their potential in 2,3-BD production. Certain strains of *B. polymyxa* were shown to be 2,3-BD producers from xylose. Yeast extract improved yields, which reached 16 g/liter (Laube et al., 1984). In the process of searching for an organism that could produce ethanol from arabinose, an *Enterobacter cloaceae* was isolated that could produce 2,3-BD from arabinose, with yields as high as 0.4 g/g of arabinose. Besides the pure sugar, the organism could use sugars from acid and enzyme hydrolyzed corn fiber. The organism prefers arabinose but will also use glucose and xylose. This organism opens up the opportunity to produce 2,3-BD from a corn fiber feedstock (Saha and Bothast, 1999).

Various *Bacillus* have been studied for 2,3-BD production. Much of the focus has been on *Bacillus polymyxa*, as besides *Klebsiella*, it is considered to have industrial potential. It produces primarily pure L-isomer. A number of isolates of *B. polymyxa* were checked on media that contained xylan. One strain especially, *B. polymyxa* NRCC 9035, produced a considerable amount of diol on xylose. Yeast extract appeared to have a beneficial effect on the xylose fermentation, and diol concentrations of 4.2 g/liter were seen. *Bacillus polymyxa* also fermented other sugars in hemicellulose, including mannose, galactose and L-arabinose (Laube et al., 1984).

Bacillus amyloliquefaciens may be a promising organism for 2,3-BD production as its major products of fermentation are 2,3-BD and some minor acids. It is able to use a variety of sugars, but the best productivity and yield are on glucose. As observed in other 2,3-BD fermentations, aeration plays a critical role, with high aeration favoring biomass formation and lower aeration favoring 2,3-BD production. With this organism, approximately 33 g of diol were formed from 100 g of sugar (Alam et al., 1990).

One of the highest 2,3-BD yields was reported from *Bacillus licheniformis*. The theoretical yield of 2,3-BD from glucose is 0.5 g/g of glucose. *Bacillus licheniformis* was able to reach 94% of the theoretical yield in 72 h. The best yields were obtained when peptone and beef extract were added to the medium. This productivity was similar to that of *Klebsiella* and *Bacillus polymyxa* (Nilegaonkar et al., 1992). Additional work to characterize *B. licheniformis* strains was done by using profiles of the fermentation products formed. A number of *B. licheniformis* strains were compared with *B. polymxya* strains. The product profiles for the *B. licheniformis* strains were similar to each other; however, they differed from the *B. polymyxa* strains. Protein electrophoretic patterns were also used to classify the strains. Again, with protein electrophoretic patterns, *B. licheniformis* was mapped in a different cluster than *B. polymyxa*. In fermentation tests, some of the *B. licheniformis* strains were shown to be as promising as *B. polymyxa* for 2,3-BD production (Raspoet et al., 1991).

Very little appears in the literature on any genetic engineering of the 2,3-BD pathway. The 2,3-BD operon from *Klebsiella terrigena* and *Enterobacter aerogenes* was cloned and characterized. The genes coding for α-acetolactate decarboxylase, α-acetolactate synthase, and acetoin (diacetyl) reductase were shown to be clustered in one operon. The genes were sequenced and called "*budABC*." The *budABC* operon appears to be regulated at the transcriptional level, as the highest amount of transcript was seen under conditions that favored 2,3-BD production. A putative fumarate nitrate reduction regulatory protein (FNR) site was found at posi-

tion –6. The FNR protein can activate genes at the level of transcription that are involved in anaerobic processes. The cloning of the *budABC* operon will facilitate the study of gene regulation and improvement of the 2,3-BD process (Blomqvist et al., 1993).

A gene fragment from *K. pneumoniae* IAM1063 that contains the *meso*-2,3-butanediol dehydrogenase was cloned and transformed into *E. coli*. This transformed *E. coli* produced only *meso*-2,3-BD from racemic acetoin (Ui et al., 1996). Using sequence homology from the *Klebsiella terrigena* operon, the *meso*-2,3-butanediol dehydrogenase and the remainder of the 2,3-BD-forming operon were cloned into *E. coli*. *Escherichia coli* was shown to produce *meso*-2,3-BD from glucose with no contamination of the L-form. The *E. coli* was tested in shake flasks, and the highest productivity was with a starting glucose concentration of 100 g/liter, which yielded 17.7 g/liter of *meso*-2,3-BD (Ui et al., 1997). Clearly, this work is promising and applying techniques of molecular biology and metabolic engineering may further improve the productivity of the 2,3-BD fermentation.

Patents and Regulatory Issues

Few patents in the United States patent literature can be found that deal with 2,3-BD production via a microbial process. With commercialization efforts, one would expect to find patents that cover the microorganisms involved, genetic manipulations, fermentation process improvements, and downstream separation of the product from broth. This may indicate that a bioprocess has not yet attracted the interest of industry.

Prospects

The study of the formation of 2,3-butanediol by bacteria has spanned quite a number of years. Early work included the elucidation of the pathway and some of the basic biochemistry of the enzymes. More recently, much of the research has focused on the fermentation process and understanding the parameters that would lead to a fermentation of high yield and productivity.

As raw materials are likely a large part of the fermentation production cost of 2,3-BD, the variety of work with biomass shows promise in helping to reduce the costs of the fermentation. The cloning of the 2,3-BD genes will help open up opportunities for improving the host organism using techniques of metabolic engineering. One of the major challenges to the economics of this process will be the development of an efficient and cheap downstream process for separation of the material from fermentation broth.

As with any chemical intermediate that can be made by a biological process, several key challenges will remain. First, there must be a sufficient market demand to drive the research and development costs. Secondly, the cost of competing petrochemicals must be taken into account, as it will be difficult for a bio-based material to compete with very cheap petrochemical-derived products. Thirdly, it must be determined whether the unique properties of the bio-based material open up new end uses and markets. Examples of this would be the use of chiral molecules in special end uses. This prospect may exist for 2,3-BD with its three racemic forms. Another example would be whether the public finds the features of biodegradability, or use of renewable materials, attractive enough to possibly pay more for a product.

It appears that 2,3-BD achieved its peak of commercial interest around World War II due to the shortage of natural rubber. It remains to be seen whether evolving industrial needs will cause renewed interest in a bioprocess for production of 2,3-butanediol.

Literature Cited

Abbad-Andaloussi, S., C. Manginot-Durr, J. Amine, E. Petitdemange, and H. Petitdemange. 1995. Isolation and Characterization of Clostridium butyricum DSM 5431 mutants with increased resistance to 1,3-propanediol and altered production of acids. Appl. Environ. Microbiol. 61:4413–4417.

Abbad-Andaloussi, S., C. Durr, G. Raval, and H. Petitdemange. 1996. Carbon and electron flow in Clostridum butyricum grown in chemostat culture on glycerol and glucose. Microbiol. 142:1149–1158.

Abbad-Andaloussi, S., E. Guedon, E. Spiesser, and H. Petitdemange. 1996. Glycerol dehydratase activity: The limiting step for 1,3-propanediol production by Clostridium butyricum DSM 5431. Letts. Appl. Microbiol. 22:311–314.

Abrini, J., H. Naveau, and E.-J. Nyns. 1994. Clostridium autoethanogenum, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch. Microbiol. 16:345–351.

Adamse, A. D. 1980. New isolation of Clostridium aceticum, (Wieringa). Ant v. Leeuwenhoek 46:523–531.

Adler, H. I., and W. Crow. 1987. A technique for predicting the solvent-producing ability of Clostridium acetobutylicum. Appl. Environ. Microbiol. 53:2496–2499.

Afschar, A. S., K. H. Bellgardt, C. E. Vaz Rossell, A. Czok, and K. Schaller. 1991. The production of 2,3-butanediol by fermentation of high test molasses. Appl. Microbiol. Biotechnol. 34:582–585.

Afschar, A. S., C. E. Vas Rossell, R. Jonas, A. Quesada Chanto, and K. Schaller. 1993. Microbial production and downstream processing of 2,3-butanediol. J. Biotechnol. 27:317–329.

Agreda, V. H., and J. R. Zoeller. 1993. Acetic Acid and Its Derivatives. Dekker. New York, NY.

Ahn, W. K., S. J. Park, and S. Y. Lee. 2000. Production of poly(3-hydroxybutyrate) by fed-batch culture of recom-

binant Escherichia coli with a highly concentrated whey solution. Appl. Environ. Microbiol. 66:3624–3627.

Ahn, W. S., S. J. Park, and S. Y. Lee. 2001. Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant Escherichia coli. Biotechnol. Lett. 23:235–240.

Ahrens, K., K. Menzel, A. P. Zeng, and W. D. Deckwer. 1998. Kinetic, dynamic, and pathway studies of glycerol metabolism by Klebsiella pneumoniae in anaerobic continuous culture. III: Enzymes and fluxes of glycerol dissimilation and 1,3-propanediol formation. Biotechnol. Bioengin. 59:544–552.

Alam, S., D. Stevens, and R. Bajpai. 1988. Production of butyric acid by batch fermentation of cheese whey with Clostridum beijerinckii. J. Indust. Microbiol. 2:359–364.

Alam, S., F. Capit, W. A. Weigand, and J. Hong. 1990. Kinetics of 2,3-butanediol fermentation by Bacillus amyloliquefaciens: Effect of initial substrate concentration and aeration. J. Chem. Tech. Biotechnol. 47:71–84.

Albertsson, A.-C., and R. H. Marchessault. 1994. Governmental policy, regulations and standards. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 313–318.

Aldrich, H. C., L. McDowell, M. de. F. S. Barbosa, L. P. Yomano, R. K. Scopes, and L. O. Ingram. 1992. Immunocytochemical localization of glycolytic and fermentative enzymes in Zymomonas mobilis. J. Bacteriol. 174:4504–4508.

Allcock, E. R., and D. R. Woods. 1981a. Carboxymethyl cellulase and cellobiase production by Clostridium acetobutylicum in an industrial fermentation medium. Appl. Environ. Microbiol. 41:539–541.

Allcock, E. R., S. J. Reid, D. T. Jones, and D. R. Woods. 1981b. Autolytic activity and an autolysis-deficient mutant of Clostridium acetobutylicum. Appl. Environ. Microbiol. 42:929–935.

Allgeier, R. J., and F. M. Hildebrandt. 1960. Developments in vinegar manufacture. Adv. Appl. Microbiol. 8:163–182.

Alpers, J. 1999. Engineering metabolism for commercial gains. Science 283:1625–1626.

Altaras, N. E., and D. C. Cameron. 1999. Metabolic engineering of a 1,2-propanediol pathway in Escherichia coli. Appl. Environ. Microbiol. 65:1180–1185.

Altaras, N. E., and D. C. Cameron. 2000. Enhanced production of (R)-1,2-propanediol by metabolically engineered Escherichia coli. Biotechnol. Prog. 16:940–946.

Altaras, N. E., M. R. Etzel, and D. C. Cameron. 2001. Conversion of sugars to 1,2-propanediol by Thermoanaerobacterium thermosaccharolyticum HG-8. Biotechnol. Prog. 17:52–56.

Althouse, J. W., and L. L. Tavlarides. 1992. Analysis of organic extractant systems for acetic acid removal for calcium magnesium acetate production. Ind. Eng. Chem. Res. 31:1971–1981.

Aminabhavi, T. M., and R. H. Balundgi. 1990. A review of biodegradable plastics. Polym. Plast. Technol. Eng. 29:235–262.

Amrane, A., and Y. Prigent. 1996. A novel concept of bioreactor: Specialized function two-stage continuous reactor, and its application to lactose conversion into lactic acid. J. Biotechnol. 45:195–203.

Anastassiadis, S., A. Aivasidis, and C. Wandrey. 1999. Process for the production of gluconic acid with a strain of Aureobasidium pullulans (deBray) Arnaud. US Patent 5692286.

Van Andel, J. G., G. R. Zoutberg, P. M. Crabbendam, and A. M. Breure. 1985. Glucose fermentation by Clostridium butyricum grown under a self-generated gas atmosphere in chemostat culture. Appl. Microbiol. Biotechnol. 23:21–26.

Anderson, A. J., and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.

Andreesen, J. R., G. Gottschalk, and H. G. Schlegel. 1970. Clostridium formicoaceticum nov. spec. isolation description, and distinction from C. aceticum and C. thermoaceticum. Arch. Mikrobiol. 72:154–174.

Andrianova, Y. E., T. L. Bakuridze, V. G. Yargunov, I. V. Zhurov, and V. G. Vinter. 1998. Effects of succinate on the growth rates of potato, Rauwolfia, and ginseng in vitro. Prikl. Biokhim. Mikrobiol. 34:435–438.

Annous, B., and H. P. Blaschek. 1991. Isolation and characterization of Clostridium acetobutylicum mutants with enhanced amylolytic activity. Appl. Environ. Microbiol. 57:2544–2548.

Anonymous. 1956. Trends of world solvents production for the past ten years and the management of the Chiai (Taiwan) Solvents Works [in Chinese]. Petroleum Communications. China Petroleum Corporation. Taiwan, China. June, Issue 60:42–57.

Anonymous. 1958. New direction of development for the Chiai (Taiwan) Solvents Works [in Chinese]. Petroleum Communications. China Petroleum Corporation. Taiwan, China. May, Issue 83:6–7.

Anonymous. 1996a. Chemical profile: Propylene glycol. Chem. Mark. Rep. 249:37.

Anonymous. 1996b. Facts and figures for the chemical industry: Production by the US chemical industry. Chem. Eng. News June 24:41.

Anonymous. 1997. Facts and figures for the chemical industry—production: mixed in 1996. Chem. Eng. News June 23:41.

Anonymous. 1999. Shell Chemicals Research Team wins ACS 2000 award for innovation. Chem. Mark. Rep. 256.

Anonymous. 2001a. Chemical prices. Chem. Mark. Rep. 259:8–21.

Anonymous. 2001b. Chemical profile: Isopropanol. Chem. Mark. Rep. November 12:31.

Anonymous. 1979. Facts and figures for the U.S. chemical industry—production of organic chemicals. Chem. Eng. News June 11:37.

Anonymous. 1993. Chemical Marketing Reporter March 1:7.

Anonymous. 1999. All-microbial route yields chiral building blocks. Chem. Eng. News 77(8):57.

Anonymous. 2000. Chemical prices. Chemical Market Reporter 257(23):26–33.

Anonymous. 2001a. Chemical prices. Chem. Market. Rep. 160(17):23–26.

Anonymous. 2001b. Glycerol, propionic acid, butyric acid. Chem. Market. Rep. 259(13):30–33.

Arihara, K., and J. B. Luchansky. 1995. Dairy lactobacilli. *In:* Y. H. Hui and G. G. Khachatouriani (Eds.) Food Biotechnology: Microorganisms. VCH. New York, NY. 609–643.

Arzberger, C. F., W. H. Peterson, and E. B. Fred. 1920. Certain factors that influence acetone production by Bacillus acetoethylicum. J. Biol. Chem. 44:465–479.

Asai, T. 1968. Acetic Acid Bacteria: Classification and Biochemical Activities. University Press of Tokyo

and University Park Press. Tokyo, Japan. Baltimore, MD.

Attwood, M. A., J. P. van Dijken, and J. T. Pronk. 1991. Glucose metabolism and gluconic acid production by Acetobacter diazotrophicus. J. Ferm. Bioeng. 72:101–105.

Auvray, F., M. Codderville, P. Ritzenthaler, and L. Dupont. 1997. Plasmid integration in a wide range of bacteria mediated by intgrase of Lactobacillus delbrueckii bacteriophage mv4. J. Bacteriol. 179:1837–1845.

Azeddoug, H., J. Hubert, and G. Reysset. 1992. Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of Clostridium acetobutylicum. J. Gen. Microbiol. 138:1371–1378.

Babb, B. L., H. J. Collett, S. J. Reid, and D. R. Woods. 1993. Transposon mutagenesis of Clostridium acetobutylicum P262: Isolation and characterization of solvent deficient and metronidazole resistant mutants. FEMS Microbiol. Lett. 114:343–348.

Babel, W., U. Iske, M. Jechorek, and D. Miethe. 1987. Method for production of products by fermentation of methanol. German Patent GDR 251,571. Chem. Abstr. 109:36639.

Babel, W., R. Mueller, D. Miethe, and U. Iske. 1988. Continuous microbial synthesis of products of incomplete oxidation, such as gluconic acid. German Patent DD 253,836. Chem. Abstr. 109:108918.

Babel, W., D. Miethe, U. Iske, K. Sattler, H. P. Richter, J. Schmidt, and Babel, W., N. Lofhagen, D. Miethe, R. Mueller, U. Iske, M. Jechorek, and R. Dueresch. 1991. Microbial manufacture of gluconic acid. German Patent DD 293, 135. Chem. Abstr. 115:278185.

Bahl, H., W. A. Andersch, K. Braun, and G. Gottschalk. 1982a. Effect of pH and butyrate concentration on the production of acetone and butanol by Clostridium acetobutylicum grown in continuous culture. Eur. J. Appl. Microbiol. Biotechnol. 14:17–20.

Bahl, H., W. Andersch, and G. Gottschalk. 1982b. Continuous production of acetone and butanol by Clostridium acetobutylicum in a two-stage phosphate limited chemostat. Appl. Microbiol. Biotechnol. 15:201–205.

Bahl, H., H. Müller, S. Behrens, H. Joseph, and F. Naberhaus. 1995. Expression of heat shock genes in Clostridium acetobutylicum. FEMS Microbiol. Rev. 17:341–348.

Bahner, B. 1994. Chemical Marketing Reporter March 21st:14.

Baker, R. C., and R. E. Kramer. 1999. Cytotoxicity of short-chain alcohols. Ann. Rev. Pharmocol. Toxicol. 39:127–150.

Balch, W. E., S. Schoberth, R. S. Tanner, and R. S. Wolfe. 1977. Acetobacterium, a new genus of hydrogen-oxidizing carbon dioxide-reducing, anaerobic bacteria. Int. J. Syst. Bacteriol. 27:355–361.

Baldus, J. M., B. D. Green, P. Youngman, and C. P. Morgan Jr. 1994. Phosphorylation of Bacillus subtilis transcription factor Spo0A stimulates transcription from the spoIIG promoter by enhancing binding to weak 0A boxes. J. Bacteriol. 176:296–306.

Ballerini, D., J. P. Desmarquest, J. Pourquie, F. Nativel, and M. Rebeller. 1994. Ethanol production from lignocellulosics: Large scale experimentation and economics. Bioresource Technol. 50:17–23.

Ballongue, J., J. Amine, E. Masion, H. Petitdemange, and R. Gay. 1985. Induction of acetoacetate decarboxylase in Clostridium acetobutylicum. FEMS Microbiol. Lett. 29:273–277.

Barber, J. M., F. T. Robb, J. R. Webster, and D. R. Woods. 1979. Bacteriocin production by Clostridium acetobutylicum in an industrial fermentation process. Appl. Environ. Microbiol. 37:433–437.

Barbirato, F., C. Camarasa-Claret, J. P. Grivet, and A. Bories. 1995. Glycerol fermentation by a new 1,3-propanediol-producing microorganism: Enterobacter agglomerans. Appl. Microbiol. Biotechnol. 43:786–793.

Barbirato, F., J. P. Grivet, P. Soucaille, and A. Bories. 1996a. 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by enterobacterial species. Appl. Environ. Microbiol. 62:1448–1451.

Barbirato, F., P. Soucaille, and A. Bories. 1996b. Physiologic mechanisms involved in accumulation of 3-hydroxypropionaldehyde during fermentation of glycerol by Enterobacter agglomerans. Appl. Environ. Microbiol. 62:4405–4409.

Barbirato, F., D. Chedaille, and A. Bories. 1997. Propionic acid fermentation from glycerol: Comparison with conventional substrates. Appl. Microbiol. Biotechnol. 47:441–446.

Barbirato, F., S. Astruc, P. Soucaille, C. Camarasa, J. M. Salmon, and A. Bories. 1997a. Anaerobic pathways of glycerol dissimilation by Enterobacter agglomerans CNCM 1210: Limitations and regulations. Microbiology 143:2423–2432.

Barbirato, F., A. Larguier, T. Conte, S. Astruc, and A. Bories. 1997b. Sensitivity to pH, product inhibition, and inhibition by NAD^+ of 1,3-propanediol dehydrogenase purified from Enterobacter agglomerans CNCM 1210. Arch. Microbiol. 168:160–163.

Barbirato, F., E. H. Himmi, T. Conte, and A. Bories. 1998. 1,3-propanediol production by fermentation: An interesting way to valorize glycerin from the ester and ethanol industries. Indust. Crops Prod. 7:281–289.

Barik, S., S. Prieto, S. B. Harrison, E. C. Clausen, and J. L. Gaddy. 1988. Biological production of alcohols from coal through indirect liquefaction: Scientific note. Appl. Biochem. Biotechnol. 18:363–378.

Barker, H. A. 1972. Co-enzyme B_{12}-dependent mutases causing carbon chain rearrangements. *In:* P. D. Boyer (Ed.) The Enzymes. Academic Press. New York, NY. 6:509–537.

Barnard, G. N., and J. K. M. Sanders. 1989. The poly-β-hydroxybutyrate granule in vivo: A new insight based on NMR spectroscopy of whole cells. J. Biol. Chem. 264:3286–3291.

Basu, R., J. S. Bershears, and E. C. Clausen. 1999. Calcium Magnesium Acetate at Lower Production Costs: Production of CMA Deicer from Biomass. US DOT. Publication No. FHWA-RD-98-055.

Bata, R. M., A. C. Elrod, and T. P. Lewandowski. 1991. Butanol as a blending agent with gasoline for I. C. engines. *In:* R. W. Hurn, W. F. Marshall, and J. R. Allsup (Eds.) Oxygenates in Motor Fuel Formulation. Society of Automotive Engineers. Warrendale, PA. 35–40.

Bates, E. E. M., H. J. Gilbert, G. P. Hazelwood, J. Huckle, J. I. Laurie, and S. P. Mann. 1989. Expression of a Clostridium thermocellum endoglucanase gene in Lactobacillus plantarum. Appl. Environ. Microbiol. 55:2095–2097.

Beall, D. S., and L. O. Ingram. 1993. Genetic engineering of soft-rot bacteria for ethanol production from lignocellulose. J. Ind. Microbiol. 11:151–155.

Beesch, S. C. 1952. Acetone-butanol fermentation of sugars. Ind. Engin. Chem. 44:1677–1682.

Beesch, S. C. 1953. Acetone-butanol fermentation of starches. Appl. Microbiol. 1:85–95.

Benda, I. 1982. Wine and brandy. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 293–402.

Bennett, G. N., and F. B. Rudolph. 1995. The central metabolic pathway from acetyl-CoA to butyryl-CoA in Clostridium acetobutylicum. FEMS Microbiol. Rev. 17:241–249.

Bennett, G. N., and K. Y. San. 2001. Microbial formation, biotechnological production and application of 1,2-propanediol. Appl. Microbiol. Biotechnol. 55:1–9.

Benninga, H. 1990. A History of Lactic Acid Making. Kluwer Academic Publishers. Dordrecht, Germany. Boston, MA.

Beppu, T. 1993. Genetic organization of Acetobacter for acetic acid fermentation. Ant. v. Leeuwenhoek 64:121–135.

Berglund, K. A., P. Elankovan, and D. A. Glassner. 1991. Carboxylic acid purification and crystallization process. US Patent. 5,034,105.

Berglund, K. A., S. Yedur, and D. D. Dunuwila. 1999. Succinic acid production and purification. US Patent. 5,958,744.

Bermejo, L. L., N. E. Welker, and E. T. Papoutsakis. 1998. Expression of Clostridium acetobutylicum ATCC 824 genes in Escherichia coli for acetone production and acetate detoxification. Appl. Environ. Microbiol. 64: 1079–1085.

Bernhauer, K. 1924. Zum Problem der Säurebildung durch A. niger. Biochem. Z. 153:517–521.

Bernhauer, K. 1928. Über die Characterisierung der Stämme von A. niger auf Grund ihres biochemischen Verhaltens. Biochem. Z 197:278–287.

Beronio Jr., P. B., and G. T. Tsao. 1993. Optimization of 2,3-butanediol production by Klebsiella oxytoca through oxygen transfer rate control. Biotechnol. Bioengin. 42:1263–1269.

Bertram, J., and P. Dü. 1989. Conjugal transfer and expression of streptococcal transposons in Clostridium acetobutylicum. Arch. Microbiol. 151:551–557.

Bertram, J., A. Kuhn, and P. Dü. 1990. Tn916-induced mutants of Clostridium acetobutylicum defective in regulation of solvent formation. Arch. Microbiol. 153:373–377.

Bhowmik, T., and J. L. Steele. 1994. Cloning, characterization, and insertional inactivation of Lactobacillus helveticus D(−)-lactate dehydrogenase. Appl. Microbiol. Biotechnol. 41:432–439.

Biebl, H. 1991. Glycerol fermentation of 1,3-propanediol by Clostridium butyricum: Measurement of product inhibition by use of a pH-auxostat. Appl. Microbiol. Biotechnol. 35:701–705.

Biebl, H., S. Marten, H. Hippe, and W. D. Deckwer. 1992. Glycerol conversion to 1,3-propanediol by newly isolated clostridia. Appl. Microbiol. Biotechnol. 36:592–597.

Biebl, H., and S. Marten. 1995. Fermentation of glycerol to 1,3-propanediol: Use of cosubstrates. Appl. Microbiol. Biotechnol. 44:15–19.

Biebl, H., A. P. Zeng, K. Menzel, and W. D. Deckwer. 1998. Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by Klebsiella pneumoniae. Appl. Microbiol. Biotechnol. 50:24–29.

Biebl, H., K. Menzel, A. P. Zeng, and W. D. Deckwer. 1999. Microbial production of 1,3-propanediol. Appl. Microbiol. Biotechnol. 52:289–297.

Bigelis, R., and S. P. Tsai. 1995. Microorganisms for organic acid production. *In:* Y. H. Hui and G. G. Khachatourians (Eds.) Food Biotechnology: Microorganisms. VCH. New York, NY. 239–280.

Billig, E., and D. R. Bryant. 1991. Oxoprocess. Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 17:903–919.

Billig, E. 1992. Butyl alcohols. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 4:691–700.

Birrer, G. A., W. R. Chesbro, and R. M. Zsigray. 1994. Electro-transformation of Clostridium beijerinckii NRRL B-592 with shuttle plasmid pHR106 and recombinant derivatives. Appl. Microbiol. Biotechnol. 41:32–38.

Blaschek, H., B. Annous, J. Formanek, and C.-K. Chen. 2002. Method of producing butanol using a mutant strain of Clostridium beijerinckii. US Patent. 6358717.

Blom, R. H., V. F. Pfeiffer, A. J. Moyer, D. H. Traufler, H. F. Conway, C. K. Crocker, R. E. Farison, and D. V. Hannibal. 1952. Sodium gluconate production-fermentation with A. niger. Ind. Eng. Chem. 44:435–440.

Blomqvist, K., M. Nikkola, P. Lehtovaara, M.-L. Suihko, U. Airaksinen, K. B. Straby, J. K. C. Knowles, and M. E. Penttila. 1993. Characterization of the genes of the 2,3-butanediol operons from Klebsiella terrigena and Enterobacter aerogenes. J. Bacteriol. 175:1392–1404.

Bock, A., and G. Sawers. 1996. "Fermentation" in Escherichia coli and Salmonella. *In:* F. C. Neidhardt (Eds.) Cellular and Molecular Biology. ASM Press. 262–282.

Boenigk, R., S. Bowien, and G. Gottschalk. 1993. Fermentation of glycerol to 1,3-propanediol in continuous cultures of Citrobacter freundii. Appl. Microbiol. Biotechnol. 38:453–457.

Bolt, J. A. 1980. A Survey of alcohol as a motor fuel. Alcohols as Motor Fuels. Society of Automotive Engineers. Warrendale, PA. 21–33.

Boutroux, L. 1880. Sur une fermentation nouvelle du glucose. CR Acad. Sci. 91:236–238.

Bouvet, O. M. M., P. Lenormand, J. P. Carlier, and P. A. D. Grimont. 1994. Phenotypic diversity of anaerobic glycerol dissimilation shown by seven enterobacterial species. Res. Microbiol. 145:129–139.

Bowles, L. K., and W. L. Ellefson. 1985. Effects of butanol on Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:1165–1170.

Bowring, S. N., and J. G. Morris. 1985. Mutagenesis of Clostridium acetobutylicum. J. Appl. Bacteriol. 58:577–584.

Boyaval, P., J. Seta, and C. Gavach. 1993. Concentrated propionic acid production by electrodialysis. Enzyme Microb. Technol. 15:683–686.

Boyaval, P., C. Corre, and M. N. Madec. 1994. Propionic acid production in a membrane bioreactor. Enzyme Microb. Technol. 16:883–886.

Boyaval, P., and C. Corre. 1995. Production of propionic acid. Lait 75:453–461.

Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1996a. Cloning, sequencing and expression of clustered genes encoding β-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from Clostridium acetobutylicum ATCC 824. J. Bacteriol. 178:3015–3024.

Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1996b. Cloning, sequencing, and expression of genes encoding

phosphotransacetylase and acetate kinase from Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 62:2758–2766.

Boynton, Z. L., J. J. Koon, E. M. Brennan, J. D. Clouart, D. M. Horowitz, T. U. Gerngross, and G. W. Huisman. 1999. Reduction of cell lysate viscosity during processing of poly(3-hydroxyalkanoates) by chromosomal integration of the Staphylococcal nuclease gene in Pseudomonas putida. Appl. Environ. Microbiol. 65:1524–1529.

Brandt, D. A. 1982. Distilled beverage alcohol. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 468–491.

Brau, B., and H. Sahm. 1986. Cloning and expression of the sturctural gene for pyruvate decarboxylase of Zymomonas mobilis in Escherichia coli. Arch. Microbiol. 144: 296–301.

Braun, M., F. Mayer, and G. Gottschalk. 1981. Clostridium aceticum (Wieringa) a microorganism producing acetic acid from molecular hydrogen and carbon dioxide. Arch. Microbiol. 128:288–293.

Bringer-Meyer, S., K.-L. Schmiz, and H. Sahm. 1986. Pyruvate decarboxylase from Zymomonas mobilis: Isolation and partial characterization. Arch. Microbiol. 146:105–110.

Brown, A. T., and L. C. Breeding. 1980. Carbon dioxide metabolism by Actinomyces viscosus: Pathways for succinate and aspartate production. Infect. Immun. 28:82–91.

Brown, D. P., L. Ganova-Raevn, B. D. Green, S. R. Wilkinson, M. Young, and P. Youngman. 1994. Characterization of spoOA homologues in diverse Bacillus and Clostridium species identifies a probable DNA-binding domain. Molec. Microbiol. 14:411–426.

Bryant, F. O., J. Wiegel, and L. G. Ljungdahl. 1988. Purification and properties of primary and secondary alcohol dehydrogenases from Thermoanaerobacter ethanolicus. Appl. Environ. Microbiol. 54:460–465.

Bryant, R. S. 1990. Microbial enhanced oil recovery and compositions therefor. US Patent. 4905761.

Bryn, K., and F. C. Stormer. 1976. Decreased riboflavin formation in mutants of Aerobacter (Enterobacter) aerogenes deficient in the butanediol pathway. Biochim. Biophys. Acta 428:257–259.

Buchholz, S. E., M. M. Dooley, and D. E. Eveleigh. 1987. Zymomonas—an alcoholic enigma. Trends Biotechnol. 5:199–204.

Bucholz, K., and B. Gödelmann. 1978. Macrokinetics and operational stability of immobilized glucose oxidase and catalase. Biotechnol. Bioeng. 20:1201–1220.

Buchta, K. 1983. Lactic acid. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. Verlag Chemie. Weinheim, Germany. 409–417.

Bulthuis, B. A., A. A. Gatenby, S. L. Laynie, A. K. Hsu, and R. D. Lareau. 1998. Method for the production of glycerol by recombinant organisms. Patent Cooperation Treaty (PCT) Application WO 98/21340.

Burdette, D., and J. G. Zeikus. 1994. Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from Thermoanaerobacter ethanolicus 39E and characterization of the secondary-alcohol dehydrogenase (2°dh) as a bifunctional alcohol dehydrogenase-acetyl-CoA reductive thioesterase. Biochem. J. 302: 163–170.

Burdette, D. S., C. Vieille, and J. G. Zeikus. 1996. Cloning and expression of the gene encoding the Thermoanaerobacter ethanolicus 39E secondary-alcohol dehydrogenase and biochemical charaterization of the enzyme. Biochem. J. 316:115–122.

Busche, R. M. 1985. Acetic acid manufacture—fermentation alternatives. *In:* P. N. Cherenisinoff and R. P. Ouellette (Eds.) Biotechnology Applications and Research. Technomic. Lancaster, PA. 88–102.

Busche, R. M. 1991. Extractive fermentation of acetic acid: Economic tradeoff between yield of Clostridium and concentration of Acetobacter. Appl. Biochem. Biotechnol. 28/29:605–621.

Buschhorn, H., P. Dü, and G. Gottschalk. 1989. Production and utilization of ethanol by homoacetogen Acetobacterium woodii. Appl. Environ. Microbiol. 55:1835–1840.

Byrom, D. 1992. Production of poly-β-hydroxybutyrate: Poly-β-hydroxyvalerate copolymers. FEMS Microbiol. Rev. 103:247–250.

Byrom, D. 1994. Polyhydroxyalkanoates. *In:* D. P. Mobley (Ed.) Plastics from Microbes. Hanser Publishers. Munich, Germany. 5–33.

Cameron, D. C., and C. L. Cooney. 1986. A novel fermentation: The production of R(−)-1,2-propanediol and acetol by Clostridium thermosaccharolyticum. Biotechnology 4:651–654.

Cameron, D. C., I. T. Tong, and F. A. Skraly. 1993. Metabolic engineering for the production of 1,3-propanediol. *In:* Chianelli, R. R. and Davison, B. H.ACS Symposium on Bioremediation and Bioprocessing. American Chemical Society. Denver, CO. 38:294–295.

Cameron, D. C., N. E. Altaras, M. L. Hoffman, and A. J. Shaw. 1998. Metabolic engineering of propanediol pathways. Biotechnol. Prog. 14:16–125.

Cameron, D. C., A. J. Shaw, and N. E. Altaras. 2000. Microbial production of 1,2-propanediol from sugar. US Patent 6087140.

Cann, I. K. O., P. G. Stroot, K. R. Mackie, B. A. White, and R. I. Mackie. 2001. Characterization of two novel saccharolytic, anaerobic thermophiles, Thermoanaerobacterium polysaccharolyticum sp. nov. and Thermoanaerobacterium zeae sp. nov., and emendation of the genus Thermoanaerobacterium. Int. J. Syst. Evol. Microbiol. 51:293–302.

Carr, N. G. 1966. The occurrence of poly-β-hydroxybutyrate in the blue-green alga, Chloroglea fritschii. Biochim. Biophys. Acta 120:308–310.

Cato, E. P., W. L. George, and S. M. Finegold. 1986. Genus Clostridium Prazmowski 1880. *In:* H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 2:1141–1200.

Cauvin, B., and J. B. Luchansky. 1992. Advances in electrotransformation of Gram-positive bacteria. Bio-rad Laboratories Bulletin 1350A.

Cayol, J.-L., B. Ollivier, B. K. C. Patel, G. Ravot, M. Magot, E. Ageron, P. A. D. Grimont, and J.-L. Garcia. 1995. Description of Thermoanaerobacter brockii subsp. lactiethylicus subsp. nov., isolated from a deep subsurface french oil well, a proposal to reclassify Thermoanaerobacter finnii as Thermoanaerobacter brockii subsp. finnii comb. nov., and an emended description of Thermoanaerobacter brockii. Int. J. Syst. Bacteriol. 45:783–789.

Champluvier, B., J. Decallonne, and P. G. Rouxhet. 1989. Influence of sugar source (lactose, glucose, galactose) on 2,3-butanediol production by Klebsiella oxytoca NRRL-B199. Arch. Microbiol. 152:411–414.

Chang, H. N., Y. C. Kim, S. Y. Lee, and B. S. Kim. 1994. Current status of biodegradable plastics in Korea: Research, commercial production and government policy. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 286–297.

Chang, D.-E., H.-C. Jung, J.-S. Rhee, and J.-G. Pan. 1999. Homofermentative production of D- or L-lactate in metabolically engineered Escherichia coli RR1. Appl. Environ. Microbiol. 65:1384–1389.

Chen, J.-S., and S. F. Hiu. 1986. Acetone-butanol-isopropanol production by Clostridium beijerinckii (synonym, Clostridium butylicum). Biotechnol. Lett. 8:371–376.

Chen, J.-S. 1987. Electron transport in anaerobes. *In:* T. J. Montville (Ed.) Concepts in Physiology and Metabolism. CRC Press. Boca Raton, FL. 1:61–101.

Chen, J.-S. 1993. Properties of acid- and solvent-froming enzymes of clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. Stoneham, MA. 51–76.

Chen, J. S. 1995. Alcohol dehydrogenase: Multiplicity and relatedness in the solvent-producing clostridia. FEMS Microbiol. Rev. 17:263–273.

Chen, C.-K., and H. P. Blaschek. 1999a. Acetate enhances solvent production and prevents degeneration in Clostridium beijerinckii BA101. Appl. Microbiol. Biotechnol. 52:170–173.

Chen, C.-K., and H. Blaschek. 1999b. Effect of acetate on molecular and physiological aspects of Clostridium beijerinckii NCIMB 8052 solvent production and strain degeneration. Appl. Environ. Microbiol. 65:499–505.

Cheng, P., R. E. Mueller, S. Jaeger, R. Bajpai, and E. L. Iannotti. 1991. Lactic acid production from enzyme-thinned corn starch using Lactobacillus amylovorus. J. Indust. Microbiol. 7:27–34.

Cheryan, M., S. Paretkh, M. Shah, and K. Witjitra. 1997. Production of acetic acid by Clostridium thermoaceticum. Adv. Appl. Microbiol. 43:1–33.

Cheryan, M. 1999. Acetic acid production. *In:* J. Lederberg (Ed.) Encyclopedia of Microbiology. Academic Press. San Diego, CA.

Chiellini, E. 1994. Status of government policy, regulation and standards on the issue of biodegradable plastic materials in Italy. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 273–285.

Choi, J., and S. Y. Lee. 1997. Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. Bioprocess Eng. 17:335–342.

Choi, J., and S. Y. Lee. 1999a. Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals. Biotechnol. Bioeng. 62:546–553.

Choi, J., and S. Y. Lee. 1999b. High-level production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fed-batch culture of recombinant Esherichia coli. Appl. Environ. Microbiol. 65:4363–4368.

Choi, J., and S. Y. Lee. 2000. Economic consideration in the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by bacterial fermentation. Appl. Microbiol. Biotechnol. 53:646–649.

Chotani, G., T. Dodge, A. Hsu, M. Kumar, R. LaDuca, D. Trimbur, W. Weyler, and K. Sanford. 2000. The commercial production of chemicals using pathway engineering. Biochim. Biophys. Acta 1543:434–455.

Chukwu, U. N., and M. Cheryan. 1996. Concentration of vinegar by electrodialysis. J. Food Sci. 61:1223–1226.

Chun, V. H., and P. L. Rogers. 1988. The simultaneous production of sorbitol from fructose and gluconic acid from glucose using an oxidoreductase of Zymomonas mobilis. Appl. Microbiol. Biotechnol. 29:19–24.

Clark, D. P., P. R. Cunningham, S. G. Reams, F. Mat-Jan, R. Mohammedkhani, and C. R. Williams. 1988. Mutants of Escherchia coli defective in acid fermentation. Appl. Biochem. Biotechnol. 17:163–173.

Clark, D. P. 1989. The fermentation pathways of Escherichia coli. FEMS Microbiol. Rev. 63:223–234.

Clark, S. W., G. N. Bennett, and F. B. Rudolph. 1989. Isolation and characterization of mutants of Clostridium acetobutylicum ATCC 824 deficient in acetoacetyl-coenzyme A: Acetate/butyrate:coenzyme A-transferase (EC 2.8.3.9) and in other solvent pathway enzymes. Appl. Environ. Microbiol. 55:970–976.

Clausen, E. C., and J. L. Gaddy. 1996. Ethanol from biomass by gasification/fermentation. *In:* M. R. Khan (Ed.) Conversion and Utilization of Waste Materials. Taylor & Francis. Washington DC, 157–167.

Cocconcelli, P. S., M. J. Gasson, L. Morelli, and V. Bottazzi. 1991. Single-stranded DNA plasmid vector construction and cloning of Bacillus stearothermophilus α-amylase in Lactobacillus. Res. Microbial 142:643–652.

Cocks, G. T., J. Aguilar, and E. C. C. Lin. 1974. Evolution of L-1,2-propanediol catabolism in Escherichia coli by recruitment of enzymes for L-fucose and L-lactate metabolism. J. Bacteriol. 118:83–88.

Colby, G. D., and J.-S. Chen. 1992. Purification and properties of 3-hydroxybutyryl-coenzyme A dehydrogenase from Clostridium beijerinckii ("Clostridium butylicum") NRRL B593. Appl. Environ. Microbiol. 58:3297–3302.

Colby, G. D. 1993. CoA-transferase and 3-hydroxybutyryl-CoA Dehydrogenases: Acetoacetyl-CoA-reacting Enzymes from Clostridium beijerinckii NRRL B593 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Colin, T., A. Bories, and G. Moulin. 2000. Inhibition of Clostridium butyricum by 1,3-propanediol and diols during glycerol fermentation. Appl. Microbiol. Biotechnol. 54:201–205.

Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow. 1994. The phylogeny of the genus Clostridium: Proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44:812–826.

Colomban, A., L. Roger, and P. Boyaval. 1993. Production of propionic acid from whey permeate by sequential fermentation, ultrafiltration, and cell recycling. Biotechnol. Bioeng. 42:1091–1098.

Compere, A. L., and W. L. Griffith. 1979. Evaluation of substrates for butanol production. Devel. Ind. Microbiol. 20:509–517.

Compere, A. L., W. L. Griffith, and J. M. Googin. 1985. Solvents production by clostridia as a function of wood stream organic toxicant concentration. Devel. Ind. Microbiol. 26:535–541.

Conway, T., Y. A. Osman, J. I. Konnan, E. M. Hoffmann, and L. O. Ingram. 1987. Promoter and nucleotide sequences of the Zymomonas mobilis pyruvate decarboxylase. J. Bacteriol. 169:949–954.

Conway, T., and L. O. Ingram. 1989. Similarity of Escherichia coli propanediol oxidoreductase (fucO product) and an

unusual alcohol dehydrogenase from Zymomonas mobilis and Saccharomyces cerevisiae. J. Bacteriol. 171:3754–3759.

Cook, G. M., F. A. Rainey, B. K. C. Patel, and H. W. Morgan. 1996. Characterization of a new obligately anaerobic thermophile, Thermanaerobacter wiegelii sp. nov. Int. J. Syst. Bacteriol. 46:123–127.

Cornillot, E., and P. Soucaille. 1996. Solvent-forming genes in clostridia. Nature 380:489.

Cornillot, E., C. Croux, and P. Soucaille. 1997a. Physical and genetic map of the Clostridium acetobutylicum ATCC 824 chromosome. J. Bacteriol. 179:7426–7434.

Cornillot, E., R. V. Nair, E. T. Papoutsakis, and P. Soucaille. 1997b. The genes for butanol and acetone formation in Clostridium acetobutylicum ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. J. Bacteriol. 179:5442–5447.

Croux, C., and J. L. Garcia. 1991. Sequence of the lyc gene encoding the autolytic lysozyme of Clostridium acetobutylicum ATCC 824: Comparison with other lytic enzymes. Gene 104:25–31.

Croux, C., and J. L. Garcia. 1992a. Reconstruction and expression of the autolytic gene from Clostridium acetobutylicum ATCC 824 in Escherichia coli. FEMS Microbiol. Lett. 95:13–20.

Croux, C., B. Canard, G. Goma, and P. Soucaille. 1992b. Autolysis of Clostridium acetobutylicum ATCC 824. J. Gen. Microbiol. 138:861–869.

Croux, C., B. Canard, G. Goma, and P. Soucaille. 1992c. Purification and characterization of an extracellular muramidase of Clostridium acetobutylicum ATCC 824 that acts on non-N-acetylated peptidoglycan. Appl. Environ. Microbiol. 58:1075–1081.

Crow, V. L. 1987. Citrate cycle intermediates in the metabolism of aspartate and lactate by Propionibacterium freudenreichii subsp. shermanii. Appl. Environ. Microbiol. 53:2600–2602.

Cueto, P. H., and B. S. Mendez. 1990. Direct selection of Clostridium acetobutylicum fermentation mutants by a protein suicide method. Appl. Environ. Microbiol. 56: 578–580.

Cummins, C., and J. L. Johnson. 1971. Taxonomy of the clostridia: Wall composition and DNA homologies in Clostridium butyricum and other butyric acid-producing clostridia. J. Gen. Microbiol. 67:33–46.

Cummins, C. S., and J. L. Johnson. 1986. The genus Propionibacterium. *In:* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 2:1346–1353.

Currie, J. N., and R. H. Carter (Charles Pfizer, and Co.). 1930. Gluconic Acid. US Patent 1896811. Chem. Abstr. 27:2757.

Currie, J. N., and A. Finlay. 1933. Fermentation such as the production of D-gluconic acid. US Patent 1908225. Chem. Abstr. 27:3774.

Dabrock, B., H. Bahl, and G. Gottschalk. 1992. Parameters affecting solvent production by Clostridium pasteurianum. Appl. Environ. Microbiol. 58:1233–1239.

Daniel, R., and G. Gottschalk. 1992. Growth temperature-dependent activity of glycerol dehydratase in Escherichia coli expressing the Citrobacter freundii dha regulon. FEMS Microbiol. Lett. 100:281–286.

Daniel, R., R. Boenigk, and G. Gottschalk. 1995a. Purification of 1,3-propanediol dehydrogenase from Citrobacter freundii and cloning, sequencing, and overexpression of the corresponding gene in Escherichia coli. J. Bacteriol. 177:2151–2156.

Daniel, R., K. Stuertz, and G. Gottschalk. 1995b. Biochemical and molecular characterization of the oxidative branch of glycerol utilization by Citrobacter freundii. J. Bacteriol. 177:4392–4401.

Daniel, R., Bobik, T. A., and G. Gottschalk. 1999. Biochemistry of coenzyme B12-dependent glycerol and diol dehydratases and organization of the encoding genes. FEMS Microbiol. Rev. 22:553–566.

Datta, R. 1989. Recovery and purification of lactate salts from whole fermentation broth by electrodialysis. US Patent to Michigan Biotech. Inst. 4,885,247.

Datta, R. 1992a. Process for the production of succinic acid by anaerobic fermentation. US Patent. 5,143,833.

Datta, R., D. A. Glassner, M. K. Jain, and J. R. Vick Roy. 1992b. Fermentation and purification process for succinic acid. US Patent. 5,168,055.

Datta, R., S.-P. Tsai, P. Bonsignore, S.-H. Moon, and J. R. Frank. 1995. Technological and economic potential of poly (lactic acid) and lactic acid derivitives. FEMS Microbiol. Rev. 16:221–231.

Davidson, B. E., R. M. Llanos, M. R. Cancilla, N. C. Redman, and A. J. Hillier. 1995. Current research on the genetics of lactic acid production in lactic acid bacteria. Int. Dairy J. 5:763–784.

Dawes, E. A., and P. J. Senior. 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. 10:135–266.

Deckwer, W. D. 1995. Microbial conversion of glycerol to 1,3-propanediol. FEMS Microbiol. Rev. 16:143–149.

de F. S. Barbosa, M., and L. O. Ingram. 1994. Expression of the Zymomonas mobilis alcohol dehydrogenase II (adhB) and pyruvate decarboxylase (pdc) genes in Bacillus. Curr. Microbiol. 28:279–282.

De Koning, G., M. Kellerhals, C. van Meurs, and B. Witholt. 1997. *In:* G. Eggink, A. Steinbuchel, Y. Poirer, and B. Witholt (Eds.) Proceedings of the 1996 International Symposium on Bacterial Polyhdroxyalknoates. NRC Research Press. 137–142.

Demain, A. L., E. L. Rickes, D. Hendlin, and E. C. Barnes. 1961. Nutritional studies on Lactobacillus heterohiochi. J. Bacteriol. 81:147–153.

de Mas, C., N. B. Jansen, and G. T. Tsao. 1988. Production of optically active 2,3-butanediol by Bacillus polymyxa. Biotechnol. Bioengin. 31:366–377.

Demirci, A., and A. L. Pometto 3rd. 1992. Enhanced production of D(−) lactic acid by mutants of Lactobacillus delbrueckii ATCC 9649. J. Ind. Microbiol. 11:23–28.

Demirci, A., and A. L. Pometto 3rd. 1995. Repeated-batch fermentation in biofilm reactors with plastic composite supports for lactic acid production. Appl. Microbiol. Biotechnol. 43:585–589.

Dennis, D., and N. O. Kaplan. 1960. D- and L-lactic acid dehydrogenases in Lactobacillus plantarum. J. Biol. Chem. 235:810–818.

Depasse, E. 1945. Vue d'ensemble d'une production industrielle de cétones. Bull. Assoc. Chim. Sucr. Distill. Fr. 62:317–339.

Desai, R. P., and E. T. Papoutsakis. 1999. Antisense RNA stragies for metabolic engineering of Clostridium acetobytylicum. Appl. Environ. Microbiol. 65:936–945.

DeVos, W. M., and G. F. M. Simons. 1994. Gene cloning and expression systems in Lactococci. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lac-

tic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 52–105.

DeWilt, H. G. J. 1972. Oxidation of glucose to gluconic acid. Ind. Eng. Chem. Prod. Res. Dev. 11:370–378.

Diaz-Torres, M., N. S. Dunn-Coleman, M. W. Chase, and D. Trimbur. 2000. Method for the recombinant production of 1,3-propanediol. US Patent 6136576.

Djordjevic, G. M., D. J. O'Sullivan, S. A. Walker, M. A. Conkling, and T. R. Kaenhammer. 1997. Triggered-suicide system designed for bacteriophage defense of Lactococcus lactis. J. Bacteriol. 179:6741–6748.

Doi, Y. 1990a. Microbial Polyesters. VCH. New York, NY.

Doi, Y., A. Segawa, Y. Kawaguchi, and M. Kunioka. 1990b. Cyclic nature of poly(3-hydroxyalkanoate) metabolism in Alcaligenes eutrophus. FEMS Microbiol. Lett. 67:165.

Doi, Y., and K. Fukuda (Eds.). 1994. Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands.

Donnelly, M. I., C. S. Millard, D. P. Clark, M. J. Chen, and J. W. Rathke. 1998a. A novel fermentation pathway in an Escherichia coli mutant producing succinic acid, acetic acid, and ethanol. Appl. Biochem. Biotechnol. 70–72: 187–198.

Donnelly, M., C. S. Millard, and L. Stols. 1998b. Mutant *E. coli* strain with increased succinic acid production. US Patent. 5,770,435.

Doran, J. B., and L. O. Ingram. 1993. Fermentation of crystalline cellulose to ethanol by Klebsiella oxytoca containing chromosomally integrated Zymomonas mobilis genes. Biotechnol. Progr. 9:533–538.

Doran, J. B., H. C. Aldrich, and L. O. Ingram. 1994. Saccharification and fermentation of sugar cane bagasse by Klebsiella oxytoca P2 containing chromosomally integrated genes encoding the Zymomonas mobilis ethanol pathway. Biotechnol. Bioengin. 44:240–247.

Drake, H. L. 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: Past and current perspectives. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 3–60.

Dubos, R. J. 1988. Pasteur and Modern Science. Science Tech Publishers. Madison, WI.

Dueresch, R. 1986. Fermentative maufacture of gluconic acid. German Patent DD236, 754. Chem. Abstr. 106:31387.

Dumsday, G. J., B. Zhou, W. Yaqin, G. A. Stanley, and N. B. Pamment. 1999. Comaprative stability of ethanol production by Escherichia coli KO11 in batch and chemostat culture. J. Ind. Microbiol. Biotechnol. 23:701–708.

Dü, P., A. Kuhn, M. Gottwald, and G. Gottschalk. 1987. Enzymatic investigations on butanol dehydrogenase and butyraldhyde dehydrogenase in extracts of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 26:268–272.

Dürre, P., R. J. Fischer, A. Kuhn, K. Lorenz, W. Schreiber, B. Stürzenhofecker, S. Ullmann, K. Winzer, and U. Sauer. 1995. Solventogenic enzymes of Clostridium acetobutylicum, catalytic properties, genetic organization and transcriptional regulation. FEMS Microbiol. Rev. 17: 251–262.

Dürre, P., and H. Bahl. 1996. Microbial production of acetone/butanol/isopropanol. *In:* M. Roehr (Ed.) Products of Primary Metabolism, 2nd ed. VCH Publisher. Weinheim, Germany. 6:229–268.

Dürre, P. 1998. New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl. Microbiol. Biotechnol. 49:639–648.

Dürre, P., M. Bohringer, S. Nakotte, S. Schaffer, K. Thormann, and B. Zickner. 2002. Transcriptional regulation of solventogenesis in Clostridium acetobutylicum. J. Molec. Microbiol. Biotechnol. 4:295–300.

Ebner, H., and H. Follmann. 1983. Acetic acid. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. VCH. Weinheim, Germany. 3:387–407.

Ebner, H. 1985. Process for the production of vinegar with more than 12g/100ml acetic acid. US Patent. 4,503,078.

Ebner, H., S. Sellmer, and H. Follmann. 1996. Acetic acid. *In:* M. Roehr (Ed.) VCH. Weinheim, Germany. 6:381–401.

Eden, G., and G. Fuchs. 1982. Total synthesis of acetyl CoA involved in autotrophic CO_2 fixation in Acetobacterium woodii. Arch. Microbiol. 133:66–74.

Eden, G., and G. Fuchs. 1983. Autotrophic CO_2 fixation in Acetobacterium woodii. II: Demonstration of enzymes involved. Arch. Microbiol. 135:68–73.

Eiteman, M. A., and J. H. Miller. 1995. Effect of succinic acid on 2,3-butanediol production by Klebsiella oxytoca. Biotechnol. Lett. 17:1057–1062.

Ennis, B. M., and I. S. Maddox. 1985. Use of Clostridium acetobutylicum P262 for production of solvents from whey permeate. Biotechol. Lett. 7:601–606.

Entani, E., S. Ohmori, H. Masai, and K.-I. Suzuki. 1985. Acetobacter polyoxogenes, sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity. J. Gen. Appl. Microbiol. 31:475–490.

Evans, P. J., and H. Y. Wang. 1990. Effects of extractive fermentation on butyric acid production by Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 32:393–397.

Evans, J. D., and S. A. Martin. 1997. Factors affecting lactate and malate utilization by Selenomonas ruminantium. Appl. Environ. Microbiol. 63:4853–4858.

Evans, V. J., H. Liyanage, A. Ravagnani, M. Young, and E. R. Kashket. 1998. Truncation of peptide deformylase reduces the growth rate and stabilizes solvent production in Clostridium beijerinckii NCIMB 8052. Appl. Environ. Microbiol. 64:1780–1785.

Fayolle, F., R. Marchal, and D. Ballerini. 1990. Effect of controlled substrate feeding on butyric acid production by Clostridium tyrobutyricum. J. Indust. Microbiol. 6:179–183.

Feldmann, S., G. A. Sprenger, and H. Sahm. 1989. Ethanol production from xylose with a pyruvate-formate-lyase mutant of Klebsiella planticola carrying a pyruvate-decarboxylase gene from Zymomonas mobilis. Appl. Microbiol. Biotechnol. 31:152–157.

Fernbach, A., and E. H. Strange. 1911. Acetone and higher alcohols (amyl, butyl or ethyl alcohols and butyric, propionic or acetic acid) from starches, sugars and other carbohydrates. British Patent. 15203–15204.

Fiedler, S., A. Steinbüchel, and B. Rehm. 2000. PhaG-mediated synthesis of poly(3-hydroxyalkanoates) consisting of medium-chain-length constituents from nonrelated carbon sources in recombinant Pseudomonas fragi. Appl. Environ. Microbiol. 66:2117–2124.

Fischer, R. J., J. Helms, and P. Dü. 1993. Cloning, sequencing and molecular analysis of the sol operon of Clostridium acetobutylicum, a chromosomal locus involved in solventogenesis. J. Bacteriol. 175:6959–6969.

Fitz, A. 1878. Über spaltpilzgärungen, IV. Bericht der Deutsch. Chem. Ges. 11:1890.

Flores-Encarnacion, M., and M. Contreras-Zentella. 1999. The respiratory system and diazotrophic activity of Ace-

tobacter diazotrophicus PAL5. J. Bacteriol. 181:6987–6995.

Fond, O., N. B. Jansen, and G. T. Tsao. 1985. A model of acetic acid and 2,3-butanediol inhibition of the growth and metabolism of Klebsiella oxytoca. Biotechnol. Lett. 7:727–732.

Fond, O., J.-M. Engasser, G. Matta-El-Amouri, and H. Petitdemange. 1986a. The acetone butanol fermentation on glucose and xylose. I: Regulation and kinetics in batch cultures. Biotechnol. Bioengin. 28:160–166.

Fond, O., J.-M. Engasser, G. Matta-El-Amouri, and H. Petitdemange. 1986b. The acetone butanol fermentation on glucose and xylose. II: Regulation and kinetics in fed-batch cultures. Biotechnol. Bioengin. 28:167–175.

Fontaine, F., W. H. Peterson, E. McCoy, M. J. Johnson, and G. Ritter. 1942. A new type of glucose fermentation by Clostridium thermoaceticum, n. sp. J. Bacteriol. 43:701–715.

Fontaine, L., I. Meynial-Salles, L. Girbal, X. Yang, C. Croux, and P. Soucaille. 2002. Molecular characterization and transcriptional analysis of adhE2, the gene encoding the NADH-dependent aldehyde/alcohol dehydrogenase responsible for butanol production in alcohologenic cultures of Clostridium acetobutylicum ATCC 824. J. Bacteriol. 184:821–830.

Forage, R. G., and M. A. Foster. 1982a. Glycerol fermentation in Klebsiella pneumoniae: Functions of the coenzyme B12-dependent glycerol and diol dehydratases. J. Bacteriol. 149:413–419.

Forage, R. G., and E. C. C. Lin. 1982b. Dha system mediating aerobic and anaerobic dissimilation of glycerol in Klebsiella pneumoniae NCIB 418. J. Bacteriol. 151:591–599.

Forde, A., C. Daly, and G. F. Fitzgerald. 1999. Identification of four phage resistance plasmids from Lactococcus lactis subsp. cremoris HO2. Appl. Environ. Microbiol. 65:1540–1547.

Frazer, F. R., and T. A. McCaskey. 1991. Effect of components of acid-hydrolyzed hardwood on conversion of D-xylose to 2,3-butanediol by Klebsiella pneumoniae. Enz. Microb. Technol. 13:110–115.

Freier-Schroeder, D., J. Wiegel, and G. Gottschalk. 1989. Butanol formation by Clostridium thermosaccharolyticum at neutral pH. Biotechnol. Lett. 11:831–836.

Fujimaki, T. 1998. Processability and properties of aliphatic polyesters, "BIONELLE," synthesized by polycondensation reaction. Polymer. Degrad. Stabil. 59:209–214.

Fukaya, M., H. Tagami, K. Tayama, H. Okumura, Y. Kawamura, and T. Beppu. 1989. Spheroplast fusion of Acetobacter aceti and its application to the breeding of strains for vinegar production. Agric. Biol. Chem. 53:2435–2440.

Fukaya, M., Y. S. Park, and K. Toda. 1992. Improvement of acetic acid fermentation by molecular breeding. J. Appl. Bacteriol. 73:447–454.

Gabriel, C. L. 1928. Butanol fermentation process. Indust. Engin. Chem. 20:1063–1067.

Gabriel, C. L., and F. M. Crawford. 1930. Development of the butyl-acetonic fermentation indistry. Indust. Engin. Chem. 22:1163–1165.

Gapes, J. R., D. Nimcevic, and A. Friedl. 1996. Long-term continuous cultivation of Clostridium beijerinckii in a two-stage chemostat with on-line solvent removal. Appl. Environ. Microbiol. 62:3210–3219.

Gapes, J. R. 2000a. The economics of the acetone-butanol fermentation: Theoretical and market considerations. J. Molec. Microbiol. Biotechnol. 2:27–32.

Gapes, J. R. 2000b. The history of the acetone-butanol project in Austria. J. Molec. Microbiol. Biotechnol. 2:5–8.

Garg, S. K., and A. Jain. 1995. Fermentative production of 2,3-butanediol: A review. Biores. Technol. 51:103–109.

Gasson, M. J., and W. M. DeVos (Eds.). 1994. Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY.

Gauss, W. F., S. Suzuki, and M. Takagi. 1976. US Patent. 3,990,944.

George, H. A., and J.-S. Chen. 1983a. Acidic conditions are not obligatory for onset of butanol formation by Clostridium beijerinckii (synonym, C. butylicum). Appl. Environ. Microbiol. 46:321–327.

George, H. A., J. L. Johnson, W. E. C. Moore, L. V. Holdeman, and J.-S. Chen. 1983b. Acetone, isopropanol, and butanol production by Clostridium beijerinckii (syn. Clostridium butylicum) and Clostridium aurantibutyricum. Appl. Environ. Microbiol. 45:1160–1163.

Gerischer, U., and P. Dü. 1990. Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from Clostridium acetobutylicum. J. Bacteriol. 172:6907–6918.

Gerngross, T. U., and D. P. Martin. 1995. Enzyme-catalyzed synthesis of poly[R-(-)-3-hydroxybutyrate]: Formation of macroscopic granules in vitro. Proc. Natl. Acad. Sci. USA 92:6279–6283.

Gerngross, T. U. 1999. Can biotechnology move us toward a sustainable society? Nature Biotechnol. 17:541–544.

Ghazvinizadeh, H., G. C. Turtura, and C. Zambonelli. 1972. The fermentation of L-rhamnose by clostridia. Ann. Microbiol. 22:155–158.

Ghommidh, C., J. M. Navarro, and G. Durand. 1981. Acetic acid production by immobilized Acetobacter cells. Biotechnol. Lett. 3:93–98.

Ghose, T. K., and A. Bhadra. 1985. Acetic acid. *In:* M. Moo-Young (Ed.) Biotechnology. Pergamon Press. New York, NY. 3:701–729.

Gibbs, D. F. 1983. The rise and fall (. . . and rise?) of acetone/butanol fermentations. Trends Biotechnol. 1:12–15.

Gilles, M., K. Kerstens, B. Hoste, D. Janssens, R. M. Kroppenstedt, M. P. Stephens, K. R. S. Teixeira, J. Dobereiner, and J. DeLey. 1989. Acetobacter diazotrophicus sp. nov. a nitrogen-fixing acetic acid bacterium associated with sugar cane. Int. J. Syst. Bacteriol. 39:361–364.

Girbal, L., and P. Soucaille. 1994. Regulation of Clostridium acetobutylicum metabolism as revealed by mixed-substrate steady-state continuous cultures: Rle of NADH/NAD ratio and ATP pool. J. Bacteriol. 176:6433–6438.

Girbal, L., C. Croux, I. Vasconcelos, and P. Soucaille. 1995a. Regulation of metabolic shifts in Clostridium acetobutylicum ATCC 824. FEMS Microbiol. Rev. 17:287–297.

Girbal, L., I. Vasoncelos, S. Saint-Amans, and P. Soucaille. 1995b. How neutral red modified carbon and electron flow in Clostridium acetobutylicum grown in chemostat culture at neutral pH. FEMS Microbiol. Rev. 16:151–162.

Girbal, L., and P. Soucaille. 1998. Regulation of solvent production in Clostridium acetobutylicum. Trends Biotechnol. 16:11–16.

Glassner, D. A., and R. Datta. 1992. Process for the production and purification of succinic acid. US Patent. 5,143, 834.

Godin, C., and J. M. Engasser. 1990. Two-stage continuous fermentation of Clostridium acetobutylicum: Effects of pH and dilution rate. Appl. Microbiol. Biotechnol. 33: 269–273.

Gokarn, R. R., M. A. Eiteman, S. A. Martin, and K. E. L. Eriksson. 1997a. Production of succinate from glucose, cellobiose, and various cellulosic materials by the ruminal anaerobic bacteria Fibrobacter succinogenes and Ruminococcus flavefaciens. Appl. Biochem. Biotechnol. 68:69–80.

Gokarn, R. R., M. A. Eiteman, and J. Sridhar. 1997b. Production of succinate by anaerobic microorganisms. American Chemical Society Symposium Series 666:237–253.

Gokarn, R. R., M. A. Eiteman, and E. Altman. 1998. Expression of pyruvate carboxylase enhances succinate production in Escherichia coli without affecting glucose uptake. Biotechnol. Lett. 20:795–798.

Gokarn, R. R., M. A. Eiteman, and E. Altman. 1999. Pyruvate carboxylase overexpression for enhanced production of oxalacetate-derived biochemicals in microbial cells. PCT International Patent Application. WO 99/53035.

Goldberg, I., K. Lonberg-Holm, E. A. Bagley, and B. Stieglitz. 1983. Improved conversion of fumarate to succinate by Escherichia coli strains amplified for fumarate reductase. Appl. Env. Microbiol. 45:1838–1847.

Goodlove, P. E., P. R. Cunningham, J. Parker, and D. P. Clark. 1989. Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of Escherichia coli. Gene 85:209–214.

Gosalbes, M. J., C. D. Esteban, J. L. Galan, and G. Perez-Martinez. 2000. Integrative food-grade expression system based on the lactose regulon of Lactobacillus casei. Appl. Environ. Microbiol. 66:4822–4828.

Gottschalk, G. 1985. Bacterial Metabolism, 2nd ed. Springer-Verlag. New York, NY. 247.

Gottschalk, G. 1986. Bacterial Metabolism, 2nd ed. Springer-Verlag. New York, NY. 97–98.

Gottschalk, G., and B. Averhoff. 1992. Process for the microbiological preparation of 1,3-propane-diol from glycerol by Citrobacter. US Patent 5164309.

Gottwald, M., H. Hippe, and G. Gottschalk. 1984. Formation of n-butanol from D-gucose by strains of the "Clostridium tetanomorphum" group. Appl. Environ. Microbiol. 48:573–576.

Gottwald, M., and G. Gottschalk. 1985. The internal pH of Clostridium acetobutylicum and its effect on the shift from acid to solvent formation. Arch. Microbiol. 143:42–46.

Goupil-Feuillerat, N., M. Cocaign-Bousquet, J.-J. Godon, S. D. Ehrlich, and P. Renault. 1997. Dual role of a-acetolactate decarboxylase in Lactococcus lactis subsp. lactis. J. Bacteriol. 179:6285–6293.

Green, E. M., and G. N. Bennett. 1996a. Inactivation of an aldehye/alcohol dehydrogenase gene from Clostridium acetobutylicum ATCC 824. Appl. Biochem. Biotechnol. 57/58:213–221.

Green, E. M., Z. L. Boynton, L. M. Harris, F. B. Rudolph, E. T. Papoutsakis, and G. N. Bennett. 1996b. Genetic manipulation of acid formation pathways by gene inactivation in Clostridium acetobutylicum ATCC 824. Microbiology 142:2079–2086.

Greenberg, K. 1999. New PDO technologies create opportunities. Chem. Mark. Rep. 255:4 and 9.

Grethlein, A. J., R. M. Worden, M. K. Jain, and R. Datta. 1990. Continuous production of mixed alcohols and acids from carbon monoxide. Appl. Biochem. Biotechnol. 24–25:875–884.

Grove, L. H. 1982. Process for the production of organic fuel. US Patent. 4326032.

Gruber, P. R., E. S. Hall, J. J. Kolstad, M. L. Iwen, R. D. Benson, and R. L. Borchardt. 1992. Continuous process for manufacture of lactide polymers with controlled optical purity. US Patent to Cargill, Inc. 5,142,023.

Gruber, P. R., E. S. Hall, J. J. Kolstad, M. L. Iwen, R. D. Benson, and R. L. Borchardt. 1994. Continuous process for manufacture of lactide polymers with purification by distillation. US Patent to Cargill, Inc. 5,357,035.

Grupe, H., and G. Gottschalk. 1992. Physiological events in Clostridium acetobutylicum during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Appl. Environ. Microbiol. 58:3896–3902.

Gu, Z., B. A. Glatz, and C. E. Glatz. 1998. Propionic acid production by extractive fermentation. I: Solvent consideration. Biotechnol. Bioeng. 57:454–461.

Gu, Z., D. A. Rickert, B. A. Glatz, and C. E. Glatz. 1999. Feasibility of propionic acid production by extractive fermentation. Lait 79:137–148.

Guettler, M. V., and M. K. Jain. 1996a. Method for making succinic acid, Anaerobiospirillum succiniciproducens variants for use in process and methods for obtaining variants. US Patent. 5,521,075.

Guettler, M. V., M. K. Jain, and D. Rumler. 1996b. Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. US Patent. 5,573, 931.

Guettler, M. V., M. K. Jain, and B. K. Soni. 1996c. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US Patent. 5,504,004.

Guettler, M. V., M. K. Jain, and B. K. Soni. 1998. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US Patent. 5,723,322.

Guettler, M. V., D. Rumler, and M. K. Jain. 1999. Actinobacillus succinogenes sp. nov., a novel succinic-acid-producing strain from the bovine rumen. Int. J. Syst. Bacteriol. 49:207–216.

Gunzel, B., S. Yonsel, and W. D. Deckwer. 1991. Fermentative production of 1,3-propanediol from glycerol by Clostridium butyricum up to a scale of 2 m^3. Appl. Microbiol. Biotechnol. 36:289–294.

Gupta, A., V. Verma, and G. N. Quazi. 1997. Transposon induced mutation in Gluconobacter oxydans with special reference to its direct glucose oxidation metabolism. FEMS Microbiol. Lett. 147:181–188.

Gupta, A., M. Felder, Y. Yerma, J. Cullum, and G. N. Qazi. 1999. A mutant of Gluconobacter oxydans deficient in gluconic acid dehydrogenase. FEMS Microbiol. Lett. 179:501–506.

Gutierrez, N. A., and I. S. Maddox. 1992. Product inhibition in a nonmotile mutant of Clostridium acetobutylicum. Enz. Microbiol. Technol. 14:101–105.

Hahn, J. J., A. C. Eschenlauer, M. H. Narrol, D. A. Somers, and F. Sriene. 1997. Growth kinetics, nutrient uptake, and expression of the Alcaligenes eutrophus poly(β-

hydroxybutyrate) synthesis pathway in transgenic maize cell suspension cultures. Biotechnol. Prog. 13:347–354.

Hamilton, G. A., and F. H. Westheimer. 1959. A crystalline decarboxylase without biotin. J. Am. Chem. Soc. 8:2277.

Han, I. S., and M. Cheryan. 1996. Downstream processing of acetate fermentation broths by nanofiltration. Appl. Biochem. Biotechnol. 57/58:19–28.

Hancock, K. R., E. Rockman, C. A. Young, L. Pearce, I. S. Maddox, and D. B. Scott. 1991. Expression and nucleotide sequence of the Clostridium acetobutylicum β-galactosidase gene cloned in Escherichia coli. J. Bacteriol. 173:3084–3095.

Harlander, S. K. 1987. Transformation of Streptococcus lactis by electroporation. *In:* J. Ferretti and R. Curtiss (Eds.) Streptococcal Genetics. American Society for Microbiology. Washington DC, 229–233.

Harris, J., R. Mulder, D. B. Kell, R. P. Walter, and J. G. Morris. 1986. Solvent production by Clostridium pasteurianum in media of high sugar content. Biotechnol. Lett. 8:889–892.

Harris, L. M., R. P. Desai, N. E. Welker, and E. T. Papoutsakis. 2000. Characterization of recombinant strains of the Clostridium acetobutylicum butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition? Biotechnol. Bioengin. 67:1–11.

Harris, L. M., N. E. Welker, and E. T. Papoutsakis. 2002. Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in Clostridium acetobutylicum ATCC 824. J. Bacteriol. 184:3586–3597.

Hartmanis, M. G. N. 1987. Butyrate kinase from Clostridium acetobutylicum. J. Biol. Chem. 262:617–621.

Hastings, J. J. H. 1971. Development of the fermentation industries in Great Britain. Adv. Appl. Microbiol. 14:1–45.

Hastings, J. J. H. 1978. Acetone-butyl alcohol fermentation. *In:* A. H. Rose (Ed.) Primary Products of Metabolism. Academic Press. London, UK. 31–45.

Haynie, S. L., and L. W. Wagner. 1997. Process for making 1,3-propanediol from carbohydrates using mixed microbial cultures. US Patent 5599689.

Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes. 1988. The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism Alcaligenes eutrophus. FEMS Microbiol. Lett. 52:259–264.

Hein, S., H. Tran, and A. Steinbüchel. 1998. Synechocystis sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. Arch. Microbiol. 170:162–170.

Helbert, J. R. 1982. Beer. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 403–467.

Hermann, M., F. Fayolle, R. Marchal, L. Podvin, M. Sebald, and J.-P. Vandecasteele. 1985. Isolation and characterization of butanol-resistant mutants of Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:1238–1243.

Herrick, H. T., and O. E. May. 1928. The production of gluconic acid by the Penicillium luteum purpurogenum group II: Some optimal conditions for acid formation. J. Biol. Chem. 77:185–195.

Heyndrickx, M., P. De Vos, M. Vancanneyt, and J. De Ley. 1991. The fermentation of glycerol by Clostridium butyricum LMG 1212t2 and 1213t1 and C. pasteurianum LMG 3285. Appl. Microbiol. Biotechnol. 34:637–642.

Himmel, M. E., W. S. Adney, K. Grohmann, and M. P. Tucker. 1994. US Patent. 5,275,944.

Himmel, M. E., W. S. Adney, J. O. Baker, R. Elander, J. D. McMillan, R. A. Nieves, J. J. Sheehan, S. R. Thomas, T. B. Vinzant, and M. Zhang. 1997. Advanced bioethanol production technologies: A perspective. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:2–45.

Himmi, E. H., A. Bories, and F. Barbirato. 1999. Nutrient requirements for glycerol conversion to 1,3-propanediol by Clostridium butyricum. Biores. Technol. 67:123–138.

Himmi, E. H., A. Bories, A. Boussaid, and L. Hassani. 2000. Propionic acid fermentation of glycerol and glucose by Propionibacterium acidipropionici and Propionibacterium freudenreichii ssp. shermanii. Appl. Microbiol. Biotechnol. 53:435–440.

Hippe, H., J. R. Andresen, and G. Gottschalk. 1991. The genus Clostridium, non-medical. *In:* Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes. Springer. New York, NY. 1799–1866.

Hiu, S. F., C.-X. Zhu, R.-T. Yan, and J.-S. Chen. 1987. Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: Different alcohol dehydrogenases in two strains of Clostridium beijerinckii (Clostridium butylicum). Appl. Environ. Microbiol. 53:697–703.

Hlasiwetz, H., and J. Habermann. 1870. Zur Kenntnis einiger Zuckerarten (Glucose, Rhorzucker, Levulose, Sorbin, Phloroglucin). Ann Chem. Pharm. 155:128–144.

Ho, K.-L. G., A. L. Pometto 3rd, and P. N. Hinz. 1997a. Optimization of L-(+)-lactic acid production by ring and disc plastic composite supports through repeated-batch biofilm fermentation. Appl. Environ. Microbiol. 63: 2533–2542.

Ho, K.-L. G., A. L. Pometto 3rd, P. N. Hinz, and A. Demirci. 1997b. Nutrient leaching and end product accumutation in plastic composite supports for L-(+)-lactic acid biofilm fermentation. Appl. Environ. Microbiol. 63:2524–2532.

Ho, K.-L. G., A. L. Pometto 3rd, P. N. Hinz, J. S. Dickson, and A. Demirci. 1997c. Ingredients selection for plastic composite supports for L-(+)-lactic acid biofilm fermentation by Lactobacillus casei subsp. rhamnosus. Appl. Environ. Microbiol. 63:2516–2523.

Hocking, P. J., and R. H. Marchessault. 1994. Biopolymers. *In:* G. J. L. Griffin (Ed.) Chemistry and Technology of Biodegradable Polymers. Chapman and Hall. London, 48–96.

Hoffman, J. 2001. Butadiene hit hard by sharp drop in derivatives demand. Chem. Mark. Rep. 260:1 and 12.

Holland-Staley, C. A., K. Lee, D. P. Clark, and P. R. Cunningham. 2000. Aerobic activity of Escherichia coli alcohol dehydrogenase is determined by a single amino acid. J. Bacteriol. 18(2):6049–6054.

Holt, R. A., G. M. Stephens, and J. G. Morris. 1984. Production of solvents by Clostridium acetobutylicum cultures maintained at neutral pH. Appl. Environ. Microbiol. 48:1166–1170.

Holt, R. A., A. J. Cairns, and J. G. Morris. 1988. Production of butanol by Clostridium puniceum in batch and continuous culture. Appl. Microbiol. Biotechnol. 27:319–324.

Holten, C. M., A. Muller, and D. Rehbinder (Eds.). 1971. Lactic Acid. Weinheim/Bergstr. Germany.

Homann, T., C. Tag, H. Biebl, W. D. Deckwer, and B. Schink. 1990. Fermentation of glycerol to 1,3-propanediol by Klebsiella and Citrobacter strains. Appl. Microbiol. Biotechnol. 33:121–126.

Hong, R. 1999. The Cloning of a Putative Regulatory Gene and the sol Region from Clostridium beijerinckii M.S. thesis. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Hongo, M., and A. Murata. 1965a. Bacteriophages of Clostridium saccharoperbutylacetonicum. Part I: Some characteristics of the twelve phages obtained from the abnormally fermented broths. Agric. Biol. Chem. 29: 1135–1139.

Hongo, M., and A. Murata. 1965b. Bacteriophages of Clostridium saccharoperbutylacetonicum. Part II: Enumeration of phages by the application of the plaque count technique and some factors influencing the plaque formation. Agric. Biol. Chem. 29:1140–1145.

Hoppner, T. C., and H. W. Doelle. 1983. Purification and kinetic characteristics of pyruvate decarboxylase and ethanol dehydrogenase from Zymomonas mobilis in relation to ethanol production. Eur. J. Appl. Microbiol. Biotechnol. 17:152–157.

Houben, M. C. M. 1995. Oxygenated blending components for gasoline-alcohols and ethers. *In:* E. L. Marshall and K. Owen (Ed.) Motor Gasoline: Critical Reports on Applied Chemistry. Royal Society of Chemistry. Cambridge, UK. 34:45–71.

Houge, C. 2000. Getting the MTBE out. Chem Eng. News 6.

Howaldt, M., A. Gottlob, K. D. Kulba, and H. Chmiel. 1988. Simultaneous conversion of glucose/fructose mixtures in a membrane reactor. Ann. NY Acad. Sci. 542:400–405.

Howaldt, M., K. D. Kulbe, and H. Chmiel. 1990. A continous enzyme membrane reactor retaining native nicotinamide cofactor NAD (H). Ann. NY Acad. Sci. 589:253–260.

Howard, W. L. 1991. Acetone. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 1:176–194.

Hrabak, O. 1992. Industrial production of poly-β-hydroxybutyrate. FEMS Microbiol. Rev. 103:251–256.

Hromatka, O., and H. Ebner. 1949. Investigations on vinegar fermentation: Generator for vinegar fermentation and aeration procedures. Enzymologia 13:369.

Hsu, S. T., and S. T. Yang. 1991. Propionic acid fermentation of lactose by Propionibacterium acidipropionici—effects of pH. Biotechnol. Bioeng. 38:571–578.

Huang, Y., and S. T. Yang. 1998a. Acetate production from whey lactose using co-immobilized cells of homolactic and homoacetic bacteria in a fibrous-bed bioreactor. Biotechnol. Bioeng. 60:498–507.

Huang, Y. L., K. Mann, and J. M. Mavak. 1998b. Acetic acid production from fructose by Clostridium formicaceticum immobilized in a fibrous bed bioreactor. Biotechnol. Prog. 800–806.

Huang, K.-X., F. B. Rudolph, and G. N. Bennett. 1999. Characterization of methylglyoxal synthase from Clostridium acetobutylicum ATCC 824 and its use in the formation of 1,2-propanediol. Appl. Environ. Microbiol. 65:3244–3247.

Huang, K.-X., S. Huang, F. B. Rudolph, and G. N. Bennett. 2000. Identification and characterization of a second butyrate kinase from Clostridium acetobutylicum ATCC 824. J. Molec. Microbiol. Biotechnol. 2:33–38.

Huff, G. F., and N. Yata. 1976. US Patent. 3,990,945.

Husemann, M., and E. T. Papoutsakis. 1986. Effect of acetoacetate, butyrate, and uncoupling ionophores on growth and product formation of Clostridium acetobutylicum. Biotechnol. Lett. 8:37–42.

Husemann, M. H. W., and E. T. Papoutsakis. 1988. Solventoggenesis in Clostridium acetobutylicum fermentations related to carboxylic acid and proton concentrations. Biotechnol. Bioengin. 32:843–852.

Husemann, M. H. W., and E. T. Papoutsakis. 1989. Comparison between in vivo and in vitro enzyme activities in continuous and batch fermentations of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 30: 585–595.

Ingram, L. O., and T. M. Buttke. 1984. Effects of alcohols on micro-organisms. Adv. Microb. Physiol. 25:253–300.

Ingram, L. O. 1986. Microbial tolerance to alcohols: Role of the cell membrane. Trends Biotechnol. 4:40–44.

Ingram, L. O., T. Conway, D. P. Clark, G. W. Sewell, and J. F. Preston. 1987. Genetic engineering of ethanol production in Escherichia coli. Appl. Environ. Microbiol. 53:2420–2425.

Ingram, L. O., and T. Conway. 1988. Expression of different levels of ethanologenic enzymes from Zymomonas mobilis in recombinant strains of Escherichia coli. Appl. Environ. Microbiol. 54:397–404.

Ingram, L. O., and K. M. Dombek. 1989. Effects of ethanol on Escherichia coli. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 227–237.

Ingram, L. O. 1990. Ethanol tolerance in bacteria. Crit. Rev. Biotechnol. 9:305–319.

Ingram, L. O., F. Alterthum, and T. Conway. 1991. US Patent. 5,000,000.

Ingram, L. O., and D. C. Clark. 1992. . US Patent. 5,028,539.

Ingram, L. O., and J. B. Doran. 1995a. Conversion of cellulosic materials to ethanol. FEMS Microbiol. Rev. 16:235–241.

Ingram, L. O., D. S. Beall, G. F. H. Burchhardt, W. V. Guimaraes, K. Ohta, B. E. Wood, K. T. Shanmugam, D. E. Fowler, and B.-B. A. 1995b. US Patent. 5,424,202.

Ingram, L. O., and M. D. F. Barbosa-Alleyne. 1996. US Patent. 5,482,846.

Ingram, L. O., and K. W. Ohta, B. E. 1998a. US Patent. 5821093.

Ingram, L. O., P. F. Gomez, X. Lai, M. Moniruzzaman, B. E. Wood, L. P. Yamano, and S. W. York. 1998b. Metabolic engineering of bacteria for ethanol production. Biotechnol. Bioengin. 58:204–214.

Ingram, L. O., H. C. Aldrich, A. C. C. Borges, T. B. Causey, A. Martinez, F. Morales, A. Saleh, S. A. Underwood, L. P. Yomano, S. W. York, J. Zaldivar, and S. Zhou. 1999. Enteric bacterial catalysts for fuel ethanol production. Biotechnol. Progr. 15:855–866.

Inoue, Y., and A. Kimura. 1995. Methylglyoxal and regulation of its metabolism in microorganisms. Adv. Microb. Physiol. 37:177–227.

Inskeep, G. C., G. G. Taylor, and W. C. Breitzke. 1952. Lactic acid from corn sugar. Ind. Eng. Chem. 44:1955–1966.

Isbell, H. S., H. L. Frush, and F. J. Bates. 1932. Manufacture of calcium gluconate by electrolytic oxidation of glucose. Ind. Eng. Chem. 24:375–378.

Ishizaki, A., N. Taga, T. Takeshita, T. Sugimoto, T. Tsuge, and K. Tanaka. 1997. Microbial production of biodegradable plastics from carbon dioxide and agricultural waste material. *In:* B. C. Saha and J. Woodward (Eds.) Fuels

and Chemicals from Biomass. American Chemical Society. Washington DC, 295–306.

Ismaiel, A. A., C.-X. Zhu, G. D. Colby, and J.-S. Chen. 1993. Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of Clostridium beijerinckii. J. Bacteriol. 175:5097–5105.

Ismaiel, A. A., and J. S. Chen. 1998. Purification of the aldehyde-alcohol dehydrogenase encoded by the aad gene from Clostridium acetobutylicum ATCC 824. *In:*Abstracts of the 98th General Meeting of the American Society of Microbiology. American Society for Microbiology. Washington, D.C., O-40:400.

Iversen, T.-G., R. Standal, T. Pederson, and D. H. Caucheron. 1994. IS 1032 from Acetobacter xylinum, a new mobile insertion sequence. Plasmid 32:46–54.

Jabalquinto, A. M., M. Laivenieks, J. G. Zeikus, and E. Cardemil. 1999. Characterization of the oxaloacetate decarboxylase and pyruvate kinase-like activities of Saccharomyces cerevisiae and Anaerobiospirillum succiniciproducens phosphoenolpyruvate carboxykinases. J. Prot. Chem. 18:659–664.

Jackson, M. D., and C. B. Moyer. 1991. Alcohol fuels. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 1:826–864.

Jan, G., A. Rouault, and J. L. Maubois. 2000. Acid stress susceptibility and acid adaptation of Propionibacterium freudenreichii subsp. shermanii. Lait 80:325–336.

Jan, G., P. Leverrier, V. Pichereau, and P. Boyaval. 2001. Changes in protein synthesis and morphology during acid adaptation of Propionibacterium freudenreichii. Appl. Environ. Microbiol. 67:2029–2036.

Jansen, N. B., and G. T. Tsao. 1983. Bioconversion of pentoses to 2,3-butanediol by Klebsiella pneumoniae. *In:* A. Fiechter and T. W. Jeffries (Eds.) Advances in Biochemical Engineering/Biotechnology. Springer-Verlag. New York, NY. 27:85–99.

Jansen, N. B., M. C. Flickinger, and G. T. Tsao. 1984. Production of 2,3-butanediol from D-xylose by Klebsiella oxytoca ATCC 8724. Biotechnol. Bioengin. 26:362–369.

Jarvis, L. 2001. Lactic acid outlook up as polylactide nears market. Chemical Market Reporter 259(9):5, 14.

Jendrossek, D., M. Backhaus, and M. Andermann. 1995. Characterization of the Comamonas sp. poly(3-hydroxybutyrate) (PHB) depolymerase and of its structural gene. Can. J. Microbiol. 41:160–169.

Jendrossek, D., A. Schirmer, and H. G. Schlegel. 1996. Biodegradation of polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 46:451–463.

Jin, Z. W., and S. T. Yang. 1998. Extractive fermentation for enhanced propionic acid production from lactose by Propionibacterium acidipropionici. Biotechnol. Prog. 14:457–465.

Joachimsthal, E., K. D. Haggett, J.-H. Jang, and P. L. Rogers. 1998. A mutant of Zymomonas mobilis ZM4 capable of ethanol production from glucose in the presence of high acetate concentrations. Biotechnol. Lett. 20:137–142.

Johansen, L., Bryn, K., and F. C. Stormer. 1975. Physiological and biochemical role of the butanediol pathway in Aerobacter (Enterobacter) aerogenes. J. Bacteriol. 123:1124–1130.

John, M. E., and G. Keller. 1996. Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. USA 93:12768–12773.

Johnson, J. L. 1984. Bacterial classification. III: Nucleic acids in bacterial classification. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:8–11.

Johnson, J. L., and J.-S. Chen. 1995. Taxonomic relationships among strains of Clostridium acetobutylicum and other phenotypically similar organisms. FEMS Microbiol. Rev. 17:233–240.

Johnson, J. L., J. Toth, S. Santiwatanakul, and J.-S. Chen. 1997. Cultures of "Clostridium acetobutylicum" from various collections comprise Clostridium acetobutylicum, Clostridium beijerinckii, and two other distinct types based on DNA-DNA reassociation. Int. J. Syst. Bacteriol. 47:420–424.

Jones, R. P., and P. Greenfield. 1982. Effect of carbon dioxide on yeast growth and fermentation. Enz. Microbiol. Technol. 4:210–223.

Jones, D. T., W. A. Jones, and D. R. Woods. 1985. Production of recombinants after protoplast fusion in Clostridium acetobutylicum P262. J. Gen. Microbiol. 131:1213–1216.

Jones, D. T., and D. R. Woods. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50:484–524.

Jones. D. T. 1993. Mutagenesis and its application to biotechnology. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 77–98.

Jones, D. T., and S. Keis. 1995. Origins and relationships of industrial solvent-producing clostridial strains. FEMS Microbiol. Rev. 17:223–232.

Jones, D. T., M. Shirley, X. Wu, and S. Keis. 2000. Bacteriophage infections in the industrial AB fermentation process. J. Molec. Microbiol. Biotechnol. 2:21–26.

Jossek, R., and A. Steinbüchel. 1998. In vitro synthesis of poly-(3-hydroxybutyric acid) by using an enzymatic coenzyme A recycling system. FEMS Microbiol. Lett. 168:319–324.

Junelles, A.-M., R. Janati-Idrissi, A. El Kanouni, H. Petitdemange, and R. Gay. 1987. Acetone-butanol fermentation by mutants selected for resistance to acetate and butyrate halogen analogues. Biotechnol. Lett. 9:175–178.

Juni, E., and G. A. Heym. 1956. A cyclic pathway for the bacterial dissimilation of 2,3-butanediol, acetylmethylcarbinol, and diacetyl. I: General aspects of the 2,3-butanediol cycle. J. Bacteriol. 71:425–432.

Kandler, O., and N. Weiss. 1986. *In:* P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 2:1208–1234.

Kaneuchi, C., M. Seki, and K. Komagata. 1988. Production of succinic acid from citric acid and related acids by Lactobacillus strains. Appl. Environ. Microbiol. 54: 3053–3056.

Kansiz, M., H. Billman-Jacobe, and D. McNaughton. 2000. Quantitative determination of the biodegradable polymer poly(β-hydroxybutyrate) in a recombinant Escherichia coli strain by use of mid-infrared spectroscopy and multivariative statistics. Appl. Environ. Microbiol. 66:3415–3420.

Kashket, E. R., and Z. Y. Cao. 1995. Clostridial strain degeneration. FEMS Microbiol. Rev. 17:307–315.

Kawaguchi, Y., and Y. Doi. 1990. Structure of native poly(3-hydroxybutyrate) granules characterized by X-ray diffraction. FEMS Microbiol. Lett. 70:151–156.

Kawaguchi, Y., and Y. Doi. 1992. Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in Alcaligenes eutrophus. Macromolecules 25:2324–2329.

Keis, S., C. F. Bennett, V. K. Ward, and D. T. Jones. 1995. Taxonomy and phylogeny of industrial solvent-producing clostridia. Int. J. Syst. Bacteriol. 45:693–705.

Keis, S., R. Shaheen, and D. T. Jones. 2001a. Emended description of Clostridium acetobutylicum and Clostridium beijerinckii and descriptions of Clostridium saccharoperbutylacetonicum sp. nov. and Clostridium saccharobutylicum sp. nov. Int. J. Syst. Evol. Microbiol. 51:2095–2103.

Keis, S., J. T. Sullivan, and D. T. Jones. 2001b. Physical and genetic map of the Clostridium saccharobutylicum (formerly Clostridium acetobutylicum) NCP 262 chromosome. Microbiology 147:1909–1922.

Kelly, F. C. 1936. One Thing Leads To Another. Houghton Mifflin. Boston, MA.

Kennedy, J. F., J. D. Humphreys, S. A. Barker, and R. N. Greenshields. 1980. Application of living immobilized cells to the acceleration of the continuous conversions of ethanol (wort) to acetic acid (vinegar) by hydrous titanium (IV) oxide-immobilized Acetobacter species. Enz. Microbiol. Technol. 2:209–216.

Keshav, K. F., L. P. Yomano, H. An, and L. O. Ingram. 1990. Cloning of the Zymomonas mobilis structural gene encoding alcohol dehydrogenase I (adhA): Sequence comparison and expression in Escherichia coli. J. Bacteriol. 172:2491–2497.

Kessler, D., I. Leibrecht, and J. Knappe. 1991. Pyruvate-formate-lyase-deactivase and acetyl-CoA reductase activities of Escherichia coli reside on a polymeric protein particle encoded by adhE. FEBS Lett. 281:59–63.

Kessler, D., W. Herth, and J. Knappe. 1992. Ultrastructure and pyruvate formate-lyase radical quenching property of the multienzymic AdhE protein of Escherichia coli. J. Biol. Chem. 267:18073–18079.

Kiatpapan, P., Y. Hashimoto, H. Nakamura, Y.-Z. Piao, H. Ono, M. Yamashita, and Y. Murooka. 2000. Characterization of pRG01, a plasmid from Propionibacterium acidipropionici, and its use for development of a host-vector system in propionibacteria. Appl. Environ. Microbiol. 66:4688–4695.

Killeffer, D. H. 1927. Butanol and acetone from corn. Indust. Engin. Chem. 19:46–50.

Kim, D. M., and H. S. Kim. 1992a. Continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an oxido reductase from Zymomonas mobilis and inulinase. Biotechnol. Bioeng. 39:336–342.

Kim, B. S., S. Y. Lee, and H. N. Chang. 1992b. Production of poly-beta-hydroxybutyrate by fed-batch culture of recombinant Escherichia coli. Biotechnol. Lett. 14:811–816.

Kim, B. S., S. C. Lee, S. Y. Lee, H. N. Chang, Y. K. Chang, and S. I. Woo. 1994. Production of poly(3-hydroxybutyric acid) by fed-batch culture of Alcaligenes eutrophus with glucose concentration control. Biotechnol. Bioeng. 43:892–898.

Kim, S. W., P. Kim, H. S. Lee, and J. H. Kim. 1996. High production of poly-beta-hydroxybutyrate (PHB) from Methylobacterium organophilum under potassium limitation. Biotechnol. Lett. 18:25–30.

Kim, G. J., I. Y. Lee, S. C. Yoon, Y. C. Shih, and Y. H. Park. 1997. Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of Pseudomonas putida by combined use of glucose and octanoate. Enz. Microb. Technol. 20:500–505.

Kinoshita, S., T. Kakizono, K. Kadota, K. Das, and H. Taguchi. 1985. Purification of two alcohol dehydrogenases from Zymomonas mobilis and their properties. Appl. Microbiol. Biotechnol. 22:249–254.

Kirk Othmer. 1983. Succinic acid and succinic anhydride. Kirk-Othmer Encyclopedia of Chemical Technology, 3rd ed. 21:848–864.

Kirschner, E. M. 1996. Growth of top 50 chemicals slowed from very high 1994 rate. Chem. Eng. News 74(15):16–19.

Kitahara, K., T. Kaneko, and O. Goto. 1957. Taxonomic studies on the hiochi-bacteria specific saprophytes of sake. I: Isolation and grouping of bacterial strains. J. Gen. Appl. Microbiol. (Japan) 3:102–110.

Klaenhammer, T. R., and G. F. Fitzgerald. 1994. Bacteriophages and bacteriophage resistance. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 106–168.

Klapatch, R. R., M. L. Guerinot, and L. R. Lynd. 1996. Electrotransformation of Clostridium thermosaccharolyticum. J. Ind. Microbiol. 16:342–347.

Klasson, K. T., M. D. Ackerson, E. C. Clausen, and J. L. Gaddy. 1992. Bioconversion of synthesis gas into liquid or gaseous fuels. Enzyme Microbiol. Technol. 14:602–608.

Klinke, S., G. de Roo, B. Witholt, and B. Kessler. 2000. Role of phaD in accumulation of medium-chain-length poly(3-hydroxyalkanoates) in Pseudomonas olevorans. Appl. Environ. Microbiol. 66:3705–3710.

Knauf, H. J., R. F. Vogel, and W. P. Hammes. 1992. Cloning, sequence, and phenotypic expression of KatA which encodes the catalase of Lactobacillus sake LTH677. Appl. Environ. Microbiol. 58:832–839.

Kok, J., and J. M. DeVos. 1994. The proteolytic system of lactic acid bacteria. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 169–210.

Konda, T., and M. Konda. 1996. Efficient production of acetic acid from glucose in a mixed culture of Zymomonas mobilisand Acetobacter sp. J. Ferment. Bioeng. 81:42–46.

Kondo, K., and S. Horinouchi. 1997. A new insertion sequence IS 1452 from Acetobacter pasteurianus. Microbiology 143:539–546.

Korkhin, Y., A. J. Kalb(Gilboa), M. Peretz, O. Bogin, Y. Burstein, and F. Frolow. 1998. NADP-dependent bacterial alcohol dehydrogenases: Crystal structure, cofactor-binding and cofactor specificity of the ADHs of Clostridium beijerinckii and Thermoanaerobacter brockii. J. Molec. Biol. 278:967–981.

Kosaric, N. 1996. Ethanol-potential source of energy and chemical products. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology. VCH. New York, NY. 6:123–203.

Kusano, K., H. Yamada, M. Niwa, and K. Yamasoto. 1997. Propionibacterium cyclohexanicum sp nov., a new acid-tolerant omega-cyclohexy/fatty acid-containing propionibacterium isolated from spoiled orange juice. Int. J. Syst. Bacteriol. 47:825–831.

Kutzenok, A., and M. Aschner. 1952. Degenerative processes in a strain of Clostridium butylicum. J. Bacteriol. 64:829–836.

Kutzing, F. T. 1837. Prakt. Chem. J. 11:385.

Kwon, Y. J., R. Kaol, and B. Mattiasson. 1996. Extractive lactic acid fermentation in Poly (ethyleneimine)-based

aqueous two-phase system. Biotechnol. Bioeng. 50:280–290.

Ladisch, M. R. 1991. Fermentation-derived butanol and scenarios for its uses in energy-related applications. Enz. Microbiol. Technol. 13:280–283.

Laffend, L. A., V. Nagarajan, and C. E. Nakamura. 1997. Bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism. US Patent. 5686276.

Laffend, L. A., V. Nagarajan, and C. E. Nakamura. 2000. Bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism. US Patent. 6025184.

Laivenieks, M., C. Vielle, and J. G. Zeikus. 1997. Cloning, sequencing, and overexpression of the Anaerobiospirillum succiniciproducens phosphoenolpyruvate carboxykinase (pckA) gene. Appl. Env. Microbiol. 63:2272–2280.

Lamed, R. J., and J. G. Zeikus. 1981. Novel NADP-linked alcohol-aldehyde/ketone oxidoreductase in thermophilic ethanologenic bacteria. Biochem. J. 195:183–190.

Landucci, R., B. Goodman, and C. Wyman. 1994. Methodology for evaluating the economics of biologically producing chemicals and materials from alternative feedstocks. Appl. Biochem. Biotechnol. 45/46:677–696.

Largier, S. T., S. Long, J. D. Santangelo, D. T. Jones, and D. R. Woods. 1985. Immobilized Clostridium acetobutylicum P262 mutants for solvent production. Appl. Environ. Microbiol. 50:477–481.

Larsen, S. H., and F. C. Stormer. 1973. Diacetyl (Acetoin) reductase from Aerobacter aerogenes. Eur. J. Biochem. 34:100–106.

Larsen, L., P. Nielsen, and B. K. Ahring. 1997. Thermoanaerobacter mathranii sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. Arch. Microbiol. 168:114–119.

Laube, V. M., D. Groleau, and S. M. Martin. 1984. 2,3-butanediol production from xylose and other hemicellulosic components by Bacillus polymyxa. Biotechnol. Lett. 6:257–262.

Layman, P. 1998. Acetic acid industry gears up for possible building boom. Chem. Eng. News 76(31):17–18.

Leaf, T. A., M. S. Peterson, S. K. Stoup, D. Somers, and F. Srienc. 1996. Saccharomyces cerevisiae expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. Microbiol. 142:1169–1180.

Lee, S. F., C. W. Forsberg, and L. N. Gibbins. 1985. Cellulolytic activity of Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:220–228.

Lee, H. K., and I. S. Maddox. 1986. Continuous production of 2,3-butanediolfrom whey permeate using Klebsiella pneumoniae immobilized in calcium alginate. Enz. Microb. Technol. 8:409–411.

Lee, S. Y., G. N. Bennett, and E. T. Papoutsakis. 1992. Construction of Escherichia coli-Clostridium acetobutylicum shuttle vectors and transfromation of Clostridium acetobutylicum strains. Biotechnol. Lett. 14:427–432.

Lee, Y.-E., M. K. Jain, C. Lee, S. E. Lowe, and J. G. Zeikus. 1993. Taxonomic distinction of saccharolytic thermophilic anaerobes: Description of Thermoanaerobacterium xylanolyticum gen. nov., sp. nov., and Thermoanaerobacterium saccharolyticum gen. nov., sp. nov.; reclassification of Thermoanaerobium brockii, Clostridium thermosulfurogenes, and Clostridium thermohydrosulfuricum E100-69 as Thermoanaerobacter brockii comb. nov., and Thermoanaerobacter thermosulfurigenes comb. nov., and Thermoanaerobacter thermohydrosulfuricus comb. nov., respectively; and transfer of Clostridium thermohydrosulfuricum 39E to Thermoanaerobacter ethanolicus. Int. J. Syst. Bacteriol. 43:41–51.

Lee, S. Y., and H. N. Chang. 1995a. Production of poly(hydroxyalkanoic acid). Adv. Biochem. Eng. Biotechnol. 52:27–58.

Lee, S. Y., and H. N. Chang. 1995b. Production of poly(3-hydroxybutyric acid) by recombinant Escherichia coli strains: Genetic and fermentation studies. Can. J. Microbiol. 41, Suppl. 1:207–215.

Lee, S. Y., Y. K. Lee, and H. N. Chang. 1995c. Stimulatory effects of amino acids and oleic acid on poly(3-hydroxybutyric acid) synthesis by recombinant Escherichia coli. J. Ferment. Bioeng. 79:177–180.

Lee, S. Y. 1996a. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49:1–14.

Lee, S. Y. 1996b. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol. 14:431–438.

Lee, I. Y., M. K. Kim, Y. H. Park, and S. Y. Lee. 1996c. Regulatory effects of cellular nicotinamide nucleotides and enzyme activities on poly(3-hydroxybutyrate) synthesis in recombinant Escherichia coli. Biotechnol. Bioeng. 52:707–712.

Lee, S. Y. 1997. E. coli moves into the plastic age. Nature Biotechnol. 15:17–18.

Lee, P. C., W. G. Lee, S. Kwon, S. Y. Lee, and H. N. Chang. 1999a. Succinic acid production by Anaerobiospirillum succiniciproducens: Effects of the H_2/CO_2 supply and glucose concentration. Enz. Microbiol. Technol. 24:549–554.

Lee, P. C., W. G. Lee, S. Y. Lee, and H. N. Chang. 1999b. Effects of medium components on the growth of Anaerobiospirillum succiniciproducens and succinic acid production. Proc. Biochem. 35:49–55.

Lee, S. Y., Y. Lee, and F. L. Wang. 1999c. Chiral compounds from bacterial polyesters: Sugars to plastics to fine chemicals. Biotechnol. Bioeng. 65:363–368.

Lefranc, L. et Cie. 1923. A process for the manufacture of butyric acid and other fatty acids with recovery of the gases of fermentation. British Patent 186-572. Chem Abstr. 17:324.

Leh, M. B., and M. Charles. 1989. The effect of whey protein hydrolyzate average molecular weight on the lactic acid fermentation. J. Ind. Microbiol. 4:77–80.

Leigh, J. A., F. Mayer, and R. S. Wolfe. 1981. Acetogenium kivui a new thermophilic hydrogen-oxidizing, acetogenic bacterium. Arch. Microbiol. 129:275–280.

Lemme, C. J., and J. R. Frankiewicz. 1985. Strains of Clostridium acetobutylicum and process for its preparation. US Patent. 4521516.

Lemmel, S. A. 1985. Mutagenesis in Clostridium acetobutylicum. Biotechnol. Lett. 7:711–716.

Lemmel, S. A., R. Datta, and J. R. Frankiewicz. 1986. Fermentation of xylan by Clostridium acetobutylicum. Enz. Microbiol. Technol. 8:217–221.

Lemoigne, M. 1925. Etudes sur l'autolyze microbienne acidification par formation d'acide β-oxybutyrique. Ann. Inst. Pasteur 39:144–173.

Lemoigne, M. 1926. Produits de déshydration et de polymérisation de l'acide β-oxybutyrique. Bull. Soc. Chim. Biol. 8:770–782.

Lemoigne, M., and H. Girard. 1943. Réserves lipidiques β-hydroxybutyriques chez Azotobacter chroococcum. Comp. Rend. Acad. Sci. 217:557–558.

Lenz, T. G., and A. R. Moreira. 1980. Economic evaluation of the acetone-butanol fermentation. Indust. Engin. Chem. Prod. Res. Devel. 19:478–483.

Lepage, C., F. Fayolle, M. Hermann, and J.-P. Vandecasteele. 1987. Changes in membrane liquid composition of Clostridium acetobutylicum during acetone-butanol fermentation: Effects of solvents, growth temperature and pH. J. Gen. Microbiol. 133:103–110.

Lewis, V. P., and S. T. Yang. 1992. Continuous propionic acid fermentation by immobilized Propionibacterium acidipropionici in a novel packed bed bioreactor. Biotechnol. Bioeng. 40:465–474.

Li, G. 1998. Development of a Reporter System for the Study of Gene Expression for Solvent Production in Clostridium beijerinckii NRRL B582 and Clostridium acetobutylicum ATCC 824 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Liebergesell, M., E. Hustede, A. Timm, A. Steinbüchel, R. C. Fuller, R. W. Lenz, and H. G. Schlegel. 1991. Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. Arch. Microbiol. 155:415–421.

Lin, Y.-L., and H. P. Blaschek. 1984. Transformation of heat-treated Clostridium acetobutylicum protoplasts with pUB110 plasmid DNA. Appl. Environ. Microbiol. 48:737–742.

Lin, R. W., E. E. Atkinson, and D. E. Balhoff. 1996. Process for producing (S,S)-ethylenediamine-N,N′-disuccinic acid. US Patent. 5554791.

Lin, M.-Y., S. Harlander, and D. Saviano. 1999. Construction of an integrative food-grade cloning vector for Lactobacillus acidophilus. Appl. Microb. Biotechnol. 45:484–489.

Linden, J. C., and R. H. Kuhn. 1989. Biochemistry of alcohol effects on Clostridia. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 271–291.

Lindsay, S. E., R. J. Bothast, and L. O. Ingram. 1995. Improved strains of recombinant Escherichia coli for ethanol production from sugar mixture. Appl. Microbiol. Biotechnol. 43:70–75.

Lipinsky, E. S., and R. G. Sinclair. 1986. Is lactic acid a commodity chemical? Chem. Eng. Prog. 82(8):26–32.

Litchfield, J. H. 1996. Microbial production of lactic acid. Adv. Appl. Microbiol. 42:45–95.

Liu, S.-Y., F. A. Rainey, H. W. Morgan, F. Mayer, and J. Wiegel. 1996. Thermoanaerobacterium aotearoense sp. nov., a slightly acidophilic, anaerobic thermophile isolated from various hot springs in New Zealand, and emendation of the genus Thermoanaerobacterium. Int. J. Syst. Bacteriol. 46:388–396.

Liu, S.-J., and A. Steinbüchel. 2000a. Exploitation of butyrate kinase and phosphotransbutyrylase from Clostridium acetobutylicum for the in vitro biosynthesis of poly(hydroxyalkanoic acid). Appl. Microbiol. Biotechnol. 53:545–552.

Liu, S.-J., and A. Steinbüchel. 2000b. A novel genetically engineered pathway for synthesis of poly(hydroxyalkanoic acids) in Escherichia coli. Appl. Environ. Microbiol. 66:739–743.

Ljungdahl, L. G. 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Ann. Rev. Microbiol. 40:415–450.

Ljungdahl, L. G. 1994. The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 63–85.

Lockwood, L. B., D. E. Yoder, and M. Zienty. 1965. Lactic acid. Ann. NY Acad. Sci. 119:854–867.

Logsdon, J. E. 1994. Ethanol. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley & Sons. New York, NY. 9:812–860.

Logsdon, J. E., and R. A. Loke. 1996. Isopropyl alcohol. *In:* J. I. Kroschwitz and M. Howe-Grant (Eds.) Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 20:216–240.

Logsdon, J. E., and R. A. Loke. 1999. Isopropyl alcohols. *In:* J. I. Kroschwitz (Ed.) Kirk-Othmer Concise Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 1654–1656.

Loos, H., R. Krämer, H. Sahm, and G. A. Sprenger. 1994. Sorbitol promotes growth of Zymomonas mobilis in environment with high concentrations of sugar: Evidence for a physiological function of glucose-fructose oxidoreductase in osmoprotection. J. Bacteriol. 176: 7688–7693.

Lorowitz, W. H., and M. P. Bryant. 1984. Peptostreptococcus productusstrain that grows rapidly with CO as an energy source. Appl. Environ. Microbiol. 47:961–964.

Lowe, S. E., M. K. Jain, and J. G. Zeikus. 1993. Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. Microbiol. Rev. 57:451–509.

Lowenheim, F. A., and M. K. Moran. 1975. Industrial Chemicals, 4th ed. John Wiley & Sons. New York, NY.

Luchansky, J. B., P. M. Muriana, and T. R. Klaenhammer. 1988. Application of electroporation for transfer of plasmid DNA to Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Bacillus, Staphylococcus, Enterococcus, and Propionibacterium. Molec. Microbiol. 2:637–646.

Luers, F. M., R. Seyfried, R. Daniel, and G. Gottschalk. 1997. Glycerol conversion to 1,3-propanediol by Clostridium pasteurianum: Cloning and expression of the gene encoding 1,3-propanediol dehydrogenase. FEMS Microbiol. Lett. 154:337–345.

Lugar, R. G., and R. J. Woolsey. 1999. The new petroleum. Foreign Affairs 78:88–102.

Lund, B. M., T. F. Brocklehurst, and G. M. Wyatt. 1981. Characterization of strains of Clostridium puniceum sp. nov., a pink-pigmented, pectolytic bacterium. J. Gen. Microbiol. 122:17–26.

Luttik, M. A. H., R. Van Spanning, D. Schipper, J. P. Van Dijken, and J. T. Pronk. 1997. The low biomass yields of the acetic acid bacterium Acetobacter pasteurianus are due to a low stoichiometry of respiration-coupled proton translocation. Appl. Envron. Microbiol. 63:3345–3351.

Lynd, L. R. 1989. Ethanol production from lignocellulosic substrates using thermophilic bacteria. Adv. Biochem. Eng. Biotechnol. 38:1–52.

Lynd, L. R., J. H. Cushman, R. J. Nichols, and C. E. Wyman. 1991. Fuel ethanol from cellulosic biomass. Science 251:1318–1323.

Lynd, L. R., S. Baskaran, and S. Casten. 2001. Salt accumulation resulting from base added for pH control, and not ethanol, limits growth of Thermoanaerobacterium thermosaccharolyticum HG-8 at elevated feed xylose concentrations in continuous culture. Biotechnol. Progr. 17:118–125.

Lyon, W. J., and B. A. Glatz. 1995. Propionibacteria. *In:* Y. H. Hui and G. G. Khachatourians (Eds.) Food Biotechnology, Microorganisms. VCH Publishers. New York, NY. 703–719.

Macis, L., R. Daniel, and G. Gottschalk. 1998. Properties and sequence of the coenzyme B12-dependent glycerol dehydratase of Clostridium pasteurianum. FEMS Microbiol. Lett. 164:21–28.

Mackenzie, K. F., C. K. Eddy, and L. O. Ingram. 1989. Modulation of alcohol dehydrogenase isoenzyme levels in Zymomonas mobilis by iron and zinc. J. Bacteriol. 171:1063–1067.

Maddox, I. S. 1980. Production of n-butanol from whey filtrate using Clostridium acetobutylicum NCIB 2951. Biotechnol. Lett. 2:493–498.

Maddox, I. S. 1996. Microbial production of 2, 3-butanediol. *In:* H.-J. Rehm and G. Reed (Eds.) Biotechnology, 2nd ed. VCH. New York, NY. 6:270–291.

Maddox, I. S., E. Steiner, S. Hirsch, S. Wessner, N. A. Gutierrez, J. R. Gapes, and K. C. Schuster. 2000. The cause of "acid crash" and "acidogenic fermentations" during the batch acetone-butanol-ethanol (ABE-) fermentation process. J. Molec. Microbiol. Biotechnol. 2:95–100.

Madison, L. L., and G. W. Huisman. 1999. Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic. Microbiol. Molec. Biol. Rev. 63:21–53.

Mai, V., W. W. Lorenz, and J. Wiegel. 1997. Transformation of Thermoanaerobacterium sp. strain JW/SL-YS485 with plasmid pIKM1 conferring kanamycin resistance. FEMS Microbiol. Lett. 148:163–167.

Mai, V., and J. Wiegel. 2000. Advances in development of a genetic system for Thermoanaerobacterium spp.: Expression of genes encoding hydrolytic enzymes, development of a second shuttle vector, and integration of genes into chromosome. Appl. Environ. Microbiol. 66:4817–4821.

Malinowski, J. J. 1999. Evaluation of liquid extraction potentials for downstream separation of 1,3-propanediol. Biotechnol. Tech. 13:127–130.

Malinowski, J. J. 2000. Reactive extraction for downstream separation of 1,3-propanediol. Biotechnol. Prog. 16:76–79.

Marchal, R., M. Ropars, J. Pourquie, F. Fayolle, and J. P. Vandecasteele. 1992. Large-scale enzymatic hydrolysis of agricultural lignocellulosic biomass. Part II: Conversion into acetone-butanol. Biores. Technol. 42:205–217.

Martin, M. E., M. Wayman, and G. Graf. 1961. Fermentation of sulfite waste liquor to produce organic acids. Can. J. Microbiol. 7:341–346.

Martin, M. C., J. C. Alonso, J. E. Suarez, and M. A. Alvarez. 2000. Generation of food-grade recombinant lactic acid bacterium strains by site-specific recombination. Appl. Environ. Microbiol. 66:2599–2604.

Mat-Jan, F., Alam, K. Y., and D. P. Clark. 1989. Mutants of Escherichia coli deficient in the fermentative lactate dehydrogenase. J. Bacteriol. 171:342–348.

Matsusaki, H., H. Abe, K. Taguchi, T. Fukui, and Y. Doi. 2000. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene phaC1 from Pseudomonas sp. 61-3. Appl. Microbiol. Biotechnol. 53:401–409.

Matsushita, K., H. Toyama, and O. Adachi. 1994. Respiratory chains and bioenergetics of acetic acid bacteria. Adv. Microb. Physiol. 36:247–301.

Mattsson, D. M., and P. Rogers. 1994. Analysis of Tn916-induced mutants of Clostridium acetobutylicum altered in solventogenesis and sporulation. J. Ind. Microbiol. 13:258–268.

May, O. E., H. T. Herrick, A. J. Moyer, and P. A. Wells. 1934. Gluconic acid-production by submerged mold growths under increased air pressure. Ind. Eng. Chem. 26:575–578.

McCoy, E., E. B. Fred, W. H. Peterson, and E. G. Hastings. 1926. A cultural study of the acetone butyl alcohol organism. J. Infect. Dis. 39:457–483.

McCoy, E., and L. S. McClung. 1935. Studies on anaerobic bacteria. J. Infect. Dis. 56:333–346.

McCoy, E., and E. B. Fred. 1941. The stability of a culture for industrial fermentation. J. Bacteriol. 41:90–91.

McCoy, M. 1998. Biomass ethanol inches forward. Chem. Eng. News 76:29–32.

McCutchan, W. N., and R. J. Hickey. 1954. The butanol-acetone fermentations. *In:* L. A. Underkofler and R. J. Hickey (Eds.) Industrial Fermentations. Chemical Publishing. New York, NY. 1:347–388.

McGee, H. 1984. On Food and Cooking: The Science and Lore of the Kitchen. Scribner's. New York, NY. 36.

McLellan, P. J., A. J. Daugulis, and J. Li. 1999. The incidence of oscillatory behavior in the continuous fermentation of Zymomonas mobilis. Biotechnol. Progr. 15:667–680.

McNeil, B., and B. Kristiansen. 1986. The acetone butanol fermentation. Adv. Appl. Microbiol. 31:61–92.

Meinecke, B., H. Bahl, and G. Gottschalk. 1984. Selection of an asporogenous strain of Clostridium acetobutylicum in continuous culture under phosphate limitation. Appl. Environ. Microbiol. 48:1064–1065.

Menzel, K., A. P. Zeng, and W. D. Deckwer. 1997. High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by Klebsiella pneumoniae. Enz. Microbiol. Technol. 20:82–86.

Mercenier, A., P. H. Pouwels, and B. M. Chassy. 1994. Genetic engineering of lactobacilli, leuconostocs, and Streptococcus thermophilus. *In:* M. J. Gasson and W. M. DeVos (Eds.) Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional Publishers. London, New York, NY. 252–293.

Mercier, P., L. Yerushalmi, D. Rouleau, and D. Dochain. 1992. Kinetics of lactic acid fermentation on glucose and corn by Lactobacillus amylophilus. J. Chem. Tech. Biotechnol. 55:111–121.

Mermelstein, L. D., N. E. Welker, G. N. Bennett, and E. T. Papoutsakis. 1992. Expression of cloned homologous fermentative genes in Clostridium acetobutylicum ATCC 824. Biotechnology 10:190–195.

Mermelstein, L. D., and E. T. Papoutsakis. 1993a. Evaluation of macrolide and lincosamide antibiotics for plasmid maintenance in low pH Clostridium acetobutylicum ATCC 824 fermentations. FEMS Microbiol. Lett. 113:71–76.

Mermelstein, L. D., and E. T. Papoutsakis. 1993b. In vivo methylation in Escherichia coli by the Bacillus subtilis phage 3T I methyltransferase to protect plasmids from restriction upon transformation of Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 59:1077–1081.

Mermelstein, L. D., E. T. Papoutsakis, D. J. Petersen, and G. N. Bennett. 1993c. Metabolic engineering of Clostridium acetobutylicum ATCC 824 for increased solvent

production by enhancement of acetone formation enzyme activities using a synthetic acetone operon. Biotechnol. Bioengin. 42:1053–1060.

Mermelstein, L. D., N. E. Welker, D. J. Petersen, G. N. Bennett, and E. T. Papoutsakis. 1994. Genetic and metabolic engineering of Clostridum acetobutylicom. ATCC 824. Ann. NY Acad. Sci. 721:54–68.

Mesa, M. M., I. Caro, and D. Cantero. 1996. Viability reduction of Acetobacter aceti due to the absence of oxygen in submerged culture. Biotech. Progress 12:709–712.

Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990a. Control of the selectivity of butyric acid production and improvement of fermentation performance with Clostridium tyrobutyricum. Appl. Microbiol. Biotechnol. 32:387–392.

Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990b. Butyrate production in continuous culture of Clostridum tyrobutyricum. Appl. Microbiol. Biotechnol. 33: 127–131.

Michel-Savin, D., R. Marchal, and J. P. Vandecasteele. 1990c. Butyric fermentation: Metabolic behavior and production performance of Clostridium tryobutyricum in a continuous culture with cell recycle. Appl. Microbiol. Biotechnol. 34:172–177.

Mickelson, M. N., and C. H. Werkman. 1940. Formation of trimethyleneglycol from glycerol by Aerobacter. Enzymologia 8:252–256.

Millard, C. S., Y. P. Chao, J. C. Liao, and M. I. Donnelly. 1996. Enhanced production of succinic acid by overexpression of phosphoenolpyruvate caboxylase in Escherichia coli. Appl. Env. Microbiol. 62:1808–1810.

Milson, P. E., and J. L. Meers. 1985. Gluconic and itaconic acids. *In:* M. Moo-Young (Ed.) Comprehensive Biotechnology. Pergamon Press. Oxford, 3:681–700.

Minton, N. P., and J. D. Oultram. 1988. Host:vector systems for gene cloning in Clostridium. Microbiol. Sci. 5:310–315.

Minton, N. P., J. K. Brehm, T. J. Swinfield, S. W. Whelan, M. L. Mauchline, N. Bodsworth, and J. D. Oultrum. 1993. Clostridial cloning vectors. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 120–150.

Mishra, P., and S. Kaur. 1991. Lipids as modulators of ethanol tolerance in yeast. Appl. Microbiol. Biotechnol. 34:697–702.

Misoph, M., and H. L. Drake. 1996. Effect of CO_2 on the fermentation capacities of the acetogen Peptostreptococcus productus U-1. J. Bacteriol. 178:3140–3145.

Mitchell, W. J. 1998. Physiology of carbohydrate to solvent conversion by clostridia. Adv. Microb. Physiol. 39:31–130.

Mobley, D. P. (Ed.). 1994. Plastics from Microbes. Hanser. Munich, Germany.

Möller, B., R. Ossmer, B. H. Howard, G. Gottschalk, and H. Hippe. 1984. Sporomusa, a new genus of Gram negative anaerobic bacteria including Sporomusa sphaeroides spec. nov. and Sporomusa ovata spec. nov. Arch. Microbiol. 139:388–396.

Molliard, M. 1922. Survene nouvelle fermentation acide produite par le Sterigmatcystis nigra. C.R. Hebd. Seances Acad. Sci. 174:881–883.

Monot, F., J.-M. Engasser, and H. Petitdemange. 1984. Influence of pH and undissociated butyric acid in the production of acetone and butanol in batch cultures of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 19:422–426.

Montoya, D., C. Arevalo, S. Gonzales, F. Aristizabal, and W. H. Schwarz. 2001. New solvent-producing Clostridium sp. strains, hydrolyzing a wide range of polysaccharides, are closely related to Clostridium butyricum. J. Ind. Microbiol. Biotechnol. 27:329–335.

Moore, S. K. 1999. DuPont, Genencor close in on biological propanediol route. Chem. Week 161:17.

Moreira, A. R., D. C. Ulmer, and J. C. Linden. 1981. Butanol toxicity in butylic fermentation. Biotechnol. Bioengin. Symp. 11:567–579.

Mori, A., and G. Tervi. 1972. Kinetic studies on submerged acetic acid fermentation. III: Efficiency of energy metabolism in acetic acid fermentation using Acetobacter rancens. J. Ferment. Technol. 50:510–517.

Mori, A. 1993. Vinegar production in a fluidized-bed reactor with immobilized bacteria. *In:* A. Tanaka, T. Tosa, and T. Kobayashi (Eds.) Industrial Application of Immobilized Biocatalysts. Dekker. New York, NY. 291–313.

Morris, J. G. 1993. History and future potential of the clostridia in biotechnology. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 1–23.

Moyer, A. J., P. A. Wells, J. J. Stubbs, H. T. Herrick, and O. E. May. 1937. Gluconic acid production-development of innoculum and composition of fermentation solution for gluconic acid production by submerged mold growths under increased air pressure. Ind. Eng. Chem. 29:777–781.

Muller, H. M., and D. Seebach. 1993. Poly(hydroxyfettsaureester) eine funfte Klasse von physiologisch bedeutsamen organischen Biopolymeren? Agnew. Chem. 105:483–459.

Müller, V., and G. Gottschalk. 1994. The sodium ion cycle in acetogenic and methanogenic bacteria: Generation and utilization of a primary electrochemical sodium ion gradient. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 125–156.

Muraoka, H., Y. Watabe, and N. Ogasawara. 1982. Effect of oxygen deficiency on acid production and morphology of bacterial cells in submerged acetic fermentation by Acetobacter aceti. J. Ferment. Technol. 60:171–180.

Muriana, P. M., and T. R. Klaenhammer. 1991. Cloning, phenotypic expression, and DNA sequence of the gene for lactacin F, an antimicrobial peptide produced by Lactobacillus spp. J. Bacteriol. 173:1779–1788.

Nagarajan, V., and C. E. Nakamura. 1998. Production of 1,3-propanediol from glycerol by recombinant bacteria expressing recombinant diol dehydratase. US Patent 5821092.

Nair, R. V., G. N. Bennett, and E. T. Papoutsakis. 1994. Molecular characterization of an alcohol/aldehyde dehydrogenase gene of Clostridium acetobutylicum ATCC 824. J. Bacteriol. 176:871–885.

Nakamura, C. E., A. A. Gatenby, A. K. Hsu, R. D. LaReau, S. L. Haynie, R. Diaz-Torres, D. E. Trimbur, G. M. Whited, A. Nagarajan, M. S. Payne, S. K. Picataggio, and R. V. Nair. 2000. Method for the production of 1,3-propanediol by recombinant microorganisms. US Patent 6013494.

Nakano, M. M., Y. P. Dailly, P. Zuber, and D. P. Clark. 1997. Characterization of anaerobic fermentative growth of Bacillus subtilis: Identification of fermentation end products and genes required for growth. J. Bacteriol. 179:6749–6755.

Nakas, J. P., M. Schaedle, C. M. Parkinson, C. E. Coonley, and S. W. Tanenbaum. 1983. System development for

linked-fermentation production of solvents from algal biomass. Appl. Environ. Microbiol. 46:1017–1023.

Nakashimada, Y., K. Kanai, and N. Nishio. 1998. Optimization of dilution rate, pH and oxygen supply on optical purity of 2,3-butanediol produced by Paenibacillus polymyxa in chemostat culture. Biotechnol. Lett. 20:113–1138.

Nakashimada, Y., B. Marwoto, T. Kashiwamura, T. Kakizono, and N. Nishio. 2000. Enhanced 2,3-butanediol production by addition of acetic acid in Paenibacillus polymyxa. J. Biosci. Bioeng. 90:661–664.

Nakotte, S., S. Schaffer, M. Bohringer, and P. Dü. 1998. Electroporation of, plasmid isolation from and plasmid conservation in Clostridium acetobutylicum DSM 792. Appl. Microbiol. Biotechnol. 50:564–567.

Narayan, R. 1994. Impact of governmental policies, regulations, and standards activities on an emerging biodegradable plastics industry. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 261–272.

Nativel, F., J. Pourquie, D. Ballerini, J. P. Vandecasteele, and P. Renault. 1992. The biotechnology facilities at Soustons for biomass conversion. Int. J. Solar Energy 11:219–229.

Nawrath, C., Y. Poirier, and C. Somerville. 1994. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of Arabidopsis thaliana results in high levels of polymer accumulation. Proc. Natl. Acad. Sci. USA 91:12760–12764.

Neale, A. D., R. K. Scopes, J. M. Kelly, and R. E. H. Wettenhall. 1986. The two alcohol dehyrogenases of Zymomonas mobilis: Purification by differential dye ligand chromatography, molecular characterisation and physiological roles. Eur. J. Biochem. 154:119–124.

Neale, A. D., R. K. Scopes, R. E. H. Wettenhall, and N. J. Hoogenraad. 1987. Nucleotide sequence of the pyruvate decarboxylase gene from Zymomonas mobilis. Nucleic Acids Res. 15:1753–1761.

Neale, A. D., R. K. Scopes, and J. M. Kelly. 1988. Alcohol production from glucose and xylose using Escherichia coli containing Zymomonas mobilis genes. Appl. Microbiol. Biotechnol. 29:162–167.

Nghiem, N. P., B. H. Davison, J. E. Thompson, B. E. Suttle, and G. R. Richardson. 1996. The effect of biotin on the production of succinic acid by Anaerobiospirillum succiniciproducens. Appl. Biochem. Biotechnol. 57/58:633–638.

Nghiem, N. P., B. H. Davison, B. E. Suttle, and G. R. Richardson. 1997. Production of succinic acid by Anaerobiospirillum succiniciproducens. Appl. Biochem. Biotechnol. 63–65:565–576.

Nghiem, N. P., M. I. Donnelly, and C. S. Millard. 1999. Method for the production of dicarboxylic acids. US Patent. 5,869,301.

Nickol, G. B. 1979. Vinegar. *In:* H. J. Peppler and D. Pearlman (Eds.) Microbial Technology. Academic Press. New York, NY. 2:155–172.

Nidetzky, B., M. Furlinger, and D. Gollhofer. 1997. Improved operational stability of cell-free glucose-fructose oxidoreductase from Zymomonas mobilis for the efficient synthesis of gluconic acid and sorbitol, in acoutin ultra filtration membrane reactor. Biotechnol. Bioeng. 53:623–629.

Nilegaonkar, S., S. B. Bhosale, D. C. Kshirsagar, and A. H. Kapadi. 1992. Production of 2,3-butanediol from glucose by Bacillus licheniformis. World J. Microbiol. Biotechnol. 8:378–381.

Nimcevic, D., M. Schuster, and J. R. Gapes. 1998. Solvent production by Clostridium beijerinckii NRRL B592 growing on different potato media. Appl. Microbiol. Biotechnol. 50:426–428.

Nimcevic, D., and J. R. Gapes. 2000. The acetone-butanol fermentation in pilot plant and pre-industrial scale. J. Molec. Microbiol. Biotechnol. 2:15–20.

Nishikawa, M., R. M. R. Brancon, K. L. Pinder, and G. A. Strasdine. 1970. Fermentation of spent sulfite liquor to produce acetic acid, propionic acid and vitamin B12. Pulp and Paper Magazine of Canada 71(3):T59–T64.

Nishioka, M., T. Tuzuki, Y. Wanajyo, H. Oonami, and T. Horiuchi. 1994. Biodegradation of Bionolle. Stud. Polym. Sci. 12:584–590.

Nölling, J., G. Breton, M. V. Omelchenko, K. S. Markarova, Q. Zeng, R. Gibson, H. M. Lee, J. DuBois, D. Qiu, J. Hitti, GTC Sequencing Center Production, Finishing, and Bioinformatics Teams, Y. I. Wolf, R. L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M. J. Daly, G. N. Bennett, E. V. Koonin, and D. R. Smith. 2001. Genome sequence and comparitive analysis of the solvent-producing bacterium Clostridium acetobutylicum. J. Bacteriol. 183:4823–4838.

Noon, R. 1982. Power-grade butanol. Chemtech 12:681–683.

Northrop, J. H., L. H. Ashe, and J. K. Senior. 1919. Biochemistry of Bacillus acetoethylicum with reference to the formation of acetone. J. Biol. Chem. 39:1–21.

Noury, and N. V. van der Lande. 1962. Process for preparation of gluconic acid monohydrate. British Patent 902609. Chem. Abstr. 57:13020g.

Oben, J. M., M. J. Amagro, and A. Manjon. 1996. Continuous retention of native NADP(H) in an enzyme membrane reactor for gluconate and glutamate production. J. Biotechnol. 50:27–36.

Ogata, S., and M. Hongo. 1979. Bacteriophages of the genus Clostridium. Adv. Appl. Microbiol. 25:241–273.

Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991a. Genetic improvement of Escherichia coli for ethanol production: Chromosomal integration of Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. 57:893–900.

Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991b. Metabolic engineering of Klebsilla oxytoca M5A1 for ethanol production from xylose and glucose. Appl. Environ. Microbiol. 57:2810–2815.

Oiwa, H., M. Naganuma, and S.-I. Ohnuma. 1987. Acetone-butanol production from dahlia inulin by Clostridium pasteurianum var. I-53. Agric. Biol. Chem. 51:2819–2820.

Okumura, H., H. Tagami, M. Fukaya, H. Masai, Y. Kawamura, S. Horinouchi, and T. Beppu. 1988. Cloning of the β-isopropylmalate dehydrogenase gene from Acetobacter aceti and its use for construction of a new host vector system for Acetobacter. Agric. Biol. Chem. 52:3125–3129.

Olijve, W., and J. J. Kok. 1979a. An analysis of growth of Gluconobacter oxydans in chemostat cultures. Arch. Microbiol. 121:291–297.

Olijve, W., and J. J. Kok. 1979b. Analysis of growth of Gluconobacter oxydans in glucose containing medium. Arch. Microbiol. 121:283–290.

O'Mullan, P. J., S. E. Buchholz, T. Chase Jr., and D. E. Eveleigh. 1995. Roles of alcohol dehydrogenases of

Zymomonas mobilis (ZADH): Characterization of a ZADH-2-negative mutant. Appl. Microbiol. Biotechnol. 43:675–678.

O'Neill, H., S. G. Mayhew, and G. Butler. 1998. Cloning and analysis of the genes for a novel electron-transferring flavoprotein from Megasphaera elsdenii. J. Biol. Chem. 273:21015–21024.

Osuga, J., A. Mori, and J. Kato. 1984. Acetic acid production by immobilized Acetobacter aceti cells entrapped in a kappa-carrageenan gel. J. Ferment. Technol. 62:139–149.

Osuna, J., X. Soberon, and E. Morett. 1997. A proposed architecture for the central domain of the bacterial enhancer-binding proteins based on secondary structure prediction and fold recognition. Protein Sci. 6:543–555.

Oude Elferink, S. J. W. H., J. Krooneman, J. C. Gorrschal, S. F. Spoelstra, F. Faber, and F. Driehuis. 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by Lactobacillus buchneri. Appl. Environ. Microbiol. 67:125–132.

Ouellette, J. 2000. PO and PG markets face tight margins. Chem. Mark. Rep. 257:5 and 14.

Oultram, J. D., and M. Young. 1985. Conjugal transfer of plasmid pAMβ1 from Streptococcus lactis and Bacillus subtilis to Clostridium acetobutylicum. FEMS Microbiol. Lett. 27:129–134.

Oultram, J. D., A. Davies, and M. Young. 1987. Conjugal transfer of a small plasmid from Bacillus subtilis to Clostridium acetobutylicum by cointegrate formation with plasmid pAMβ1. FEMS Microbiol. Lett. 42:113–119.

Oultram, J. D., M. Loughlin, T.-J. Swinfield, J. K. Brehm, D. E. Thompson, and N. P. Minton. 1988. Introduction of plasmids into whole cells of Clostridium acetobutylicum by electroporation. FEMS Microbiol. Lett. 56:83–88.

Oultram, J. D., I. D. Burr, M. J. Elmore, and N. P. Minton. 1993. Cloning and sequence analysis of the genes encoding phosphotransbutyrylase and butyrate kinase from Clostridium acetobutylicum NCIMB 8052. Gene 131:107–112.

Owen, K., and T. Coley. 1990. Oxygenated blend components for gasoline. Automotive Fuels Handbook. Society of Automotive Engineers. Warrendale, PA. 221–259.

Page, W. J., and A. Cornish. 1993. Growth of Azotobacter vinelandii UWD in fish peptone medium and simplified extraction of poly-beta-hydroxybutyrate. Appl. Environ. Microbiol. 59:4236–4244.

Paik, H. D., and B. A. Glatz. 1994. Propionic acid production by immobilized cells of a propionate-tolerant strain of Propionibacterium acidipropionici. Appl. Microbiol. Biotechnol. 42:22–27.

Palosaari, N. R., and P. Rogers. 1988. Purification and properties of the inducible coenzyme A-linked butyraldehyde dehydrogenase from Clostridium acetobutylicum. J. Bacteriol. 170:2971–2976.

Pan, J. G., S. A. Soo, C. K. Park, P. Kim, D. E. Chang, and J. E. Kim. 1999. A pta ldhA double mutant Escherichia coli SS373 and the method of producing succinic acid therefrom. PCT Application. WO 99/06532.

Papanikolaou, S., P. Ruiz-Sanchez, B. Pariset, F. Blanchard, and M. Fick. 2000. High production of 1,3-propanediol from industrial glycerol by a newly isolated Clostridium butyricum strain. J. Biotechnol. 77:191–208.

Papoutsakis, E. T., and C. L. Meyer. 1985. Equations and calculations of product yields and preferred pathways for butanediol and mixed-acid fermentations. Biotechol. Bioengin. 27:50–66.

Papoutsakis, E. T., and G. N. Bennett. 1993. Cloning, structure, and expression of acid and solvent pathway genes of Clostridium acetobutylicum. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 157–200.

Papoutsakis, E. T., and G. N. Bennett. 1999. Molecular regulation and metabolic engineering of solvent production by Clostridium acetobutylicum. *In:* E. T. Papoutsakis and S. Y. Lee (Eds.) Bioprocess Technology. Marcel Dekker. New York, NY. 24:253–279.

Paquet, V., C. Croux, G. Goma, and P. Soucaille. 1991. Purification and characterization of the extracellular α-amylase from Clostridium acetobutylicum. Appl. Environ. Microbiol. 57:212–218.

Parekh, S. R., and M. Cheryan. 1991. Production of acetate by mutant strains of Clostridium thermoaceticum. Appl. Microbiol. Biotechnol. 36:384–387.

Parekh, S. R., and M. Cheryan. 1994a. Continuous production of acetate by Clostridium thermoaceticum in a cell-recycle membrane bioreactor. Enz. Microb. Technol. 16:104–109.

Parekh, S. R., and M. Cheryan. 1994b. High concentrations of acetate with a mutant strain of Clostridium thermoaceticum. Biotechnol. Lett. 16:139–142.

Park, Y. S., H. Ohtake, M. Fukaya, H. Okumura, Y. Kawamura, and K. Toda. 1989. Acetic acid production using a fermenter equipped with a hollow fiber filter module. Biotechnol. Bioeng. 33:918–923.

Park, Y. S., H. Ohtake, M. Fukaya, H. Kawamura, and K. Toda. 1991. Production of high concentration of acetic acid by Acetobacter aceti using a repeated fed-batch culture with cell recycling. Appl. Microbiol. Biotechnol. 35:149–153.

Park, D. H., and G. K. Zeikus. 1999. Utilization of electrically reduced neutral red by Actinobacillus succinogenes: Physiological function of netural red in membrane-driven fumarate reduction and energy conservation. J. Bacteriol. 181:2403–2410.

Parrer, G., G. Schroll, J. R. Gapes, W. Lubitz, and K. C. Schuster. 2000. Conversion of solvent evaporation residues from the AB-(acetone-butanol) bioprocess into bacterial cells accumulating thermoplastic polyesters. J. Molec. Microbiol. Biotechnol. 2:81–86.

Pasteur, L. 1861a. Animalcules infusoires vivant sans gaz oxygène libre et déterminant des fermentations. Comptes Rendus 52:344–347.

Pasteur, L. 1861b. Mémoire sur les corpuscles organisés qui éxistent dans l'atmosphere: Esamen de la doctrine des générations spontanées. Annales Sci. Natur., 4th series 16:5–98.

Pasteur, L. 1868. Etudes sur le vinaigre, sa fabrication se maladres, moyens de les préveair nouvelles observations sur la conservation des vinc par la châleur. Gauthier-Villars. Paris, FR.

Pasteur, L. 1879. Studies on Fermentation. London.

Pedroni, P., A. Volpe, G. Galli, G. M. Mura, C. Pratesi, and G. Grandi. 1995. Characterization of the locus encoding the [Ni-Fe] sulfhydrogenase from the archaeon Pyrococcus furiosus: Evidence for a relationship to bacterial sulfite reductases. Microbiology 141:449–458.

Peguin, S., G. Goma, P. Delorme, and P. Soucaille. 1994. Metabolic flexibility of Clostridium acetobutylicum in response to methyl viologen addition. Appl. Microbiol. Biotechnol. 42:611–616.

Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey. 1987. Biosynthetic thiolase from Zoogloea ramigera. III:

Isolation and characterization of the structural gene. J. Biol. Chem. 262:97–102.

Peoples, O. P., and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16: Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. 264:15293–15297.

Peretz, M., O. Bogin, S. Tel-Or, A. Cohen, G. Li, J.-S. Chen, and Y. Burstein. 1997. Molecular cloning, nucleotide sequencing, and expression of genes encoding alcohol dehydrogenase from the thermophile Thermoanaerobacter brockii and the mesophile Clostridium beijerinckii. Anaerobe 3:259–270.

Perozich, J., H. Nicholas, B. C. Wang, R. Lindahl, and J. Hempel. 1999. Relationships within the adehyde dehydrogenase extended family. Protein Sci. 8:137–146.

Petersen, D. J., R. W. Welch, F. B. Rudolph, and G. N. Bennett. 1991. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from Clostridium acetobutylicum ATCC 824. J. Bacteriol. 173:1831–1834.

Petersen, D. J., J. W. Cary, J. Vanderleyden, and G. N. Bennett. 1993. Sequence and arrangement of genes encoding enzymes of the acetone-production pathway of Clostridium acetobutylicum ATCC 824. Gene 123:93–97.

Petitdemange, H., C. Cherrier, G. Raval, and R. Gay. 1976. Regulation of NADH and NADPH ferredoxin oxidoreductases in clostridia of the butyric group. Biochem. Biophys. Acta 421:334–347.

Petitdemange, E., C. Durr, S. Abbad-Andalousi, and G. Raval. 1995. Fermentation of raw glycerol to 1,3-propanediol by new strains of Clostridium butyricum. J. Indust. Microbiol. 15:498–502.

Pflugmacher, U., and G. Gottschalk. 1994. Development of an immobilized cell reactor for the production of 1,3-propanediol by Citrobacter freundii. Appl. Microbiol. Biotechnol. 41:313–316.

Philips, J. R., E. C. Clausen, and J. L. Gaddy. 1994. Synthesis gas as substrate for the biological production of fuels and chemicals. Appl. Biochem. Biotechnol. 45–46:145–157.

Phillips, M. A. 1963. Catalytic hydrogenation of glucose to sorbitol using a highly active catalyst. Br. Chem. Eng. 8(11):767–769.

Pierce, S. M., and M. Wayman. 1983. Diesel fuel by fermentation of wastes. US Patent. 4368056.

Piveteau, P. 1999. Metabolism of lactate and sugars by dairy propionibacteria: A review. Lait 79:23–41.

Planas, J., P. Radström, F. Tjerneld, and B. Hahn-Hägerdal. 1996. Enhanced production of lactic acid through the use of a novel aqueous two-phase system as an extractive fermentation system. Appl. Microbiol. Biotechnol. 45:737–743.

Playne, M. J. 1985. Propionic acid and butyric acid. *In:* Moo-Young (Ed.) Comprehensive Biotechnology. Pergamon. New York, NY. 3:731–755.

Podkovyrov, S. M., and J. G. Zeikus. 1993. Purification and characterization of phosphoenolpyruvate carboxykinase, a catabolic CO_2-fixing enzyme, from Anaerobiospirillum succiniciproducens. J. Gen. Microbiol. 139: 223–228.

Poehland, H. D., V. Schierz, and R. Schumann. 1993. Optimization of gluconic acid synthesis by removing limitations and inhibitions. Acta Biotechnol. 13:257–268.

Poirier, Y., D. E. Dennis, K. Klomparens, and C. Somerville. 1992. Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science 256:520–522.

Poirier, Y., C. Nawrath, and C. Somerville. 1995. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Bio/technology 13:142–150.

Posno, M., P. T. H. M. Heuvelmans, M. J. F. van Giezen, B. C. Lokman, R. J. Leer, and P. H. Pouwels. 1991. Complimentation of the inability of Lactobacillus strains to utilize D-xylose with D-xylose catabolism-encoding genes of Lactobacillus pentosus. Appl. Environ. Microbiol. 57:2764–2766.

Potera, C. 1997. Genencor & DuPont create "green" polyester. Genet. Eng. News 17:17.

Prescott, S. C., and C. G. Dunn. 1949. The acetone-butanol fermentation. Industrial Microbiology, 2nd ed. McGraw-Hill. New York, NY. 312–351.

Prescott, S. C., and C. G. Dunn. 1949. The acetone-butanol fermentation. Industrial Microbiology, 2nd ed. McGraw-Hill. New York, NY. 477–486.

Prescott, S. C., and C. G. Dunn. 1959a. The acetone-butanol fermentation. *In:* Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 250–284.

Prescott, S. C., and C. G. Dunn. 1959b. The acetone-ethanol fermentation. *In:* Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 295–298.

Prescott, S. C., and C. G. Dunn. 1959c. The butanol-isopropanol fermentation. Industrial Microbiology, 3rd ed. McGraw-Hill. New York, NY. 285–294.

Pries, A., A. Steinbüchel, and H. G. Schlegel. 1990. Lactose-utilizing and galactose-utilizing strains of poly(hydroxyalkanoic acid)-accumulating Alcaligenes eutrophus and Pseudomonas saccharophila obtained by recombinant DNA technology. Appl. Microbiol. Biotechnol. 33:410–417.

Prieto, M. A., B. Buhler, K. Jung, B. Witholt, and B. Kessler. 1999a. PhaE, a polyhydroxyalkanoate-granule-associated protein of Pseudomonas oleovorans GPo1 involved in the regulatory expression system for pha genes. J. Bacteriol. 181:858–868.

Prieto, M. A., M. B. Kellerhals, G. B. Bozzato, D. Radnovic, B. Witholt, and B. Kessler. 1999b. Engineering of stable recombinant bacteria for production of chiral medium-chain-length poly-3-hydroxyalkanoates. Appl. Environ. Microbiol. 65:3265–3271.

Pronk, J. T., P. R. Levering, W. Olijve, and J. P. Van Dijken. 1989. The role of NADP-dependent and quino protein glucose dehydrogenase in gluconic acid production by Gluconobacter oxydans. Enz. Microb. Technol. 11:160–164.

Quesada-Chanto, A., A. S. Afschar, and F. Wagner. 1994a. Microbial production of propionic acid and vitamin B-12 using molasses or sugar. Appl. Microbiol. Biotechnol. 41:378–383.

Quesada-Chanto, A., A. S. Afschar, and F. Wagner. 1994b. Optimization of a Propionibacterium acidipropionici continuous culture utilization of sucrose. Appl. Microbiol. Biotechnol. 42:16–21.

Quesada-Chanto, A., J. P. C. L. da Costa, M. M. Silveira, A. G. Schroeder, A. C. Schmidt-Meyer, and R. Jona. 1998a. Influence of different vitamin-nitrogen sources on cell growth and propionic acid production from sucrose by Propionibacterium shermanii. Acta Biotechnologia 18:267–274.

Quesada-Chanto, A., M. M. Silveira, A. C. Schmidt-Meyer, A. G. Schroeder, J. P. C. L. da Costa, J. Lopez, M. F.

Carvalho-Jonas, M. J. Artolozaga, and R. Jona. 1998b. Effect of oxygen supply on pattern of growth and corrinoid and organic acid production of Propionibacterium shermanii. Appl. Microbiol. Biotechnol. 49:732–736.

Qureshi, N., and H. P. Blaschek. 2001a. ABE production from corn: A recent economic evaluation. J. Indust. Microbiol. Biotechnol. 27:292–297.

Qureshi, N., A. Lolas, and H. P. Blaschek. 2001b. Soy molasses as fermentation substrate for production of butanol using Clostridium beijerinckii BA101. J. Indust. Microbiol. Biotechnol. 26:290–295.

Ragsdale, S. W. 1994. CO dehydrogenase and the central role of this enzyme in fixation of carbon-dioxide by anaerobic bacteria. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 88–126.

Ragsdale, S. W., and C. G. Riordan. 1996. The role of nickel in acetyl-CoA synthesis by the bifunctional enzyme CO dehydrogenase/acetyl-CoA synthase: Enzymology and model chemistry. J. Biol. Inorg. Chem. 1:489–493.

Rakhley, G., Z. H. Zhou, M. W. W. Adams, and K. L. Kovacs. 1999. Biochemical and molecular characterization of the [NiFe] hydrogenase from the hyperthermophilic archaeon, Thermococcus litoralis. Eur. J. Biochem 266:1158–1165.

Ramachandran, K. B., and G. Goma. 1988. 2,3-Butanediol production from glucose by Klebsiella pneumoniae in a cell recycle system. J. Biotechnol. 9:39–46.

Rani, K. S., and G. Seenayya. 1999. High ethanol tolerance of new isolates of Clostridium thermocellum strains SS21 and SS22. World J. Microbiol. Biotechnol. 15:173–178.

Rao, G., and R. Mutharasan. 1986. Alcohol production by Clostridium acetobutylicum induced by methyl viologen. Biotechnol. Lett. 8:893–896.

Rao, G., and R. Mutharasan. 1987. Altered electron flow in continuous cultures of Clostridium acetobutylicum induced by viologen dyes. Appl. Environ. Microbiol. 53:1232–1235.

Rao, G., and R. Mutharasan. 1988. Altered electron flow in a reduced environment in Clostridium acetobutylicum. Biotechnol. Lett. 10:129–132.

Raspoet, D., B. Pot, D. De Deyn, P. De Vos, K. Kersters, and J. De Ley. 1991. Differentiation between 2,3-butanediol producing Bacillus licheniformis and B. polymyxa strains by fermentation product profiles and whole-cell protein electrophoretic patterns. Syst. Appl. Microbiol. 14:1–7.

Ravagnani, A., K. C. B. Jennert, E. Steiner, R. Grunberg, J. R. Jefferies, S. R. Wilkinson, D. I. Young, E. C. Tidswell, D. P. Brown, P. Youngman, J. G. Morris, and M. Young. 2000. Spo0A directly controls the switch from acid to solvent production in solvent-forming clostridia. Molec. Microbiol. 37:1172–1185.

Rayner, A. 1926. The occurrence, properties, and uses of trimethylene glycol, and the fermentation of glycerin lyes. J. Soc. Chem. Indust. 45:265T–266T.

Reed, G. 1982. Production of fermentation alcohol as a fuel source. *In:* G. Reed (Ed.) Prescott & Dunn's Industrial Microbiology, 4th ed. AVI Publishing. Westport, CT. 835–859.

Rehberger, J. L., and B. A. Glatz. 1998. Response of cultures of Propionibacterium to acid and low pH tolerance and inhibition. J. Food Protect. 61:211–216.

Rehm, B. H. A., N. Kruger, and A. Steinbüchel. 1998. A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. J. Biol. Chem. 237:24044–24051.

Rehr, B., C. Wilhelm, and H. Sahm. 1991. The production of sorbitol and gluconic acid by permeablized cells of Zymomonas mobilis. Appl. Microbiol. Biotechnol. 35:144–148.

Reid, S. J., E. R. Allcock, D. T. Jones, and D. R. Woods. 1983. Transformation of Clostridium acetobutylicum protoplasts with bacteriophage DNA. Appl. Environ. Microbiol. 45:305–307.

Reid, M. F., and C. A. Fewson. 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. 20:13–56.

Reimann, A., and H. Biebl. 1996. Production of 1,3-propanediol by Clostridium butyricum DSM 5431 and product tolerant mutants in fedbatch culture: Feeding strategy for glycerol and ammonium. Biotechnol. Lett. 18:827–832.

Reimann, A., S. Abbad-Andaloussi, H. Biebl, and H. Petitdemange. 1998a. 1,3-propanediol formation with product-tolerant mutants of Clostridium butyricum DSM 5431 in continuous culture: Productivity, carbon and electron flow. J. Appl. Microbiol. 84:1125–1130.

Reimann, A., H. Biebl, and W. D. Deckwer. 1998b. Production of 1,3-propanediol by Clostridium butyricum in continuous culture with cell recycling. Appl. Microbiol. Biotechnol. 49:359–363.

Reisch, M. S. 1999. Vision 2020 comes into focus. Chem. Eng. News 77(32):10–12.

Ren, Q., N. Sierro, M. Kellerhals, B. Kessler, and B. Witholt. 2000. Properties of engineered poly-3-hydroxyalkanoates produced in recombinant Esherichia coli strains. Appl. Environ. Microbiol. 66:1311–1320.

Reusch, R. N., and H. L. Sadoff. 1988. Putative structure and functions of a poly-β-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc. Natl. Acad. Sci. USA 85:4176–4180.

Reusch, R. N. 1992a. Biological complexes of poly-β-hydroxybutyrate. FEMS Microbiol. Rev. 103:119–130.

Reusch, R. N., and A. Sparrow. 1992b. Transport of poly-β-hydroxybutyrate in human plasma. Biochim. Biophys. Acta 1123:33–40.

Reynen, M., and H. Sahm. 1988. Comparison of the structural genes for pyruvate decarboxylase in different Zymomonas mobilis strains. J. Bacteriol. 170:3310–3313.

Reysset, G., and M. Sebald. 1985. Conjugal transfer of plasmid-mediated antibiotic resistance from streptococci to Clostridium acetobutylicum. Ann. Inst. Pasteur/Microbiol. 136B:275–282.

Reysset, G., and M. Sebald. 1993. Transformation/electrotransformation of clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 151–158.

Rickert, D. A., C. E. Glatz, and B. A. Glatz. 1998. Improved organic acid production by calcium alginate-immobilized propionibacteria. Enzyme Microb. Technol. 22:409–414.

Ro, H. S., and H. S. Kim. 1991. Continuous production of gluconic acid and sorbitol from sucrose using invertase and an oxidoreductase from Zymomonas mobilis. Enz. Microb. Technol. 13:920–924.

Roehr, M., C. P. Kubicek, and J. Kominek. 1996. Gluconic acid. *In:* M. Roehr (Ed.) Biotechnology, 2nd ed. VCH. New York, NY. 348–362.

Rogers, P. L., K. J. Lee, M. L. Skotnicki, and D. E. Tribe. 1982. Ethanol production by Zymomonas mobilis. Adv. Biochem. Engin. 23:37–84.

Rogers, P. 1986. Genetics and biochemistry of Clostridium relevant to development of fermentation processes. Adv. Appl. Microbiol. 31:1–60.

Rogers, P., and N. Palosaari. 1987. Clostridium acetobutylicum mutants that produce butyraldehyde and altered quantities of solvents. Appl. Environ. Microbiol. 53: 2761–2766.

Rogers, P. L., K. J. Lee, G. M. Smith, and K. D. Barrow. 1989. Ethanol tolerance of Zymomonas mobilis. *In:* N. van Uden (Ed.) Alcohol Toxicity in Yeasts and Bacteria. CRC Press. Boca Raton, FL. 239–256.

Rogers, P., and G. Gottschalk. 1993. Biochemistry and regulation of acid and solvent production in clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. Stoneham, MA. 25–50.

Rogers, P. 1999. Clostridia: Solvent formation. *In:* M. C. Flickinger and S. W. Drew (Eds.) Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation. John Wiley. New York, NY. 2:670–687.

Roos, J. W., J. K. McLaughlin, and E. T. Papoutsakis. 1985. The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of Clostridium acetobutylicum. Biotechnol. Bioengin. 27:681–694.

Rose, N. 1986. Chaim Weizmann. Penguin Books. New York, NY.

Rosenberg, S. L. 1980. Fermentation of pentose sugars to ethanol and other neutral products by microorganisms. Enz. Microb. Technol. 2:185–193.

Ross, D. 1961. The acetone-butanol fermentation. Progr. Indust. Microbiol. 3:71–90.

Rudolph, F. B., D. L. Purich, and H. J. Fromm. 1968. Coenzyme A-linked aldehyde dehydrogenase from Escherichia coli. I: Partial purification, properties, and kinetic studies of the enzyme. J. Biol. Chem. 243:5539–5545.

Ryden, R. 1958. Development of anaerobic fermentation processes: Acetone-butanol. *In:* R. Steel (Ed.) Biochemical Engineering. Macmillan. New York, NY. 125–148.

Sablayrolles, J. M., and G. Goma. 1984. Butanediol production by Aerobacter aerogenes NRRL B199: Effects of initial substrate concentration and aeration agitation. Biotechnol. Bioengin. 26:148–155.

Saeki, A., M. Taniguchi, K. Matsushita, H. Yoyama, G. Theeragol, N. Lotong, and O. Adachi. 1997a. Microbial aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. Biosci. Biotech. Biochem. 61:317–323.

Saeki, A., G. Theeragol, and K. Matsushita. 1997b. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at high temperatures. Biosci. Biotech. Biochem. 61:138–145.

Saha, B. C., and R. J. Bothast. 1999. Production of 2,3-butanediol by newly isolated Enterobacter cloacae. Appl. Microbiol. Biotechnol. 52:321–326.

Saint-Amans, S., P. Perlot, G. Goma, and P. Soucaille. 1994. High production of 1,3-propanediol from glycerol by Clostridium butyricum VPI 3266 in a simply controlled fed-batch system. Biotechnol. Lett. 16:831–836.

Saint-Amans, S., L. Girbal, J. Andrade, K. Ahrens, and P. Soucaille. 2001. Regulation of carbon and electron flow in Clostridium butyricum VP 13266g frown on glucose-glycerol mixtures. J. Bacteriol. 183:1748–1754.

Sakura, H., H. W. Lee, S. Sato, S. Mukataka, and J. Takahashi. 1989. Gluconic acid production by Aspergillus niger immobilized on nonwoven fabric. J. Ferment. Bioeng. 67:404–408.

Samuelov, N. S., R. Lamed, S. Lowe, and J. G. Zeikus. 1991. Influence of CO_2-HCO_3-levels and pH on growth, succinate production, and enzyme activities of Anaerobiospirillum succiniciproducens. Appl. Environ. Microbiol. 57:3013–3019.

Samuelov, N. S., R. Datta, M. K. Jain, and J. G. Zeikus. 1999. Whey fermentation by Anaerobiospirillum succiniciproducens for production of a succinate-based animal feed additive. Appl. Env. Microbiol. 65:2260–2263.

Sanchez-Riera, F., D. C. Cameron, and C. L. Cooney. 1987. Influence of environmental factors in the production of R(−)-1,2-propanediol by Clostridium thermosaccharolyticum. Biotechnol. Lett. 9:449–454.

Sass, C., J. Walter, and G. N. Bennett. 1993. Isolation of mutants of Clostridium acetobutylicum ATCC 824. Curr. Microbiol. 26:151–154.

Sauer, E. T. 1991. Carboxylic acids (economic aspects). *In:* Encyclopedia of Chemical Technology, 4th ed. John Wiley. New York, NY. 5:179–187.

Sauer, U., and P. Dürre. 1995. Differential induction of genes related to solvent formation during the shift from acidogenesis to solventogenesis in continuous culture of Clostridium acetobutylicum. FEMS Microbiol. Lett. 125:115–120.

Sauer, U., J. D. Santangelo, A. Trever, M. Bucholz, and P. Dürre. 1995. Sigma factor and sporulation genes in Clostridium. FEMS Microbiol. Rev. 17:331–340.

Scheirlinck, T., J. Mahillon, H. Joos, P. Dahaese, and F. Michiels. 1989. Integration and expression of α?amylase and endoglucanase genes in the Lactobacillus plantarum chromosome. Appl. Environ. Microbiol. 55:2130–2137.

Schilling, L. B. 1995. Chemicals from alternative feedstocks in the United States. FEMS Microbiol. Rev. 16:101–110.

Schlegel, H. G., G. Gottschalk, and R. Von Vartha. 1961. Formation and utilization of poly-β-hydroxybutyric acid by Knallgas bacteria (Hydrogenomonas). Nature 191: 463–465.

Schneider, Z., E. G. Larsen, G. Jacobson, B. C. Johnson, and J. Pawelkiewicz. 1970. Purification and properties of glycerol dehydrase. J. Biol. Chem. 245:3388–3396.

Schoutens, G. H., M. C. H. Nieuwenhuizen, and N. W. F. Kossen. 1984. Butanol from whey ultrafiltrate: Batch experiments with Clostridium beyerinckii LMD 27.6. Appl. Microbiol. Biotechnol. 19:203–206.

Schoutens, G. H., and W. J. Groot. 1985a. Economic feasibility of the production of iso-propanol-butanol-ethanol fuels from whey permeate. Process Biochem. 20:117–121.

Schoutens, G. H., M. C. H. Nieuwenhuizen, and N. W. F. Kossen. 1985b. Continuous butanol production from whey permeate with immobilized Clostridium beyerinckii LMD 27.6. Appl. Microbiol. Biotechnol. 21:282–286.

Schubert, P., A. Steinbüchel, and H. G. Schlegel. 1988. Cloning of the Alcaligenes eutrophus gene for synthesis of poly-β-hydroxybutyric acid and synthesis of PHB in Escherichia coli. J. Bacteriol. 170:5837–5847.

Schuster, K. C., R. Goodacre, J. R. Gapes, and M. Young. 2001. Degeneration of solventogenic Clostridium strains monitored by Fourier transform infrared spectroscopy of bacterial cells. J. Ind. Microbiol. Biotechnol. 27:314–321.

Scopes, R. K. 1983. An iron-activated alcohol dehydrogenase. FEBS Lett. 156:303–306.

Seifert, C., S. Bowien, G. Gottschalk, and R. Daniel. 2001. Identification and expression of the genes and purification nd characterization of the gene products involved in reactivation of coenzyme B12-dependent glycerol dehydratase of Citrobacter freundii. Eur. J. Biochem. 268:2369–2378.

Senthuran, A., V. Senthuran, B. Mathasson, and R. Kaul. 1997. Lactic acid fermentation in a recycle batch reactor using immobilized Lactobacillus casei. Biotechnol. Bioeng. 55:841–853.

Seyfried, M., R. Daniel, and G. Gottschalk. 1996. Cloning, sequencing, and overexpression of the genes encoding coenzyme B12-dependent glycerol dehydratase of Citrobacter freundii. J. Bacteriol. 178:5793–5796.

Shah, M. M., and M. Cheryan. 1995a. Acetate production by Clostridium thermoaceticum in corn steep liquor media. J. Ind. Microbiol. 15:424–428.

Shah, M. M., and M. Cheryan. 1995b. Improvement of productivity in acetic acid fermentation with Clostridium thermoaceticum. Appl. Biochem. Biotechnol. 51/52: 418–422.

Sheehan, J. 2000. The road to bioethanol: A strategic perspective of the U.S. Department of Energy's national ethanol program. *In:* H. M. E., J. O. Baker, and J. N. Saddler (Ed.) Glycosyl Hydrolases for Biomass Conversion. American Chemical Society. Washington DC, ACS Symposium Series 769:2–25.

Shelley, S., and R. L. D'Aquino. 1999. Three routes vie for the 1,3-propanediol market. Chem. Engin. 106:56.

Shen, G. J., B. A. Annons, R. W. Lovitt, M. K. Jain, and J. G. Zeikus. 1996. Biochemical route and control if butyrate synthesis in Butyribacterium methylotropicum. Appl. Microbiol. Biotechnol. 45:355–362.

Sherman, J. M., and R. H. Shaw. 1923. Process for the production of propionates and propionic acid. US Patent 1,470,885. Chem Abstr. 18:146.

Shiraishi, F., K. Kawakami, S. Kono, and A. Tamura. 1989a. Continous production of free gluconic acid by Gluconobacter suboxydans IFO 3290 immobilized by adsorption on ceramic honeycomb monolith: Effect of reactor configuration on further oxidation of gluconic acid to ketogluconic acid. Appl. Microbiol. Biotechnol. 31:445–447.

Shiraishi, F., K. Kawakami, S. Kono, A. Tamura, S. Tsuruta, and K. Kosmoki. 1989b. Characterization of production of free gluconic acid by Gluconobacter suboxydans absorbed on ceramic honeycomb monolith. Biotechnol. Bioeng. 33:1413–1418.

Shoham, Y., R. Lamed, and E. A. Bayer. 1999. The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol. 7:275–281.

Shone, C. C., and H. J. Fromm. 1981. Steady-state and pre-steady-state kinetics of coenzyme A linked aldehyde dehydrogenase from Escherichia coli. Biochemistry 20: 7494–7501.

Sievers, M., S. Sellmer, and M. Teuber. 1992. Acetobacter europaeus sp. nov., a main component of industrial vinegar fermenters in central Europe. Syst. Appl. Microbiol. 15:386–392.

Sievers, M., W. Ludwig, and M. Teuber. 1994a. Phylogenetic positioning of Acetobacter, Gluconobacter, Rhodophila, and Acidiphilium species as a branch of acidophilic bacteria in the α-subclass of proteobacteria based on 16S ribosomal DNA sequences. Syst. Appl. Microbiol. 17:189–196.

Sievers, M., W. Ludwig, and M. Teuber. 1994b. Phylogenetic positioning of Acetobacter, Gluconobacter, Rhodophila and Acidiphilium species as a branch of acidophilic bacteria in the α subclass of Proteobacteria based on 16S RNA sequences. Syst. Appl. Microbiol. 17:352–354.

Sievers, M., W. Ludwig, and M. Teuber. 1994c. Revival of the species Acetobacter methanolicus (ex: Uhlig et al., 1986) nom. rev. Syst. Appl. Microbiol. 17:352–354.

Silva-Martinez, M., D. Haltrich, S. Novalic, K. D. Kobe, and B. Nidetzky. 1998. Simultaneous enzymatic syntheses of gluconic acid and sorbitol. Appl. Biochem. Biotechnol. 70–72:863–875.

Silveira, M. M., E. Wisbeck, and C. Lemmel. 1999. Bioconversion of glucose and fructose to sorbitol and gluconic acid by untreated cells of Zymomonas mobilis. J. Biotechnol. 75:99–103.

Silveira, M. M., E. Wisbeck, I. Hoch, and R. Jonas. 2001. Production of glucose-fructose oxidoreductase and ethanol by Zymomonas mobilis ATCC 29191 in medium containing corn steep liquor as a source of vitamins. Appl. Microbiol. Biotechnol. 55:442–445.

Sinskey, A. J., M. Akedo, and C. L. Cooney. 1981. Acrylate fermentations. *In:* A. Hollaender (Ed.) Trends in the Biology of Fermentations. Plenum Press. New York, NY. 473–492.

Skraly, F. A., B. L. Lytle, and D. C. Cameron. 1998. Construction and characterization of a 1,3-propanediol operon. Appl. Env. Microbiol. 64:98–105.

Slapack, G. E., I. Russell, and G. G. Stewart. 1987. Thermophilic Microbes in Ethanol Production. CRC Press. Boca Raton, FL.

Slater, S., K. L. Houmiel, M. Tran, T. A. Mitsky, N. B. Taylor, S. R. Padgette, and K. J. Gruys. 1998. Multiple β-ketothiolase mediate poly(β-hydroxyalkanoate) copolymer synthesis in Ralstonia eutropha. J. Bacteriol. 180:1979–1987.

Slater, S., T. A. Mitsky, K. L. Houmiel, M. Hao, S. E. Reiser, N. B. Taylor, M. Tran, H. E. Valentin, J. Rodriguez, D. A. Stone, S. R. Padgette, G. Kishore, and K. J. Gruys. 1999. Metabolic engineering of Arabidopsis and Brassica for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production. Nature Biotechnol. 17:1011–1016.

Smith, W. P. 1993. Soap/Cosmetic Specialties 9:54–58.

Société Lefranc et Cie. 1925. An improved process for the manufacture of dipropylketone. British Patent 216-120. Chem. Abstr. 19:77.

Sokollek, S. J., and W. P. Hammes. 1997. Description of a starter culture preparation for vinegar fermentation. Syst. Appl. Microbiol. 20:481–491.

Sokollek, S. J., C. Hertel, and W. P. Hammes. 1998. Cultivation and preservation of vinegar bacteria. J. Biotechnol. 60:195–206.

Solichien, M. S., D. O'Brien, E. G. Hammond, and C. E. Glatz. 1995. Membrane-based extractive fermentation to produce propionic and acetic acids: toxicity and mass transfer considerations. Enzyme Microb. Technol. 17:23–31.

Solomon, B. O., A. P. Zeng, H. Biebl, A. O. Ejiofor, C. Posten, and W. D. Deckwer. 1994. Effects of substrate limitation on product distribution and H_2/CO_2 ratio in Klebsiella pneumoniae during anaerobic fermentation of glycerol. Appl. Microbiol. Biotechnol. 42:222–226.

Solomon, B. O., A.-P. Zeng, H. Biebl, H. Schlieker, C. Posten, W.-D. Deckwer. 1995. Comparison of the energetic efficiencies of hydrogen and oxychemicals formation in Klebsiella pneumoniae and Clostridium butyricum dur-

ing anaerobic growth on glycerol. J. Biotechnol. 39:107–117.

Solomons, G. L. 1976. Solvents from carbohydrates: Some economic considerations. Process Biochem. 11:32–37.

Somrutai, W., M. Takagi, and T. Yoshida. 1996. Acetone-butanol fermentation by Clostridium aurantibutyricum ATCC 17777 from a model medium for palm oil mill effluent. J. Ferment. Bioengin. 81:543–547.

Spivey, M. J. 1978. The acetone/butanol/ethanol fermentation. Process Biochem. 13:2–25.

Sprenger, G. A., B. A. Hammer, E. A. Johnson, and E. C. Lin. 1989. Anaerobic growth of Escherichia coli on glycerol by importing genes of the dha regulon from Klebsiella pneumoniae. J. Gen. Microbiol. 135:1255–1262.

Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846–849.

Steinbüchel, A. 1991. Polyhydroxyalkanoic acids. *In:* D. Byrom (Ed.) Biomaterials: Novel Materials from Biological Sources. Macmillan.

Steinbüchel, A., and G. Schmack. 1995. Large-scale production of poly(3-hydroxyvaleric acid) by fermentation of Chromobacterium violaceleum, processing, and characterization of the homopolyester. J. Environ. Polym. Degrad. 3:243–258.

Steinbüchel, A. 1996. PHB and other polyhydroxyalkanoic acids. *In:* M. Roehr (Ed.) Biotechnology, 2nd edn. VCH. Weinheim, Germany. 6:403–464.

Steinbüchel, A., and V. Gorenflo. 1997. Biosynthetic and biodegradable polyesters from renewable resources: Current state and prospects. Macromol. Symp. 123:61–66.

Steinbüchel, A., and B. Füchtenbusch. 1998. Bacterial and other biological systems for polyester production. Trends Biotechnol. 16:419–427.

Stephens, G. M., R. A. Holt, J. C. Gottschal, and J. G. Morris. 1985. Studies on the stability of solvent production by Clostridium acetobutylicum in continuous culture. J. Appl. Bacteriol. 59:597–605.

Stevens, D., S. Alam, and R. Bajpai. 1988. Fermentation of cheese whey by a mixed culture of Clostridium beijerinckii and Bacillus cereus. J. Ind. Microbiol. 3:15–19.

Stieb, M., and B. Schink. 1984. A new 3-hydroxybutyrate fermenting anaerobe, Ilyobacter polytropus, gen. nov.sp.nov., possessing various fermentation pathways. Arch Microbiol. 140:139–146.

Stim-Herndon, K. P., D. J. Petersen, and G. N. Bennett. 1995. Molecular characterization of the acetyl coenzyme A acetyltransferase (thiolase) from Clostridium acetobutylicum ATCC 824. Gene 154:81–85.

St. Martin, E. J., W. B. Freedberg, and E. C. C. Lin. 1977. Kinase replaceent by a dehydrogenase for Escherichia coli glycerol utilization. J. Bacteriol. 131:1026–1028.

Stols, L., and M. I. Donnelly. 1997a. Production of succinic acid through overexpression of NAD^+-dependent malic enzyme in an Escherichia coli mutant. Appl. Env. Microbiol. 63:2695–2701.

Stols, L., G. Kulkarni, B. G. Harris, and M. I. Donnelly. 1997b. Expression of Ascaris suum malic enzyme in a mutant Escherichia coli allows production of succinic acid from glucose. Appl. Biochem. Biotechnol. 63–65:153–158.

Stormer, F. C. 1975. 2,3-Butanediol biosynthetic system in Aerobacter aerogenes. *In* W. A. Wood (Eds.) Methods in Enzymology. Academic Press. New York, NY. 41:518–533.

Strecker, A. 1854. Über eine eigentümliche Bildungsweise der Propionsaüre und einige Salze derselben. Ann. Chem. 92:80.

Streekstra, H., M. J. Teixera de Mattos, O. M. Neijssel, and D. W. Tempest. 1987. Overflow metabolism during anaerobic growth of Klebsiella aerogenes NCTC 418 on glycerol and dihydroxyacetone in chemostat culture. Arch. Microbiol. 147:268–275.

Suzuki, T., and H. Onishi. 1968. Aerobic dissimilation of L-rhamnose and the production of L-rhamnonic acid and 1,2-propanediol by yeasts. Agric. Biol. Chem. 32:888–893.

Suzuki, T., T. Yamane, and S. Shimizu. 1986. Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. Appl. Microbiol. Biotechnol. 24:370–374.

Swick, R. W., and H. G. Wood. 1960. The role of transcarboxylation in propionic acid fermentation. Proc. Natl. Acad. Sci. USA 46:28–41.

Swings, J., and J. De Ley. 1977. The biology of Zymomonas. Bacteriol. Rev. 41:1–46.

Swings, J., and J. De Ley. 1984. Genus Zymomonas. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:576–580.

Swings, J. 1992. The genera Acetobacter and Gluconobacter. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 3:2268–2286.

Syu, M. J. 2001. Biological production of 2,3-butanediol. Appl. Microbiol. Biotechnol 55:10–18.

Szmant, H. H. 1989. Organic Building Blocks of the Chemical Industry. John Wiley. New York, NY. 347–348.

Taguchi, H., and T. Ohata. 1991. D-Lactate dehydrogenase is a member of the D-isomer- specific 2-hydrox acid dehydrogenase family. J. Biol. Chem. 266:12588–12594.

Takahashi, R., N. Fujimoto, M. Suzuki, and T. Endo. 1997. Biodegradabilities of ethylenediamine-N,N′-disuccinic acid (EDDS) and other chelating agents. Biosci. Biotech. Biochem. 61:1957–1959.

Takemura, H., S. Horinouchi, and T. Beppu. 1991. Novel insertion sequence IS1380 from Acetobacter pasteurianus is involved in loss of ethanol-oxidizing ability. J. Bacteriol. 173:7070–7076.

Takiyama, E., and T. Fujimaki. 1994. "BIONELLE" biodegradable plastic through chemical synthesis. *In:* Y. Doi and K. Fukuda (Eds.) Biodegradable Plastics and Polymers. Elsevier Science. Amsterdam, The Netherlands. 150–174.

Tanaka, K., A. Ishizaki, T. Kanamaru, and T. Kawano. 1995. Production of poly(D-3-hydroxybutyrate) from CO_2, H_2, and O_2 by high cell-density autotrophic cultivation of Alcaligenes eutrophus. Biotechnol. Bioeng. 45:268–275.

Tang, J. C. T., F. E. Ruch, and E. C. C. Lin. 1979. Purification and properties of a nicotinamide adenine dinucleotide-linked dehydrogenase that serves an Escherichia coli mutant for glyerol catabolism. J. Bacteriol. 140:182–187.

Tang, J. C. T., R. G. Forage, and E. C. C. Lin. 1982. Immunochemical properties of NAD^+-linked glycerol dehydrogenases from Escherichia coli and Klebsiella pneumoniae. J. Bacteriol. 152:1169–1174.

Tanner, R. S., L. M. Miller, and D. Yang. 1993. Clostrium ljungdahlii sp. nov., an acetogenic species in clostridial

rRNA homology group I. Int. J. Syst. Bacteriol. 43:232–236.

Taroncher-Oldenburg, G., K. Nishia, and G. Stephanopoulos. 2000. Identification and analysis of polyhydroxyalkanoate-specific β-ketothiolase and acetoacetyl coenzyme A reductase genes in cyanobacterium Synechocystis sp. strain PCC6803. Appl. Environ. Microbiol. 66:4440–4448.

Taylor, M. B., and E. Juni. 1960. Stereoisomeric specificities of 2,3-butanediol dehydrogenases. Biochim. Biophys. Acta 39:448–457.

Terracciano, J. S., and E. R. Kashket. 1986. Intracellular conditions required of initiation of solvent production by Clostridium acetobutylicum. Appl. Environ. Microbiol. 52:86–91.

Thauer, R. K., K. Jungerman, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100–180.

Thauer, R. K. 1989. Biochemistry of acetic acid metabolism in anaerobic chemotropic bacteria. Ann. Rev. Microbiol. 43:43–67.

Thayer, A. 2000. Challenges of a biobased economy. C & EN May 29:40.

Thomas, S. R., W. S. Adney, J. O. Baker, Y.-C. Chou, and M. E. Himmel. 1997. US Patent. 5,712,142.

Thompson, D. K., and J.-S. Chen. 1990. Purification and properties of an acetoacetyl coenzyme A-reacting phosphotransbutyrylase from Clostridium beijerinckii ("Clostridium butylicum") NRRL B593. Appl. Environ. Microbiol. 56:607–613.

Thomson, A. W., J. G. O'Neill, and J. F. Wilkinson. 1976. Acetone production by methlobacteria. Arch. Microbiol. 109:243–246.

Thormann, K., L. Feustel, K. Lorenz, S. Nakotte, and P. Dü. 2002. Control of butanol formation in Clostridium acetobutylicum by transcriptional activation. J. Bacteriol. 184:1966–1973.

Tobimatsu, T., T. Hara, M. Sakaguchi, Y. Kishimoto, Y. Wada, M. Isoda, T. Sakai, and T. Toraya. 1995. Molecular cloning, sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of Klebsiella oxytoca. J. Biol. Chem. 270:712–7148.

Tobimatsu, T., M. Azuma, H. Matsubara, H. Takatori, T. Niida, K. Nishinoto, H. Satoh, R. Hayashi, and T. Toraya. 1996. Cloning, sequencing, and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydrase of Klebsiella pneumoniae. J. Biol. Chem. 271:22352–22357.

Tobimatsu, T., H. Kjiura, M. Yunoki, M. Azuma, and T. Toraya. 1999. Identification and expression of the genes encoding a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. J. Bacteriol. 181:4110–4113.

Toda, K., Y. S. Park, T. Asakura, C. Y. Cheng, and H. Ohtake. 1989. High rate acetic acid production in a shallow flow bioreacter. Appl. Microbiol. Biotechnol. 30:559–563.

Tolan, J. S., and R. K. Finn. 1987a. Fermentation of D-xylose and L-arabinose to ethanol by Erwinia chrysanthemi. Appl. Environ. Microbiol. 53:2033–2044.

Tong, I.-T., H. H. Liao, and D. C. Cameron. 1991. 1,3-propanediol production by Escherichia coli expressing genes from the Klebsiella pneumoniae dha regulon. Appl. Env. Microbiol. 57:3541–3546.

Tong, I. T., and D. C. Cameron. 1992. Enhancement of 1,3-Propanediol Production by Cofermentation in Escherichia coli expression Klebsiella pneumoniae dha regulon genes. Appl. Biochem. Biotechnol. 34/35:149–159.

Toth, J., A. A. Ismaiel, and J.-S. Chen. 1999. Purification of a coenzyme A-acylating aldehyde dehydrogenase and cloning of the structural gene from Clostridium beijerinckii NRRL B593. Appl. Environ. Microbiol. 65:4973–4980.

Traeger, M., G. N. Qazi, N. Gulan, R. Buse, and V. Onken. 1992. Comparison of direct glucose oxidation by Gluconobacter oxydans ssp. suboxydans and Aspergillus niger in a pilot scale airlift reactor. J. Ferment. Bioeng. 74:274–281.

Tran-Din, K., and G. Gottschalk. 1985. Formation of D(−)-1,2-propanediol and D(−)-lactate from glucose by Clostridium sphenoides under phosphate limitation. Arch. Microbiol. 142:87–92.

Truffaut, N., J. Hubert, and G. Reysset. 1989. Construction of shuttle vectors useful for transforming Clostridium acetobutylicum. FEMS Microbiol. Lett. 58:15–20.

Trunger, V., and W. Boos. 1994. Mapping and cloning of gldA, the structural gene of the Escherichia coli glycerol dehydratase. J. Bacteriol. 176:1796–1800.

Tsai, S. P., and S.-H. Moon. 1998. An integrated bioconversion process for the production of L-lactic acid from starchy potato feed stocks. Appl. Biochem. Biotechnol. 70–72:417–428.

Tsao, G. T., N. J. Cao, J. Du, and C. S. Gong. 1999. Production of mutifunctional organic acids from renewable resources. Adv. Biochem. Eng. Biotechnol. 65:243–280.

Tullo, A. 1999. BDO market embraces new technologies. Chemical Market Reporter April 26.

Tummala, S. B., N. E. Welker, and E. T. Papoutsakis. 1999. Development and characterization of a gene expression reporter system for Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 65:3793–3799.

Turk, R. 1993. Metal free and low metal salt substitutes containing lysine. US Patent. 5229161.

Turner, K. W., and A. M. Roberton. 1979. Xylose, arabinose, and rhamnose fermentation by Bacteroides ruminicola. Appl. Environ. Microbiol. 38:7–12.

Uchida, K. 1974. Lipids of alcoholophilic lactobacilli. II: Occurrence of polar lipids with unusually long acyl chains in Lactobacillus heterochiochii. Biochim. Biophys. Acta 369:146–155.

Uchida, K. 1975a. Alteration of the unsaturated to saturated ratio of fatty acids in bacterial lipids by alcohols. Agric. Biol. Chem. 39:1515–1516.

Uchida, K. 1975b. Effects of cultural conditions on the cellular fatty acid composition of Lactobacillus heterohiochii, an alcoholophilic bacterium. Agric. Biol. Chem. 39:837–842.

Uhlig, H., K. Kahrbaum, and A. Steudel. 1986. Acetobacter methanolicus sp. nov., an acetophilic facultatively methylotrophic bacterium. Int. J. Syst. Bacteriol. 36:317–322.

Ui, S., H. Masuda, and H. Muraki. 1983. Laboratory-scale production of 2,3-butanediol isomers (D(−),L(+), and Meso) by bacterial fermentations. J. Ferment. Technol. 61:253–259.

Ui, S., T. Masuda, H. Masuda, and H. Muraki. 1986. Mechanism for the formation of 2,3-butanediol stereoisomers in Bacillus polymyxa. J. Ferment. Technol. 64:481–486.

Ui, S., M. Odagiri, A. Mimura, H. Kanai, T. Kobayashi, and T. Kudo. 1996. Preparation of a chiral acetoinic compound using transgenic Escherichia coli expressing the 2,3-butandiol dehydrogenase gene. J. Ferment. Bioeng. 81:386–389.

Ui, S., Y. Okajima, A. Mimura, H. Kanai, and T. Kudo. 1997. Molecular generation of an Escherichia coli strain producing only the meso-isomer of 2,3-butanediol. J. Ferment. Bioeng. 84:185–189.

Ui, S., T. Hosaka, K. Watanabe, and A. Mimura. 1998. Discovery of a new mechanism of 2,3-butanediol stereoisomer formation in Bacillus cereus YUF-4. J. Ferment. Bioeng. 85:79–83.

Valentin, H. E., T. A. Mitsky, D. A. Mahadeo, M. Tran, and K. J. Gruys. 2000a. Application of a propionyl coenzyme A synthetase for poly(3-hydroxypropionate-co-3-hydroxybutyrate) accumulation in recombinant Esherichia coli. Appl. Environ. Microbiol. 66:5253–5258.

Valentin, H. E., S. Reiser, and K. J. Gruys. 2000b. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) formation from γ-aminobutyrate and glutamate. Biotechnol. Bioeng. 67:291–299.

Vandak, D., M. Telgarsky, and E. Sturkik. 1995a. Influence of growth factor components on butyrate production form sucrose by Clostridum butyricum. Folia Microbiol. 40:32–42.

Vandak, D., T. M. Zigova, and E. Sturdik. 1995b. Effect of growth supplements and whey pretreatment on butyric acid production by Clostridium butyricum. World J. Microbiol. Biotechnol. 11:363.

Vandak, D., J. Zigova, E. Sturdik, and S. Schlosser. 1997. Evaluation of solvent and pH for extractive fermentation of butyric acid. Process Biochem. 32:245–251.

Van der Werf, M. J., M. V. Guettler, M. K. Jain, and J. G. Zeikus. 1997. Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by Actinobacillus sp. 130Z. Arch. Microbiol. 167:332–342.

van der Westhuizen, A., D. T. Jones, and D. R. Woods. 1982. Autolytic activity and butanol tolerance of Clostridium acetobutylicum. Appl. Environ. Microbiol. 44:1277–1281.

Van Niel, C. B. 1928. The Propionic Acid Bacteria (thesis). Labortorium voor Microbiologie der Techische Hoogeschool Delft, N.V. Uitgeversaak, J. W. Boissevain & Co.. Haarlem, The Netherlands.

Varadarajan, S., and D. J. Miller. 1999. Catalytic upgrading of fermentation-derived organic acids. Biotechnol. Prog. 15:845–854.

Vasconcelos, I., L. Girbal, and P. Soucaille. 1994. Regulation of carbon and electron flow in Clostridium acetobutylicum grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J. Bacteriol. 176:1443–1450.

Vega, J. L., S. Prieto, B. B. Elmore, E. C. Clausen, and J. L. Gaddy. 1989. The biological production of ethanol from synthesis gas. Appl. Biochem. Biotechnol. 20/21:781–797.

Verhasselt, P., F. Poncelet, K. Vits, A. van Gool, and J. Vanderleyden. 1989. Cloning and expression of a Clostridium acetobutylicum α-amylase gene in Escherichia coli. FEMS Microbiol. Lett. 59:135–140.

Verhave, T. H. 1930. Verfahren zur bacteriellen oxydation organisher Verbindugen zwecks Herstellungen von Oxydationsprodukten wie Dioxyacton usw. German Patent 563, 758. Chem. Abstr. 27:1085.

Vick Roy, T. B. 1985. Lactic acid. *In:* M. Moo Young (Ed.) Comprehensive Biotechnology. Pergamon Press. Oxford, New York, NY. 761–776.

Voget, C. E., C. F. Mignone, and R. J. Ertola. 1985. Butanol production from apple pomace. Biotechnol. Lett. 7:43–46.

Vollbrecht, and El Nawawy. 1980. Restricted oxygen supply and excretion of metabolites. Euro. J. Appl. Microbiol. Biotechnol. 9:1–8.

Voloch, M., M. R. Ladisch, V. W. Rodwell, and G. T. Tsao. 1983. Reduction of acetoin to 2,3-butanediol in Klebsiella pneumoniae: A new model. Biotechnol. Bioengin. 25:173–183.

Von Freudenreich, E., and O. Jensen. 1906. Über die im Emmentaler Käse stattfindende Propionsauregärung. Zentralblatt für Bacteriologie, Parasiten Kunde Infectious Krankheiten und Hygiene Abt. II, 17:529–546.

Wagner, T. O., D. S. Gray, B. Y. Zarah, and A. A. Kozinski. 1980. Practicality of alcohols as motor fuel. *In:* Alcohols as Motor Fuels. Society of Automotive Engineers. Warrendale, PA. 249–265.

Walker, S. A., and T. R. Klaenhammer. 2000. An explosive antisense RNA strategy for inhibition of a lactococcal bacteriophage. Appl. Environ. Microbiol. 66:310–319.

Walter, K. A., G. N. Bennett, and E. T. Papoutsakis. 1992. Molecular characterization of two Clostridium acetobutylicum ATCC 824 butanol dehydrogenase isozyme genes. J. Bacteriol. 174:7149–7158.

Walter, K. A., R. V. Nair, J. W. Cary, G. N. Bennett, and E. T. Papoutsakis. 1993. Sequence and arrangement of two genes of the butyrate-synthesis pathway of Clostridium acetobutylicum ATCC 824. Gene 134:107–111.

Walton, M. T., and J. L. Martin. 1979. Production of butanol-acetone by fermentation. *In:* H. J. Peppler and D. Perlman (Eds.) Microbial Technology, 2nd ed. Academic Press. New York, NY. 1:187–209.

Wang, T.-T., and B. H. Lee. 1997a. Plasmids in Lactobacillus. Crit. Rev. Biotechnol. 17:227–272.

Wang, F. L., and S. Y. Lee. 1997b. Poly(3-hydroxybutyrate) production with high productivity and high polymer content by a fed-batch culture of Alcaligenes latus under nitrogen limitation. Appl. Environ. Microbiol. 63:3703–3706.

Wang, F., and S. Y. Lee. 1998a. High cell density culture of metabolically engineered Escherichia coli for the production of poly(3-hydroxybutyrate) in a defined medium. Biotechnol. Bioeng. 58:325–328.

Wang, X., C. S. Gong, and G. T. Tsao. 1998b. Bioconversion of fumaric acid to succinic acid by recombinant E. coli. Appl. Biochem. Biotechnol. 70–72:919–928.

Waterson, R. M., F. J. Castellino, G. M. Hass, and R. L. Hill. 1972. Purification and characterization of crotonase from Clostridium acetobutylicum. J. Biol. Chem. 247: 5266–5271.

Wayman, M., M. E. Martin, and G. Graf. 1962. Propionic Acid Fermentation. US Patent 3,067,107. Chem. Abstr. 58:7337.

Weber, G. H., and W. A. Broich. 1986. Shelf life extension of cultured dairy foods. Cult. Dairy Prod. J. 21(4):19–23.

Webster, J. R., S. J. Reid, D. T. Jones, and D. R. Woods. 1981. Purification and characterization of an autolysin from Clostridium acetobutylicum. Appl. Environ. Microbiol. 41:371–374.

Weimer, P. J. 1984a. Control of product fromation during glucose fermentation by Bacillus macerans. J. Gen. Microbiol. 130:103–111.

Weimer, P. J. 1984b. Fermentation of 6-deoxyhexoses by Bacillus macerans. Appl. Environ. Microbiol. 47:263–267.

Weiss, N., U. Schillinger, and O. Kandler. 1983. Lactobacillus trichodes and Lactobacillus heterochiochii, subjective

synonym of Lactobacillus fruitivorans. Syst. Appl. Microbiol. 4:507–511.

Weizmann, C. 1915. Improvements in bacterial fermentation of carbohydrates and in bacterial cultures for the same. British Patent. 4845.

Welch, R. W., F. B. Rudolph, and E. T. Papoutsakis. 1989. Purification and characterization of the NADH-dependent butanol dehydrogenase from Clostridium acetobutylicum (ATCC 824). Arch. Biochem. Biophys. 273:309–318.

Welch, R. W. 1991. Purification and studies of two butanol (ethanol) dehydrogenases and the effects of rifampicin and chloramphenicol on other enzymes important in the production of butyrate and butanol in Clostridium acetobutylicum ATCC 824 [PhD thesis]. Rice University. Houston, TX.

Welling, H. 1998. Fibers industry get ready. Apparel Industry Magazine 59:65–66.

Weusthuis, R. A., G. N. M. Huijberts, and G. Eggink. 1997. *In:* G. Eggink, A. Steinbuchel, Y. Poirer, and B. Witholt (Eds.) Proceedings of the 1996 International Symposium on bacterial Polyhydroxyalkanoates. NRC Research Press. 102–109.

Weyer, E. R., and L. F. Rettger. 1927. A comparative study of six different strains of the organism commonly concerned in large-scale production of butyl alcohol and acetone by the biological process. J. Bacteriol. 14:399–424.

White, D. 1995. The Physiology and Biochemistry of Prokaryotes. Oxford University Press. New York, NY.

Whitfield, C. D., and S. G. Mayhew. 1974. Purification and properties of electron-transferring flavoprotein from Peptostreptococcus elsdenii. J. Biol. Chem. 249:2801–2810.

Wiegel, J. 1980. Formation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 36:1434–1446.

Wiegel, J., M. Braun, and G. Gottschalk. 1981. Clostridium thermoautotrophicum species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. Curr. Microbiol. 5:255–260.

Wiegel, J., and L. G. Ljungdahl. 1986. The importance of thermophilic bacteria in biotechnology. Crit. Rev. Biotechnol. 3:39–108.

Wiegel, J. 1992. The obligately anaerobic thermophilic bacteria. *In:* J. K. Kristjansson (Ed.) Thermophilic Bacteria. CRC Press. Boca Raton, FL. 105–184.

Wiegel, J. 1994. Acetate and the potential of homoacetogenic bacteria for industrial applications. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 484–504.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1988. Thiolase from Clostridium acetobutylicum ATCC 824 and its role in the synthesis of acids and solvents. Appl. Environ. Microbiol. 54:2717–2722.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1989a. Coenzyme A transferase from Clostridium acetobutylicum ATCC 824 and its role in the uptake of acids. Appl. Environ. Microbiol. 55:323–329.

Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1989b. Phosphotransbutyrylase from Clostridium acetobutylicum ATCC 824 and its role in acidogenesis. Appl. Environ. Microbiol. 55:317–322.

Wilke, D. 1995. What should and what can biotechnology contribute to chemical bulk production? FEMS Microbiol. Rev. 16:89–100.

Wilke, D. 1999. Chemicals from biotechnology: Molecular plant genetics will challenge the chemical and the fermentation industry. Appl. Microbiol. Biotechnol. 52:135–145.

Wilkinson, S. R., and M. Young. 1994. Targeted integration of genes into the Clostridium acetobutylicum chromosome. Microbiology 140:89–95.

Wilkinson, S. R., and M. Young. 1995. Physical map of the Clostridium beijerinckii (formerly Clostrdium acetobutylicum) NCIMB 8052 chromosome. J. Bacteriol. 177:439–448.

Wilkinson, S. R., D. I. Young, J. G. Morris, and M. Young. 1995. Molecular genetics and the initiation of solventogenesis in Clostridium beijerinckii (formerly Clostridium acetobutylicum) NCIMB 8052. FEMS Microbiol. Rev. 17:275–285.

Williams, D. R., D. I. Young, and M. Young. 1990. Conjugative plasmid transfer from Escherichia coli to Clostridium acetobutylicum. J. Gen. Microbiol. 136:819–826.

Wills, C., P. Kratofil, D. Londo, and T. Martin. 1981. Characterization of the two alcohol dehydrogenases of Zymomonas mobilis. Arch. Biochem. Biophys. 210:775–785.

Winston, S. J. 1981. Ethanol Fuels: Use, Production & Economics. Solar Energy Information Data Bank, 1st ed. US Government Printing Office.

Winzer, K., K. Lorenz, and P. Dü. 1997. Acetate kinase from Clostridium acetobutylicum: A highly specific enzyme that is actively transcribed during acidogenesis and solventogenesis. Microbiology 143:3279–3286.

Winzer, K., K. Lorenz, B. Zickner, and P. Dü. 2000. Differential regulation of two thiolase genes from Clostridium acetobutylicum DSM 792. J. Molec. Microbiol. Biotechnol. 2:531–541.

Wittlich, P., A. Themann, and K.-D. Vorlop. 2001. Conversion of glycerol to 1,3-propanediol by a newly isolated thermophilic strain. Biotechnol. Lett. 23:463–466.

Wong, H. H., and S. Y. Lee. 1998. Poly(3-hydroxybutyrate) production from whey by high density cultivation of recombinant Escherichia coli. Appl. Microbiol. Biotechnol. 50:30–33.

Wood, H. G., and C. H. Werkman. 1938. The utilization of CO_2 by the propionic acid bacteria. Biochem. J. 32:1262–1271.

Wood, H. G., and C. H. Werkman. 1940. The relationship of bacterial utilization of CO_2 to succinic acid formation. Biochem. J. 34:129–138.

Wood, B. E., and L. O. Ingram. 1992. Ethanol production from cellobiose, amorphous cellulose, and crystalline cellulose by recombinant Klebsiella oxytoca containing chromosomally integrated Zymomonas mobilis genes for ethanol production and plasmids expressing thermostable cellulase genes from Clostridium thermocellum. Appl. Environ. Microbiol. 58:2103–2110.

Wood, A. 2001. DuPont, Genencor extend alliance. Chem. Week 163:38.

Woods, D. R. 1995. The genetic engineering of microbial solvent production. Trends Biotechnol. 13:259–264.

Woolley, R. C., A. Pennock, R. J. Ashton, A. Davies, and M. Young. 1989. Transfer of Tn1545 and Tn916 to Clostridium acetobutylicum. Plasmid 22:169–174.

Woolley, R. C., and J. G. Morris. 1990. Stability of solvent production by Clostridium acetobutylicum in continuous culture: Strain differences. J. Appl. Bacteriol. 69:718–728.

Worden. R. M., A. J. Grethlein, J. G. Zeikus, and R. Datta. 1989. Butyrate production from carbonmonoxide by Butyribacterium methylotrophicum. App. Biochem. Biotechnol. 20/21:687–698.

Worden, R. M., G. A. J., M. K. Jain, and R. Datta. 1991. Production of butanol and ethanol from synthesis gas via fermentation. Fuel 70:615–619.

Worden, R. M., M. D. Bredwell, and A. J. Grethlein. 1997. Engineering issues in synthesis-gas fermentations. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:320–335.

Woskow, S. A., and B. A. Glatz. 1991. Propionic acid production by a propionic acid tolerant strain of Propionibacterium acidipropionici in batch and semicontinuous fermentation. Appl. Environ. Microbiol. 57:2821–2828.

Wyman, C. E. 1994. Ethanol from lignocellulosic biomass: Technology, economics, and opportunities. Bioresource Technol. 50:3–16.

Wyman, C. E. (Ed.). 1996. Handbook on Bioethanol: Production and Utilization. Taylor and Francis. Washington DC.

Wyman, C. E. 2001. Twenty years of trials, tribulations and research progress in bioethanol technology. Appl. Biochem. Biotechnol. 91–93:5–21.

Xavier, A. M. R. B., L. M. D. Gonsalvas, and J. L. Moreira. 1995. Operation patterns affecting lactic acid production in ultrafiltration cell recycle bioreactor. Biotechnol. Bioeng. 45:320–327.

Xue, Y., Y. Xu, Y. Liu, Y. Ma, and P. Zhou. 2001. Thermoanaerobacter tengcongensis sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tongcong, China. Int. J. Syst. Evol. Microbiol. 51:1335–1341.

Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi. 1995. Transfer of two Burkholderia and an Alcaligenes species to Ralstonia gen. nov.: Proposal of Ralstonia pickettii (Ralston, Palleroni and Doudoroff 1973) comb. nov., Ralstonia salanacearum (Smith 1896) comb. nov. and Ralstonia eutropha (Davis 1969) comb. nov. Microbiol. Immunol. 39:897–904.

Yamada, K. 1977. Recent advances in industrial fermentation in Japan. Biotech. Bioeng. 19:1563–1621.

Yamamoto, I., T. Saiki, S.-M. Liu, and L. G. Ljungdahl. 1983. Purification and properties of NADP-dependent formate dehydrogenase from Clostridium thermoaceticum, a tungsten-selenium-iron protein. J. Biol. Chem. 258: 1826–1832.

Yan, R.-T., C.-X. Zhu, C. Golemboski, and J.-S. Chen. 1988. Expression of solvent-forming enzymes and onset of solvent production in batch cultures of Clostridium beijerinckii ("Clostridium butylicum"). Appl. Environ. Microbiol. 54:642–648.

Yan, R.-T., and J.-S. Chen. 1990. Coenzyme A-acylating adlehyde dehydrogenase from Clostridium beijerinckii NRRL B592. Appl. Environ. Microbiol. 56:2591–2599.

Yan, R.-T. 1991. Enzymology of butanol formation in Clostridium beijerinckii NRRL B592 [PhD thesis]. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Yang, S. T., H. Zhu, Y. Li, and G. Hong. 1994. Continuous propionate production from whey permeate using a novel fibrous bed bioreactor. Biotech. Bioeng. 43:1124–1130.

Yang, S. T., Z. W. Jin, and B. H. Chollar. 1997. Production of low-cost acetate deicers from biomass and industrial wastes. Snow Removal and Ice Control Technology. Transportation Research Board, National Research Council. Washington DC, 60–69.

Yang, S. T., Y. L. Huang, Z. Jin, Y. Huang, H. Zhu, and W. Qin. 1999. Calcium Magnesium Acetate at Power-production Cost: Production of CMA Deicer from Cheese Whey. US DOT. Publication No. FHWA-RD-98-174.

Yasui, Y. 1958. Process for continuous production of vinegar. Japanese Patent 244, 905. Publication No. Sho. 33-3798/1958.

Yeh, C.-S. 1955. Butanol-acetone fermentation [in Chinese]. *In:* Petroleum Communications. China Petroleum Corporation. Taiwan, China. December, Issue 54:10–19.

Yomano, L. P., S. W. York, and L. O. Ingram. 1998. Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production. J. Ind. Microbiol. Biotechnol. 20:132–138.

Yoon, K.-H., and M. Y. Pack. 1990. Nucleotide sequence of the Zymomonas mobilis alcohol dehydrogenase II gene. Nucleic Acids Res. 18:187.

Yoon, K.-H., J.-K. Lee, and B. H. Kim. 1991. Construction of a Clostridium acetobutylicum-Escherichia coli shuttle vector. Biotechnol. Lett. 13:1–6.

Yoshino, S., T. Yoshino, S. Hara, S. Ogata, and S. Hayashida. 1990. Construction of shuttle vector plasmid between Clostridium acetobutylicum and Escherichia coli. Agric. Biol. Chem. 54:437–441.

Young, M., N. P. Minton, and W. L. Staudenbauer. 1989. Recent advances in the genetics of the clostridia. FEMS Microbiol. Rev. 63:301–326.

Young, M. 1993. Development and exploitation of conjugative gene transfer in clostridia. *In:* D. R. Woods (Ed.) The Clostridia and Biotechnology. Butterworth-Heinemann. London, 99–118.

Youngleson, J. S., J. D. Santangelo, D. T. Jones, and D. R. Woods. 1988. Cloning and expression of a Clostridium acetobutylicum alcohol dehydrogenase gene in Escherichia coli. Appl. Environ. Microbiol. 54:676–682.

Youngleson, J. S., D. T. Jones, and D. R. Woods. 1989. Homology between hydroxybutyryl and hydroxyacyl coenzyme A dehydrogenase enzymes from Clostridium acetobutylicum fermentation and vertebrate fatty acid β-oxidation pathways. J. Bacteriol. 171:6800–6807.

Youngleson, J. S., F.-P. Lin, S. J. Reid, and D. R. Woods. 1995. Structure and transcription of genes within the β-hbd-adh1 region of Clostridium acetobutylicum P262. FEMS Microbiol. Lett. 125:185–192.

Yu, E. K. C., and J. N. Saddler. 1982a. Enhanced production of 2,3-butanediol by Klebsiella pneumoniae grown on high sugar concentrations in the presence of acetic acid. Appl. Environ. Microbiol. 44:777–784.

Yu, E. K. C., N. Levitin, and J. N. Saddler. 1982b. Production of 2,3-butanediol by Klebsiella pneumoniae grown on acid-hydrolyzed wood hemicellulose. Biotechnol. Lett. 4:741–746.

Yu, E. K. C., and J. N. Saddler. 1983. Fed-batch approach to production of 2,3-butanediol by Klebsiella pneumoniae grown on high substrate concentrations. Appl. Environ. Microbiol. 46:630–635.

Yu, E. K. C., and J. N. Saddler. 1985a. Biomass conversion to butanediol by simultaneous saccharification and fermentation. Trends. Biotechnol. 3:100–104.

Yu, E. K. C., L. Deschatelets, G. Louis-Seize, and J. N. Saddler. 1985b. Butanediol production from cellulose and hemicellulose by Klebsiella pneumoniae grown in

sequential coculture with Trichoderma harzianum. Appl. Environ. Microbiol. 50:924–929.

Yu, P.-L., and L. E. Pearce. 1986. Conjugal transfer of streptococcal antibiotic resistance plasmids into Clostridium acetobutylicum. Biotechnol. Lett. 8:469–474.

Zachariou, M., and R. K. Scopes. 1986. Glucose-fructose oxidoreductase, a new enzyme isolated from Zymomonas mobilis that is responsible for sorbitol production. J. Bacteriol. 167:863–869.

Zakpaa, H. D., A. Ishizaki, and K. Shimizu. 1997. Computer-mediated addition of fresh medium in continuous culture of Zymomonas mobilis by monitoring weight changes. *In:* B. C. Saha and J. Woodward (Ed.) Fuels and Chemicals from Biomass. American Chemical Society. Washington DC, ACS Symposium Series 666:143–154.

Zanin, G. M., C. C. Santana, E. P. S. Bon, R. C. L. Giordano, F. F. de Moraes, S. R. Andrietta, C. C. de Carvalho Neto, I. C. Macedo, D. L. Fo, L. P. Ramos, and J. D. Fontana. 2000. Brazilian bioethanol program. Appl. Biochem. Biotechnol. 84–86:1147–1161.

Zappe, H., D. T. Jones, and D. R. Woods. 1986. Cloning and expression of Clostridium acetobutylicum endoglucanase, cellobiase and amino acid biosynthesis genes in Escherichia coli. J. Gen. Microbiol. 132:1367–1372.

Zappe, H., D. T. Jones, and D. R. Woods. 1987. Cloning and expression of a xylanase gene from Clostridium acetobutylicum P262 in Escherichia coli. Appl. Microbiol. Biotechnol. 27:57–63.

Zappe, H., W. A. Jones, D. T. Jones, and D. R. Woods. 1988. Structure of an endo-β-1,4-glucanase gene from Clostridium acetobutylicum P262 showing homology with endoglucanase genes from Bacillus spp. Appl. Environ. Microbiol. 54:1289–1292.

Zeikus, J. G. 1980. Chemical and fuel production by anaerobic bacteria. Ann. Rev. Microbiol. 34:423–464.

Zeikus, J. G., M. K. Jain, and P. Elankoven. 1999. Biotechnology of succinic acid production and markets for derived industrial products. Appl. Microbiol. Biotechnol. 51: 545–552.

Zeng, A.-P., H. Biebl, and W.-D. Deckwer. 1990. Effect of pH and acetic acid on growth and 2,3-butanediol production of Enterobacter aerogenes in continuous culture. Appl. Microbiol. Biotechnol. 33:485–489.

Zeng, A.-P., and W.-D. Deckwer. 1991. A model for multiproduct-inhibited growth of Enterobacter aerogenes in 2,3-butanediol fermentation. Appl. Microbiol. Biotechnol. 35:1–3.

Zeng, A.-P., H. Biebl, H. Schlieker, and W.-D. Deckwer. 1993. Pathway analysis of glycerol fermentation by Klebsiella pneumoniae: Regulation of reducing equivalent balance and product formation. Enz. Microbol. Technol. 15:770–779.

Zeng, A.-P., T.-G. Byun, C. Posten, and W.-D. Deckwer. 1994a. Use of respiratory quotient as a control parameter for optimum oxygen supply and scale-up of 2,3-butanediol production under microaerobic conditions. Biotechnol. Bioengin. 44:1107–1114.

Zeng, A.-P., A. Ross, H. Biebl, C. Tag, B. Gunzel, and W.-D. Deckwer. 1994b. Multiple product inhibition and growth modeling of Clostridium butyricum and Klebsiella pneumoniae in glycerol fermentation. Biotechnol. Bioengin. 44:902–911.

Zeng, A.-P. 1996. Pathway and kinetic analysis of 1,3-propanediol production from glycerol fermentation by Clostridium butyricum. Bioproc. Engin. 14:169–175.

Zeng, A.-P., H. Biebl, and W. D. Deckwer. 1997. Microbial conversion of glycerol to 1,3-propanediol: Recent progress. *In:* B. C. Saha and J. Woodward (Eds.) Fuels and Chemicals from Biomass. Oxford Press. Oxford, UK. ACS Symposium Series 666:264–279.

Zhang, D. X., and M. Cheryan. 1994a. Starch to lactic acid in a continuous membrane reactor. Process Biochem. 29(2):145–150.

Zhang, H., V. Obias, K. Gonyer, and D. Dennis. 1994b. Production of polyhydroxyalkanoates in sucrose-utilizing recombinant Escherichia coli and Klebsiella strains. Appl. Environ. Microbiol. 60:1198–1205.

Zhang, M., C. Eddy, K. Deanda, M. Finkelstein, and S. Picataggio. 1995. Metabolic engineering of a pentose metabolism pathway in ethanologenic Zymomonas mobilis. Science 267:240–243.

Zhou, X., and R. W. Traxler. 1992. Enhanced butanol production and reduced autolysin activity after chloramphenicol treatment of Clostridium acetobutylicum ATCC 824. Appl. Microbiol. Biotechnol. 37:293–297.

Ziffer, J. A., S. Gaffney, S. Rothenberg, and T. J. Cairney. 1971. Aldonic acid and aldonate compositions and production thereof. British Patent 1,249,347. Chem. Abstr. 79:113.

Zigova, J., E. Sturdik, D. Vandak, and S. Schlosser. 1999. Butyric acid production by Clostridium butyricum with integrated extraction and pertraction. Process Biochem. 34:835–843.

Zigova, J., and E. Sturdik. 2000. Advances in biotechnological production of butyric acid. J. Indust. Microb. Biotechnol. 24:153–160.

Prokaryotes (2006) 1:756–765
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CHAPTER 3.2

Amino Acid Production

HIDEHIKO KUMAGAI

Introduction

The water extract of a marine algae "kelp" (*Laminaria japonica*) has been used in a number of Japanese recipes as a kind of soup to flavor cooking. The tasty ("Umami" in Japanese) factor in the marine algae was identified as monosodium glutamate by Prof. Kikunae Ikeda in 1908. Soon thereafter, Ajinomoto Co. Ltd. started to produce monosodium glutamate by extraction from wheat protein after hydrolysis with hydrochloric acid.

In 1956, Kyowa Hakko Kogyo Co. Ltd. succeeded in producing sodium glutamate by using a bacterium (*Corynebacter glutamicum*). Then, production using microbial methods of various amino acids (including of L-alanine, L-aspartic acid, L-arginine, L-citrulline, L-cysteine, L-DOPA [3,4-dihydroxyphenylalanine], L-glutamic acid, L-glutamine, glutathione, L-histidine, D-hydroxyphenylglycine, L-hydroxyproline, L-isoleucine, L-lysine, L-ornithine, L-phenylalanine, D-phenylglycine, L-polylysine, L-proline, L-serine, L-threonine, L-tryptophan, and L-tyrosine) was investigated and successfully manufactured on an industrial scale. Glycine is produced by chemical methods because the molecule has no chiral center, and methionine is also produced by chemical methods in its racemic form because the main use of the amino acid is as feedstuff. D-Methionine is metabolized in animals by the action of D-amino acid oxidase. These amino acids were useful as sources of medicines, food additives, feedstuffs, and starting materials for chemical synthesis.

The microbial methods for the production of amino acids are either fermentative or enzymatic. Fermentation methods use cheap carbon and nitrogen sources as the starting materials to produce rather large amounts of amino acids. These starting materials are metabolized by a number of enzymatic reaction steps and the product accumulates in the culture medium during cell growth. Enzymatic methods require substrates that are generally expensive because they usually are produced by chemical synthesis. So this method is suitable for rather expensive, small-scale production. Figure 1 shows the difference between fermentative and enzymatic methods.

This chapter describes the microbial production of some amino acids including the producing strains, production method, product usage, and industrial production.

L-Alanine

The annual world production of L-alanine is about 500 tons. This amino acid is useful as an enteral and parenteral nutrient and as a food additive, which has a sweet taste and bacteriostatic properties.

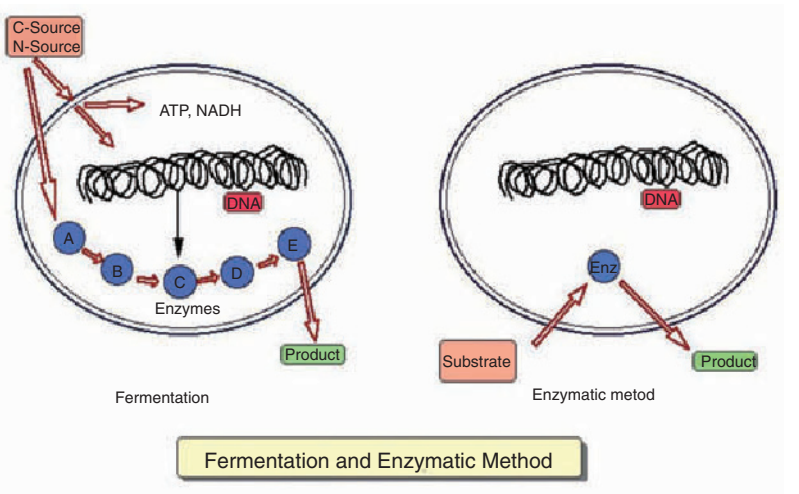
L-Alanine is produced from L-aspartate by a one-step enzymatic method using aspartate β-decarboxylase (Chibata et al., 1986).

L-Alanine production by fermentation is difficult because bacteria usually have an alanine racemase to racemize the product. Fermentative production of L-alanine with racemase-deficient strains of *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Arthrobacter oxydans* (Hashimoto and Katsumata, 1999) has been investigated and good yields have been reported, although the method is not yet industrially applicable.

Tanabe Seiyaku Co. in Japan applied on an industrial scale the enzymatic method described above, using aspartic acid produced by immobilized enzymes as the starting material. A bacterial strain selected for its strong aspartate β-decarboxylase activity was identified as *Pseudomonas dacunhae* (Chibata et al., 1965). The bacterial cells with high activity were immobilized on κ-carageenan, a polysaccharide obtained from seaweed, and packed in a column. The L-alanine was produced by this column system continuously. To prevent the evolution of carbon dioxide gas, a closed column reactor was designed and used for the production. In this column the enzyme reaction proceeds under high pressure (Chibata et al., 1986).

The substrate, L-aspartate, is produced from fumarate by an enzyme system involving aspartase, as described in the section on L-aspartate.

Fig. 1. Fermentation and enzymatic methods.



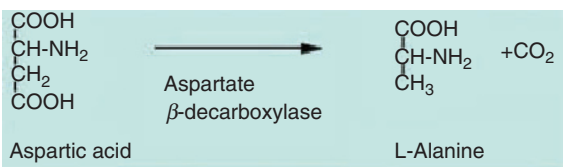


Fig. 2. Enzymatic synthesis of L-alanine.

To produce L-alanine directly from fumarate, the L-alanine-producing column was connected in tandem to an L-aspartate-producing column. In this tandem column system, side reactions caused by fumarase in *Escherichia coli* and alanine racemase in *P. dacunhae* reduced the yield. Then, both bacterial cells were separately treated with high temperature and low pH, respectively, and the enzymes responsible for the side reactions were inactivated. Immobilization of these two kind of bacterial cells with κ-carrageenan resulted in the production of L-alanine in a single reactor without the production of the side products, malate and D-alanine (Takamatsu et al., 1982; Chibata et al., 1986).

L-Aspartate

The annual world production of L-aspartate is estimated to be 7000 tons. L-Aspartate is used as an enteral and parenteral nutrient, a food additive, and a starting material for the low-calorie sweetener aspartame (aspartylphenylalanine methyl ester). It is also used as a raw material to synthesize detergent and for chelating or water treatment agents.

L-Aspartate is produced by the reaction of fumarate and ammonia catalyzed by aspartase.

L-Aspartate production began in 1960 using a batchwise process involving *E. coli* cells containing high aspartase activity. In 1973, Chibata and collaborators at the Tanabe Seiyaku Co. started producing L-aspartate using a continuous reaction system consisting of an immobilized enzyme column. In the system, aspartase extracted from *E. coli* cells was immobilized on ion exchange resin. *Escherichia coli* cells were immobilized by trapping in acrylamide gel and then the column was used in industrial production (Tosa et al., 1973). In 1978, this matrix was changed to κ-carrageenan. The production of L-aspartate was greatly improved by this method and the yield became 100 tons/month using a 1 kiloliter bioreactor (Chibata et al., 1986). In the United States, *E. coli* cells with high aspartase activity immobilized on polyurethane and polyazetidine were reported and the latter was shown to have high aspartase activity, producing aspartate at the rate of 55.9 mol/h/kg cell (wet weight; Fusee et al., 1981).

A different system for the enzymatic production of L-aspartate was proposed and used by Mitsubishi Petrochemical Co. in Japan in 1985. In this system, resting intact coryneform bacteria, *Brevibacterium flavum*, were used without immobilization in a reactor with an ultrafiltration membrane (Terasawa et al., 1985). The starting material, maleate, was converted to fumarate by maleate isomerase in the cells. The bacterial strain with high maleate isomerase and aspartase activity was obtained by the transformation of its genes. The plasmids introduced were stabilized (Zupansic et al., 1995) and the cells were reused many times without any loss of activity and lysis (Yukawa, 1999).

L-Cysteine

Annual world production of L-cysteine is 1500 tons. Its uses are as an enteral and parenteral

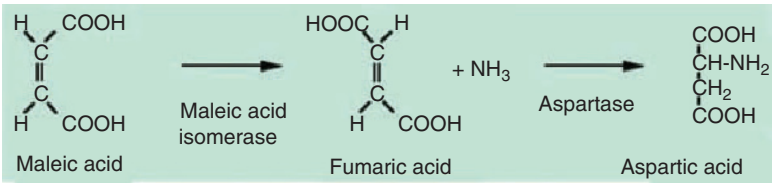


Fig. 3. Enzymatic synthesis of L-aspartate.

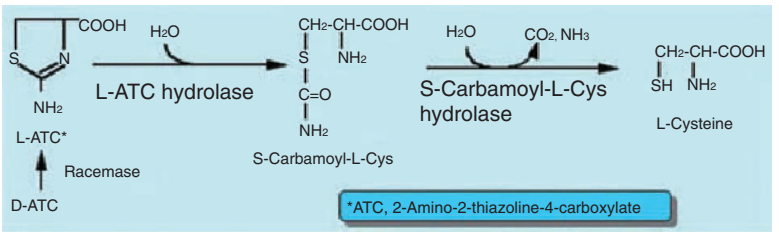


Fig. 4. Enzymatic synthesis of L-cysteine.

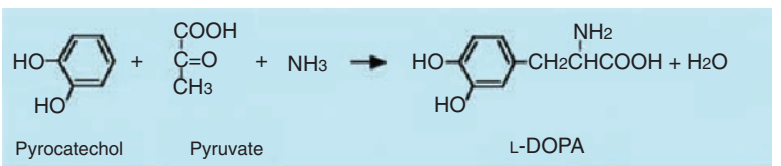


Fig. 5. Enzymatic synthesis of L-3,4-dihydroxyphenylalanine (DOPA).

nutrient, food additive, constituent of hair treatment preparations, and starting material of constituents used in cosmetics.

In 1982, Ajinomoto Co. Ltd. industrially applied a three-step enzymatic process to produce L-cysteine from DL-2-amino-2-thiazoline-4-carboxylate (DL-ATC), a starting material of the chemical synthesis of L-cysteine. The enzymes catalyzing this process are DL-ATC racemase, L-ATC hydrolase, and *S*-carabamoyl-L-cysteine (SCC) hydrolase (Sano et al., 1977; Sano et al., 1978; Sano et al., 1979; Fig. 4).

S-Carboxymethyl-L-cysteine is also produced by the same enzymatic method from the corresponding starting material (Yokozeki et al., 1988).

In screening for high-yield producers, the bacterial strain that produced the most L-cysteine from DL-ATC was isolated from soil and designated "*Pseudomonas thiazolinophilum*." The enzymes responsible for the conversion were inducible and the addition of DL-ATC to the culture medium was essential for enzyme activity. Addition of Mn^{+2} and Fe^{+2} to the medium also contributed to increasing the enzyme activity. The reaction proceeds by adding cells with high enzyme activities but no cysteine-desulfhydrase (a L-cysteine degrading enzyme) to the reaction mixture containing DL-ATC. L-Cysteine produced in the reaction mixture is oxidized to L-cystine by aeration and precipitated as crystals. This increases the efficiency of L-cysteine production, which is 31.4 g/liter obtained from 40 g/liter of DL-ATC, i.e., 95% product yield by molar ratio.

L-DOPA

The annual world production of L-DOPA is around 250 tons. L-DOPA (the precursor of the neurotransmitter dopamine) is useful as a treatment for Parkinson's disease. It had been mainly produced by a chemical synthetic method that included eight reaction steps including an optical resolution step.

L-DOPA is produced from pyrocatechol, pyruvate and ammonia by a one-step enzyme reaction using tyrosine phenol-lyase (TPL). Ajinomoto Co. Ltd. began using *Erwinia* TPL for enzymatic L-DOPA production (by a simple one-step method and one of the most economical processes to date) in 1993.

Tyrosine phenol-lyase (TPL) is a pyridoxal 5′-phosphate dependent multifunctional enzyme and catalyzes degradation of tyrosine into phenol, pyruvate and ammonia. This reaction is reversible, and the reverse reaction is available to produce L-DOPA using pyrocatechol instead of phenol (Fig. 5).

Erwinia herbicola was selected as the most favorable strain for the L-DOPA production out of 1041 microbial strains tested. No enzyme activity was found in yeasts, fungi and actinomycetes. Culture conditions for the preparation of cells containing high TPL activity and reac-

tion conditions for the synthesis of L-DOPA were optimized with *Erwinia herbicola*. Additions of yeast extract, meat extract, polypeptone, and the hydrolyzate of soybean protein to the basal medium enhanced cell growth as well as the formation of TPL. Catabolite repression of biosynthesis of TPL was observed on adding glucose, pyruvate, and α-ketoglutarate to the medium at high concentrations. Glycerol was a suitable carbon source for cell growth as well as for the accumulation of the enzyme in growing cells. TPL is an inducible enzyme and the addition of L-tyrosine to the medium is essential for formation of the enzyme. L-Phenylalanine is not an inducer of TPL biosynthesis but works as a synergistic agent for the induction by L-tyrosine. The activity of TPL increased five times by the addition of L-phenylalanine together with L-tyrosine to the medium. Cells of *E. herbicola* with high TPL activity were prepared by growing them at 28°C for 28 h in a medium containing 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 2 ppm of Fe^{+2} ($FeSO_4 \cdot 7H_2O$), 0.01% pyridoxine-HCl, 0.6% glycerol, 0.5% succinic acid, 0.1% DL-methionine, 0.2% DL-alanine, 0.05% glycine, 0.1% L-phenylalanine, and 12 ml of hydrolyzed soybean protein in 100 ml of tap water, with the pH controlled at 7.5 throughout cultivation. Under these conditions, TPL was efficiently accumulated in the cells of *E. herbicola* and made up about 10% of the total soluble cellular protein (Yamada and Kumagai, 1975).

The enzymatic synthesis reaction of L-DOPA is carried out in a batchwise system with cells of *E. herbicola* containing high activity of TPL. Since pyruvate, one of the substrates, was unstable in the reaction mixture at high temperature, low temperature was used for the synthesis of L-DOPA. The reaction was carried out at 16°C for 48 h in a reaction mixture containing various amounts of sodium pyruvate, 5 g of ammonium acetate, 0.6 g of pyrocatechol, 0.2 g of sodium sulfite, 0.1 g of EDTA, and cells harvested from 100 ml of broth in a total volume of 100 ml. The pH was adjusted to 8.0 by the addition of ammonia. At 2-h intervals, sodium pyruvate and pyrocatechol were added to the reaction mixture to maintain the initial concentrations. The maximum synthesis of L-DOPA was obtained when the concentration of sodium pyruvate was kept at 0.5%. The addition of substrates, pyrocatechol and pyruvate, was separated by a time interval to prevent the denaturation of TPL and to prevent byproduct formation. Sodium sulfite is added to keep the reactor in a reductive state and to prevent the oxidation of product L-DOPA. The L-DOPA is not soluble in the reaction medium, so it forms a crystalline precipitate (reaching 110 g/liter) during the reaction (Yamada and Kumagai, 1975; Kumagai, 1999a; Kumagai, 1999b).

Induction and repression mechanisms of TPL in *E. herbicola* were studied. It was found that TPL biosynthesis is regulated at the transcriptional level. Tyrosine phenol-lyase mRNA was increased by the addition of tyrosine and decreased by the addition of glucose in the medium. TyrR box and operator-like regions were found in the 5' flanking region of its gene, *tpl*. TyrR box is a typical binding site on DNA where a regulator protein TyrR binds and controls transcription of the regulon of enzyme genes or transporter genes responsible for biosynthesis of aromatic amino acids or transport through cell membrane (Suzuki et al., 1995; Katayama et al., 1999). Katayama et al. reported three point mutations in the *tyrR* gene that caused high-level expression of *lacZ* in *E. coli* and *tpl* in *E. herbicola* (Katayama et al., 2000). The function of the product of *tutB* gene and the gene itself, located just downstream of *tpl* in *E. herbicola*, were analyzed. It was elucidated that *tutB* encodes a tyrosine specific transporter and this is essential for maximum induction of TPL in *E. herbicola* cells (Katayama et al., 2002).

Glutamic Acid

World production of monosodium L-glutamate using so-called "coryneform bacteria" is around one million tons per annum. Monsodium glutamate is used as a flavor enhancer and an intermediate material for chemical synthesis of medicines. Its ester is used as a detergent and the polymer as artificial skin. Two Japanese companies, Ajinomoto and Kyowa Hakko Kogyo, built factories and produced it in other countries, mainly in southeast Asia. China, Korea and Taiwan also are producing large amounts of monosodium L-glutamate.

Glutamic acid is produced by *Corynebacterium glutamicum* in the presence of high concentrations of sugar and ammonium, appropriate concentrations of minerals, and a limited concentration of biotin under aerobic conditions (Kikuchi and Nakao, 1986). In 2-3 days, around 100 g of L-glutamate per liter accumulates in the medium.

Various glutamic acid-producing strains were reported after the first report on *Corynebacterium glutamicum*. They are *Brevibacterium flavum*, *Brevibacterium lactofermentum*, *Brevibacterium thiogenitalis* and *Microbacterium ammoniaphilum*, and all strains are Gram-positive, nonsporulating, nonmotile, cocci or rod-like and all require biotin for growth. Currently, these strains are thought to belong to the genus *Corynebacterium*.

The carbon source most commonly used as a starting material is glucose, which is obtained by enzymatic hydrolysis of starch from corn, potato and cassava. Waste molasses is also used since it is cheap. Acetic acid and ethanol are also good carbon sources to produce glutamate. A high concentration of a nitrogen source is necessary to accumulate glutamate, and ammonia gas, its solution, the inorganic salt, or urea is used in actual production.

Coryneform bacteria generally show high rates of sugar assimilation and highly active glutamate dehydrogenase, which is responsible for glutamate biosynthesis. Glucose incorporated in the cell is metabolized through the Embden-Meyerhof pathway (EMP) and a part of the tricarboxylic acid (TCA) cycle, and 2-oxoglutarate formed in the cycle is aminated to glutamate by the action of glutamate dehydrogenase.

Biotin is an important factor regulating the growth of the bacterium and glutamic acid production. Its suboptimal addition is essential to produce a large amount of glutamic acid in the medium. To use a starting material such as waste molasses, which contains excess biotin, the addition of penicillin to the medium during growth was found to be effective. In the production of glutamic acid, several saturated fatty acids or their esters were also found to function similarly to penicillin. A glycerol-requiring mutant of *Corynebacterium alkanolyticum* was used to produce glutamic acid in appreciable amounts without the addition of penicillin and without the need to control biotin concentration (Kikuchi and Nakao, 1986).

Since these treatments are essential for the glutamate fermentation, it has been suggested that the cell surface of the bacteria is damaged under such conditions, and consequently leaking of glutamate takes place. This leaking theory has been accepted for a long time. But recently another published theory of excretion of glutamate suggested an exporter protein of glutamate was present on the cell surface of the bacterium (Kraemer, 1994).

The amount of 2-oxoglutarate dehydrogenase complex (ODHC), which catalyzes the conversion of 2-oxo-glutarate to succinyl-CoA as the first step of succinate synthesis in the TCA cycle, was reported to be decreased in glutamate-producing bacterial cells. And recently, the enzyme activity was confirmed to be very low in the presence of detergent, or limited amounts of biotin or penicillin (Kawahara et al., 1997). These results suggest that one of the main causes for the glutamate overproduction is the decrease of 2-oxoglutarate dehydrogenase activity (ODH). A disrupted (ODH) gene-bearing bacterial strain produced as much glutamate as the wildtype strain under conditions of glutamate overproduction.

Furthermore, a novel gene *dtsR* was cloned, which rescues the detergent sensitivity of a mutant derived from a glutamate producing bacterium, *Corynebacterium glutamicum* (Kimura et al., 1996). The authors found that this gene encodes a putative component of a biotin-containing enzyme complex and has something to do with fatty acid metabolism. The disruption of this gene causes constitutive production of glutamate even in the presence of excess biotin. The authors suggested that the overproduction of glutamate is caused by an unbalance of the coupling between fatty acid and glutamate synthesis (Kimura et al., 1997). They successfully showed that inducers of glutamate overproduction such as Tween 40 and limited amounts of biotin reduced the level of DtsR, which then triggered overproduction by decreasing the activity of ODHC (Kimura et al., 1999).

Kyowa Hakko Kogyo Co. Ltd., the Research Institute for Innovative Technology for Earth in Japan, and Degussa in Germany completed the analysis of the genomic DNA nucleotide sequence of *Corynebacterium glutamicum*.

D-*p*-Hydroxyphenylglycine

Kaneka Co. Ltd. started the enzymatic production of D-*p*-hydroxyphenylglycine (D-HPG) in 1980 in Singapore, and the immobilized D-carbamoylase reactor was introduced in this process in 1995. The annual production of D-HPG by this method is around 2,000 tons. D-HPG is a starting material for the production of semi-synthetic penicillins and cephalosporins, such as amoxicillin and cephadoxel. D-HPG is produced from DL-*p*-hydroxyphenylhydantoin (DL-HPH) by a two-step enzymatic method (Takahasi, 1986).

The starting material DL-HPH is synthesized by the amidoalkylation of phenol (Ohashi et al., 1981). Only D-HPH is hydrolyzed by hydantoinase to form D-HPG via *N*-carbamoyl-D-*p*-HPG. L-HPH is spontaneously racemized at a slightly alkaline pH. Then during the reaction, only D-HPH is hydrolyzed to form D-HPG via *N*-carbamoyl-D-*p*-HPG. Finally DL-HPH in the reaction mixture is completely hydrolyzed to D-HPG.

D-Hydantoin hydrolase activity was found in some bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Aerobacter*, *Agrobacterium* and *Corynebacterium* and in actinomycetes belonging to the genera *Streptomyces* and *Actinoplanes*. D-Carbamylase activity was found in various bacteria belonging to the genera *Agrobacterium*, *Pseudomonas*, *Comamonas* and *Blastobacter*.

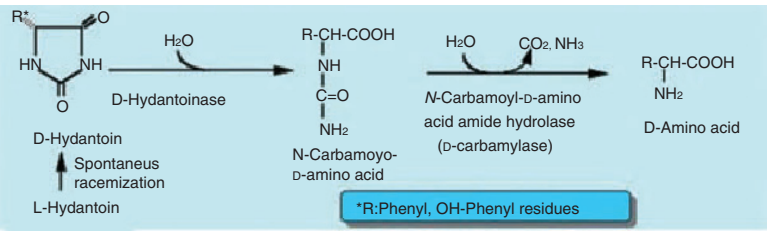


Fig. 6. Enzymatic synthesis of D-hydroxy-phenylglycine.

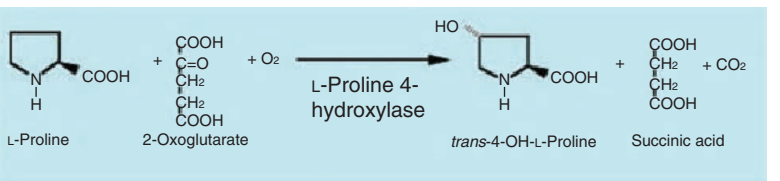


Fig. 7. Enzymatic synthesis of L-hydroxyproline.

The genes of these two enzymes were cloned, and an *E. coli* strain transformed by this gene was used as the practical enzyme source. To obtain stable D-carbamoylase for repeated use, a random mutation technique was applied to the *Agrobacterium* D-carbamoylase. Three heat stable mutant enzymes were obtained. These mutations were found at His 57, Pro203, and Val236. These mutations were combined in one molecule, and the mutant enzyme containing the triple mutation His57Tyr, Pro203Glu, and Val236Ala had 19°C higher heat stability than did the wildtype enzyme (Ikenaka, 1999). *Escherichia coli* cells containing this mutant enzyme were immobilized and used for practical industrial production of D-HPG with the simultaneous use of immobilized D-hydantoinase on line. This immobilized mutant D-carbamoylase reactor can be used for one year without any supply of new enzyme.

D-Phenylglycine is also produced by the same enzymatic method using the corresponding starting material.

Hydroxy-L-proline

The industrial production of *trans*-4-hydroxy-L-proline was started by Kyowa Hakko Kogyo Co. Ltd. in 1997. 4-Hydroxy-L-proline is useful as a chiral starting material in chemical synthesis and as a starting material of medicinals, cosmetics, and food additives. *trans*-4-Hydroxy-L-proline is a component of animal tissue protein such as collagen and was extracted from collagen after hydrolysis with strong acid before this enzyme process was industrially utilized. The discovery of L-proline hydroxylases made the microbial production of hydroxyproline possible. *trans*-4-Hydroxy-L-proline or *cis*-3-hydroxy-L-proline is produced from L-proline by the respective action of L-proline 4-hydroxylase or 3-hydroxylase. The other substrate 2-oxoglutamate is supplied from glucose added to the reaction mixture through the EMP pathway and TCA cycle.

Ozaki et al. developed a specific hydroxyproline detection method with high performance liquid chromatography (Ozaki et al., 1995) and screened strains for microbial proline hydroxylase activity. L-Proline 4-hydroxylase was found in some etamycin-producing actinomycetes belonging to the genera *Streptomyces*, *Dactylosporangium* or *Amycolatopsis* (Shibasaki et al., 1999). L-Proline 3-hydroxylase was found in some telomycin producing actinomycetes belonging to the genus *Streptomyces* and in bacteria belonging to *Bacillus* (Mori et al., 1996).

The genes of these proline hydroxylase-producing organisms were cloned in *E. coli* cells, respectively, and the cells overexpressing the enzyme were used as the enzyme source in the industrial process of L-hydroxyproline production. Since the genes obtained from actinomycetes had some difficulty in being highly expressed in *E. coli* cells, the genetic codons corresponding to the N-terminal of the enzyme protein were changed to match the codon usage in *E. coli*. Furthermore, the promoter of *trp* operon was introduced twice at the promotor site of the gene in the plasmid to achieve the overexpression. These transformants expressed 1400 times higher activity of proline 4-hydroxylase and 1000 times higher activity of proline 3-hydroxylase in comparison with the original strain.

2-Oxoglutarate, one of the substrates of hydroxylation, is made from glucose in the reaction medium via the EMP pathway and TCA cycle in *E. coli*, and the product succinate is recycled. The mutant strain of *E. coli* lacking the L-proline-degrading enzyme was obtained and used for the host cells in the production of L-hydroxyproline.

Using *E. coli* as the host cells in L-proline production, the direct production of L-hydroxyproline from glucose became possible. In this case, the derepressed genes of the L-proline biosynthetic pathway were introduced into *E. coli* cells together with the gene of L-proline hydroxylase.

L-Lysine

The estimated annual world production of L-lysine is around 500,000 tons, almost all supplied by Ajinomoto, Kyowa Hakko Kogyou, Archer Daniels Midland (ADM), and Badische Aniline and Soda-Fabrik (BASF). L-Lysine (an essential amino acid for swine and poultry) is useful as an additive to feeds such as grains and defatted soybeans, which contain less lysine.

L-Lysine is produced by some mutants derived from wild strains of glutamate-producing bacteria including *Corynebacterium glutamicum*, *Brevibacterium lactofermentum* and *B. flavum* in the presence of high concentration of sugar and ammonium, at neutral pH, and under aerobic conditions (Tosaka and Takinami, 1986).

The pathway of biosynthesis of L-lysine and L-threonine including controls of the biosynthesis in *Corynebacterium glutamicum* is shown in Fig. 8. The formation of phosphoaspartate from aspartate is the first step and is catalyzed by aspartokinase. The activity of this enzyme is controlled through concerted feedback inhibition by L-lysine and L-threonine. In 1958, Kinoshita and Nakayama of Kyowa Hakko Kogyo Co. Ltd. reported that the auxotrophic mutant of *Corynebacterium glutamicum*, which lacks homoserine dehydrogenase and is defective in L-homoserine (or L-threonine plus L-methionine) biosynthesis, produced L-lysine in the culture medium (Kinoshita et al., 1958). This was the first report on production of an amino acid by an auxotrophic mutant. Subsequently, amino acid production by auxotrophic mutants expanded greatly. Then, the mutants with the L-threonine- or L-methionine-sensitive phenotype due to the mutation in homoserine dehydrogenase (low activity) were also found to produce appreciable amounts of L-lysine in the culture medium (Tosaka and Takinami, 1986). Furthermore, a lysine analogue (*S*-aminoethylcysteine)-resistant mutant was obtained as an L-lysine producer. In this strain, aspartokinase was insensitive to feedback inhibition (Tosaka and Takinami, 1986). This is the first demonstration of amino acid production by an analogue-resistant mutant.

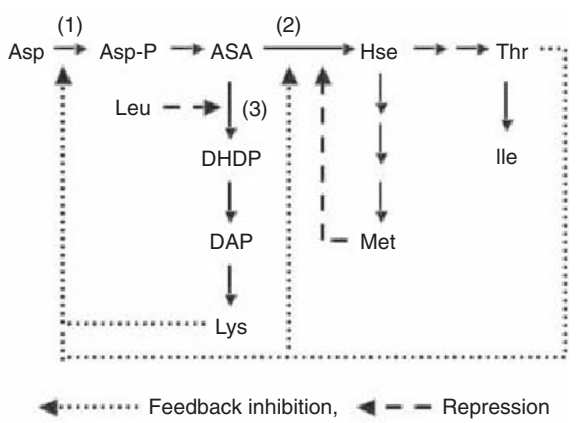


Fig. 8. Regulation of lysine biosynthesis. ASA, aspartate-β-semialdehyde; DHDP, dihydrodipicolinate; DAP, α, ε-diaminopimelate; and Hse, homoserine. (1), Aspartate kinase; (2), Homoserine dehydrogenase; and (3), Dihydrodipicolinate synthase.

These characteristics of lysine production were combined to make strains that were much more efficient producers. In addition, the introduction of a leucine requiring mutation also increases the amount of lysine, since in the mutant dihydrodipicolinate synthase is released from repression by leucine.

The precursors of lysine synthesis include phosphoenol pyruvate, pyruvate, and acetyl CoA. Many mutations are induced in lysine producer cells to supply sufficient amounts of these precursors in good balance. These are deletion mutants of pyruvate kinase, those that show low activity of pyruvate dehydrogenase, etc. Furthermore, an alanine requirement was also reported to be effective in increasing the lysine amount.

The genes of the enzymes responsible for the biosynthesis of lysine in *Corynebacterium* have been cloned and their nucleotide sequences are known. These genes include aspartokinase, aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase, dihydrodipicolinate reductase, tetrahydrodipicolinate succinylase, succinyl diaminopimelate desuccinylase, diaminopimelate dehydrogenase, and diaminopimelate decarboxylase (Tosaka and Takinami, 1986). A host-vector system of *Corynebacterium* was established, and the introduction of some genes that encode the enzymes responsible for lysine biosynthesis (i.e., aspartokinase and dihydrodipicolinate synthase) was found to be effective in increasing the amount of lysine produced (Cremer et al., 1991).

A new gene *ldc* which encodes lysine decarboxylase (formerly known as *cadA* in *E. coli*) has been identified and the enzyme was purified from the overexpressing strain. The lysine decarboxylase encoded by *ldc* is constitutively produced by *E. coli* cells, although lysine decarboxylase encoded by *cadA* is inducible (Kikuchi et al., 1997). It is interesting to note that

this new lysine decarboxylase is present in lysine producing *Corynebacterium* and to investigate the effects of deleting the gene on L-lysine production.

Vrljic et al. (1996) cloned a new gene *lysE* from *Corynebacterium glutamicum* and showed that it encodes a specific L-lysine exporter. Recently, they analyzed the membrane topology of the gene product and showed that it is a member of a protein family found in some other bacteria such as *E. coli*, *Bacillus subtilis*, *Mycobacterium tuberculosis* and *Helicobacter pylori*. The authors suggested that LtsE superfamily members would be shown to catalyze export of a variety of biologically important solutes including amino acids (Aleshin et al., 1999; Vrljic et al., 1999; Zakataeva et al., 1999).

L-Threonine

The annual worldwide production of L-threonine is around 13,000 to 14,000 tons. L-Threonine is an essential amino acid for humans and some livestock animals, such as pigs and poultry. It is used as an additive for animal feed, medicines, food, and cosmetics.

L-Threonine is produced by some auxotrophic mutants or threonine-analogue resistant mutants, and those are created by genetic engineering techniques. The bacteria used are *Escherichia coli*, *Corynebacterium glutamicum*, *Brevibacterium lactofermentum*, *B. flavum*, *Serratia marcescens* and *Proteus retgerii* (Nakamori, 1986). L-Threonine production by fermentation was started in the 1970s. The auxotrophic mutant and analogue resistant mutant strains obtained for this purpose were cultured in the presence of amino acids required by the mutant.

Auxotrophic mutants of L-lysine, diaminopimelate or L-methionine were found to produce L-threonine in the culture medium, but the amount was not high enough to justify their use in practical production. A resistant mutant to an L-threonine analogue, α-amino-β-hydroxyvaleic acid (AHV), was obtained and shown to be an L-threonine producer. In this strain homoserine dehydrogenase was insensitive to the feedback inhibition by L-threonine (Fig. 8). The much stronger L-threonine producing strains were obtained by the combination of the auxotrophic mutations and AHV resistant mutation. L-Threonine producing mutant of *S. marcescens* was induced by the techniques of phage transduction. The strain has the following properties: deficiency of L-threonine degrading enzymes; a mutation in the aspartokinase and homoserine dehydrogenase genes, making them insensitive to feedback inhibition by L-threonine; mutations in genes for L-threonine biosynthetic enzymes, releasing them from repression by L-threonine; a mutation in the aspartokinase gene, making it insensitive to feedback inhibition by L-lysine; and a mutation in the aspartokinase and homoserine dehydrogenase genes, releasing them from the repression by L-methionine.

Recombinant DNA techniques were employed to improve the L-threonine producer. Genes of the threonine operon obtained from AHV resistant and feedback insensitive mutants were introduced into a threonine deficient mutant of *E. coli* to amplify the expression of enzymes and to increase the amount of L-threonine. *Escherichia coli* mutant strains were also constructed with amplified genes of the threonine operon (obtained from AHV resistant and feedback insensitive mutants) by the action of Mu phage on the chromosomal DNA. This strain is used in France for L-threonine production. Okamoto et al. constructed an L-threonine hyper-producing *E. coli* mutant that can produce L-threonine (100 g/liter) in 77 h. They suggested that uptake of L-threonine in this strain is impaired (Okamoto et al., 1997).

Literature Cited

Aleshin, V. V., N. P. Zakataeva, and V. A. Livshits. 1999. A new family of amino-acid-efflux proteins. Trends Biochem. Sci. 24:133–135.

Chibata, I., T. Kakimoto, and J. Kato. 1965. Enzymatic production of L-alanine by Pseudomonas dachnae. Appl. Microbiol. 13:638–645.

Chibata, I., T. Tosa, and T. Kakimoto. 1986a. Alanine. *In:* K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada (Eds.) Biotechnology of Amino Acid Production. Kodansha/Elsevier. 224–232.

Chibata, I., T. Tosa, and T. Sato. 1986b. Aspartic acid. *In:* K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada (Eds.) Biotechnology of Amino Acid Production. Kodansha/Elsevier. 144–151.

Clement, Y., and G. Lanneelle. 1986. Glutamate excretion mechanism in Corynebacterium glutamicum: Triggering by biotin starvation or by surfactant addition. J. Gen. Microbiol. 132:925–929.

Cremer, J., L. Eggeling, and H. Sahm. 1991. Control of the lysine biosynthesis sequence in Corynebacterium glutamicum, as analyzed by overexpression of the individual corresponding genes. Appl. Environ. Microbiol. 57:1746–1752.

Fusee, M. C., W. E. Swann, and G. J. Carlson. 1981. Immobilization of Escherichia coli cells containing aspartase activity with polyurethane and its application for L-aspartic acid production. Appl. Environ. Microbiol. 42:672–676.

Hashimoto, S., and R. Katsumata. 1999. Mechanism of alanine hyperproduction by Arthrobacter oxydans HAP-1: Metabolic shift to fermentation under nongrowth aerobic conditions. Appl. Environ. Microbiol. 65:2781-2783.

Ikenaka,Y., H. Nanba, K. Yajima, Y. Yamada, M. Takano, and S. Takahashi. 1999. Thermostability reinforcement through a combination of thermostability-related muta-

tions of N-carbamyl-D-amino acid amidohydrolase. Biosci. Biotechnol. Biochem. 63:91–95.

Katayama, T., H. Suzuki, K. Yamamoto, and H. Kumagai. 1999. Transcriptional regulation of tyrosine phenol-lyase gene mediated through TyrR and cAMP receptor protein. Biosci. Biotechnol. Biochem. 63:1823–1827.

Katayama, T., H. Suzuki, T. Koyanagi, and H. Kumagai. 2000. Cloning and random mutagenesis of the Erwinia herbicola tyrR gene for high-level expression of tyrosine phenol-lyase. Appl. Environ. Microbiol. 66:4764–4771.

Katayama, T., H. Suzuki, T. Koyanagi, and H. Kumagai. 2002. Functional analysis of the Erwinia herbicola tutB gene and its product. J. Bacteriol. 184:3135–3141.

Kawahara, Y., K. Takahashi-Fuke, E. Shimizu, T. Nakamatsu, and S. Nakamori. 1997. Relationship between the glutamate production and the activity of 2-oxoglutarate dehydrogenase in Brevibacterium lactofermentum. Biosci. Biotechnol. Biochem. 61:109–1112.

Kikuchi, M., and Y. Nakao. 1986. Glutamic acid. *In:* K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada (Eds.) Biotechnology of Amino Acid Production. Kodansha/Elsevier. 101–120.

Kikuchi, Y. H. Kojima, T. Tanaka, Y. Takatsuka, and Y. Kamio. 1997. Characterization of a second lysine decarboxylase isolated from Escherichia coli. J. Bacteriol. 179:4486–4492.

Kimura, E., C. Abe, Y. Kawahara, and T. Nakamatsu. 1996. Molecular cloning of a novel gene, dtsR, which rescues the detergent sensitivity of a mutant derived from Brevibacterium lactofermentum. Biosci. Biotechnol. Biochem. 60:1565–1570.

Kimura, E., C. Abe, Y. Kawahara, T. Nakamatsu, and H. Tokuda. 1997. A dtsR gene-disrupted mutant of Brevibacterium requires fatty acids for growth and efficiently produces L-glutamate in the presence of an excess biotin. Biochem. Biophys. Res. Commun. 234:157–161.

Kimura, E., C. Yagoshi, Y. Kawahara, T. Ohsumi, T. Nakamatsu, and H. Tokuda. 1999. Glutamate overproduction in Corynebacterium glutamicum triggered by a decrease in the level of a complex comprising dtsR and a biotin-containing subunit. Biosci. Biotechnol. Biochem. 63:1274–1278.

Kinoshita, S., K. Nakayama, and S. Kitada. 1958. L-lysine production using microbial auxotroph. J. Gen. Appl. Microbiol. 4:128–129.

Kraemer, R. 1994. Secretion of amino acids by bacteria: physiology and mechanism. FEMS Microbiol. Rev. 13:75–79.

Kumagai, H. 1999a. Dihydroxyphenylalanine, produced by mycroorganisms. *In:* M. C. Flicinger and S. W. Drew (Eds.) Encyclopedia of Bioprocess, Technology: Fermentation, Biocatalysis and Bioseparation. John Wiley. New York, NY. 821–823.

Kumagai, H. 1999b. Tyrosine phenol-lyase. *In:* M. C. Flicinger and S. W. Drew (Eds.) Encyclopedia of Bioprocess, Technology: Fermentation, Biocatalysis and Bioseparation. John Wiley. New York, NY. 2605–2609.

Mori, H., T. Shibasaki, Y. Uozaki, K. Ochiai, and A. Ozaki. 1996. Detection of novel proline 3-hydroxylase activities in Streptomyces and Bacillus spp. by regio-and streospecific hydroxylation of L-proline. Appl. Environ. Microbiol. 62:1903–1907.

Nakamori, S. 1986. Threonine and homoserine. *In:* K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada (Eds.) Biotechnology of Amino Acid Production. Kodansha/Elsevier. 173–182.

Ohhashi,T., S. Takahashi, T. Nagamachi, K. Yoneda, and H. Yamada. 1981. A new method for 5-(4-hydroxyphenyl)hydantoin synthesis. Agric. Biol. Chem. 45: 831–838.

Okamoto, K., K. Kino, and M. Ikeda. 1997. Hyperproductin of L-threonine by an Escherichia coli mutant with impaired L-threonine uptake. Biosci. Biotechnol. Biochem. 61:1877–1882.

Ozaki, A., T. Shibasaki, and H. Mori. 1995. Specific roline and hydroxyproline detection method by post-column derivatization for high-performance liquid chromatography. Biosci. Biotechnol. Biochem. 59:1764–1765.

Sano, K., K. Yokozki, F. Tamura, N. Yasuda, I. Noda, and K. Mitsugi. 1977. Microbial conversion of DL-2-amino-Δ2-thiazoline-4-carboxylic acid to L-cysteine and L-cysteine:screening of microorganisms and identification of products. Appl. Environ. Microbiol. 34:806–810.

Sano, K., and K. Mitsugi. 1978. Enzymatic production of L-cysteine from DL-2-amino-Δ2-thiazoline-4-carboxylic acid by Pseudomonas thiazolinohilum: Optimal conditions for the enzyme formation and enzymztic reaction. Agric. Biol. Chem. 42:2315–2321.

Sano, K. C. Eguchi, N. Yasuda, and K. Mitsugi. 1979. Metabolic pathway of L-cysteine formation from DL-2-amino-Δ2-thiazoline-4-carboxylic acid by Pseudomonas. Agric. Biol. Chem. 43:2373–2374.

Shibasaki, T., H. Mori, S. Chiba, and A. Ozaki. 1999. Microbial proline 4-hydroxylase screening and gene cloning. Appl. Environ. Microbiol. 65:4028–4031.

Suzuki, H., T. Katayama, K. Yamamoto, and H. Kumagai. 1995. Transcriptional regulation of tyrosine phenol-lyase gene of Erwinia herbicola AJ2985. Biosci. Biotechnol. Biochem. 59:2028–2032.

Takahashi, S. 1986. Microbial production of D-p-hydroxyphenylglycine. *In:* K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada (Eds.) Biotechnology of Amino Acid Production. Kodansha/Elsevier. 269–279.

Takamatsu, S., I. Umemura, K. Yamamoto, T. Sato, and I. Chibata. 1982. Production of L-alanine from ammonium fumarate using two immobilized microorganisms. Eur. J. Appl. Microbiol. Biotechnol. 15:147–152.

Terasawa, M., H. Yukawa, and Y. Takayama. 1985. Production of L-aspartic acid from Brevibacterium by the cell re-using process. Process Biochem. 20:124–128.

Tosa, T., T. Sato, T. Mori, Y. Matsuo, and I. Chibata. 1973. Continuous production of L-aspartic acid by immobilized aspartase. Biotechnol. Bioeng. 15:69–84.

Tosaka, O., and K. Takinami. 1986. Lysine. *In:* K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada (Eds.) Biotechnology of Amino Acid Production. Kodansha/Elsevier. 152–172.

Vrljic, M., H. Sahm, and L. Eggeling. 1996. A new type of transporter with a new type of cellular function: L-lysine export from Corynebacterium glutamicum. Molec. Microbiol. 22:815–826.

Vrljic, M., J. Garg, A. Bellmann, S. Wachi, R. Freudl, M. J. Malecki, H. Sahm, V. J. Kozina, L. Eggeling, and M. H. Saier Jr. 1999. The LysE superfamily: Topology of the lysine exporter LysE of Corynebacterium glutamicum, a paradigm for a novel superfily of transmembrane solute translocators. J. Molec. Microbiol. Biotechnol. 1:327–336.

Yamada, H., and H. Kumagai. 1975. Synthesis of L-tyrosine related amino acids by β-tyrosinase. *In:* D. Perlman

(Ed.) Advances in Applied Microbiology. Academic Press. New York, NY. 19:249–288.

Yokozeki, K., C. Eguchi, A. Kamimura, and K. Kubota. 1988. Asymmetric synthesis of S-carboxymethyl-L-cysteine by a chemico-enzymatic method. Agric. Biol. Chem. 52:2367–2368.

Yukawa, H. 1999. L-aspartic acid. *In:* M. C. Flicinger and S. W. Drew (Eds.) Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation. John Wiley. New York, NY. 206–208.

Zakataeva, N. P., V. V. Aleshin, I. L. Tokmakova, P. V. Troshin, and V. A. Livshits. 1999. The novel transmembrane Escherichia coli proteins involved in the amino acid efflux. FEBS Lett. 452:228–232.

Zupansic, T., J. Kittle, B. Baker, C. Miller, D. Palmaer, Y. Asai, M. Inui, A. Verrttes, M. Kobayashi, M. Kurusu, and H. Yukawa. 1995. Isolation of promoters from Brevibacterium flavum strain MJ233C and comparison of their gene expression levels in B. flavum and Escherichia coli. FEMS Microbiol. Lett. 131:121–125.

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CHAPTER 3.3

Microbial Exopolysaccharides

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Introduction

The abundant nature and manifold uses of polysaccharides derived from plants and animals are well established. Application of these materials, notably chitosan and cellulose, in food and healthcare products is widespread. Unfortunately, issues of purity and seasononal availability and variability surround the use of these and other materials when obtained from environmental sources. Although several hydrocolloids used widely in the same sectors (i.e., xanthan, gellan, and dextrans) are currently available only from microbial sources, commercial and academic interest in the synthesis and properties of microbial polysaccharides has continued to grow over the past several decades for a source of more economical, consistent, and higher purity raw material (Giavasis et al., 2000). Continuing focus on environmentally benign production techniques and the desire for customizability in bacterially derived products also motivate continued investigation into the molecular biology, polymer science, and process engineering of microbial exopolymer production. While progress continues in the understanding of biosynthetic pathways, substrate transport, and polymer secretion in a variety of organisms, recombinant DNA techniques have also resulted in the elaboration of new, selectively modified polymers that offer the potential for a wide variety of novel materials with more predictable structure and function. The ongoing application of biotechnology to the synthesis, optimization, and customization of bacterial polysaccharides is the focus of this chapter.

While the production of polysaccharides by selected microorganisms was first reported in the 1880s (Whitfield, 1988), large-scale application was not achieved until the middle of the twentieth century, with the production and approval of dextran from *Leuconostoc mesenteroides* in 1947 and the United States Food and Drug Administration (FDA) approval of food grade xanthan from *Xanthamonas campestris* in 1969. Today, both materials continue to be important (although xanthan dominates) in the estimated $253 million market for microbial polysaccharides (Magnani, 1998). In the intervening years, various polysaccharides of microbial derivation have been described and, in a few cases, developed into products. While the production of bacterial polymers continues to expand, a still surprisingly small list of polymers has achieved industrial relevance (Sutherland, 1998; Table 1). This continues to be primarily motivated by factors of organismal productivity, regulatory approval, and thus in both cases, cost. Also at issue are two areas of fundamental relevance, the relatively loose understanding of structure-function relationships that slows the progress of research and development in this area (Sutherland, 2001) and the lack of template-controlled synthesis characteristic of proteins.

It should be mentioned from the outset that this chapter necessarily excludes several very important topics of microbial exopolysaccharides as outside of present scope. The most complex of these topics is that of microbial biofilms, around which an extensive literature continues to evolve. Likewise excluded are the areas of biosurfactants, the use of bacteria in the direct fermentation of food products, and capsular polysaccharides used in vaccines. Reviews on each of these topics are available in this volume.

Scientific Background

Microbiology and Genetics

PUTATIVE NATURAL ROLES OF MICROBIAL EXOPOLYMERS. While the focus of this review is the industrial use of microbial polysaccharides, an understanding of the biological roles of these molecules should lead to more rational control of their synthesis in industrial settings. Microbial exopolysaccharides are largely hypothesized to serve protective roles for the microorganism that produces them. While this hypothesis is logical, definitive experiments demonstrating the essential role in the microorganism's fitness and survival are still needed.

One role often attributed to exopolysaccharides is protection from dessication. The hydra-

Table 1. Present applications of microbial polysaccharides.

Polymer	Application
Xanthan	Emulsion stabilization and suspension agent in foods
	Foam stabilization in foods
	Crystallization inhibitor in foods
	Viscosity control in oil drilling mud and inkjet printing
Bacterial cellulose	Moisture retention in wound dressings
	High strength acoustic diaphragms in sound reproduction
Hyaluronic acid	Hydrating agent in cosmetics and pharmaceuticals
	Replacement for synovial fluid and vitreous humor in biomedicine
Emulsan	Emulsifier and vaccine adjuvant
Curdlan	Gelling agent in foods
Gellan	Gelling agent in foods
Pullulan	Food coatings
Various	Paper coating and water flocculant

Adapted from Sutherland (1998).

tion properties of exopolysaccharides clearly can allow a microorganism to survive drying and subsequent rehydration in industrial applications (Schnider-Keel et al., 2001). In the case of a *Nostoc* commune, expression of exopolysaccharides allowed survival over many years of dessication (Shirkey et al., 2000). The layer of exopolysaccharide would also likely affect the diffusion of nutrients into and waste products away from the cell. This may be especially important in antibiotic resistance of biofilms (Olson et al., 2002).

Evidence of the protective effect of exopolysaccharide is perhaps most clear in the case of pathogenic bacteria avoiding phagocytosis (Fernandez-Prada et al., 2003). This effect is likely a result of not only reduced opsonization of the microorganism, but also the masking of bacterial cell wall ligands that are recognized by the receptors of the phagocyte.

The mucoid properties of the exopolysaccharide capsules formed by many bacteria allow the adhesion and stabilization of biofilms and bacterial colonies. This manifests itself as an important step in the survival of bacteria in many scenarios including biofilm formation in periodontal disease (Kaplan et al., 2003), binding to host lectins in cystic fibrosis patients (von Bismarck et al., 2001), stabilization of the symbiosis of *Rhizobium* bacteria and legumes, and the fouling of pipelines (Fraysse et al., 2003).

COMMON METABOLIC PRECURSORS. The wide variety of exopolysaccharides requires an equally diverse pool of precursor molecules. The most basic of these compounds are energy-rich monosaccharides. Usually nucleoside diphosphate-modified monosaccharides serve this role, although nucleoside monophosphate sugars are utilized in some pathways (Steenbergen and Vimr, 2003). These monosaccharide-nucleoside diphosphates provide the basic assembly unit of the exopolysaccharide as well as the energy necessary for its synthesis. For the most part, the presence of a monosaccharide in an exopolysaccharide indicates the necessity of an analogous nucleoside diphosphate sugar. However, several interconversions of sugars can occur via mechanisms such as epimerization, oxidation, decarboxylation, reduction, and rearrangement.

Aside from the obvious need for activated monosaccharide components, exopolysaccharides often contain modified sugars or are decorated with several non-sugar components. O-methyl sugars have been described in a *Rhodococcus* strain (Neu et al., 1992), but the mechanism of synthesis and the identity of the precursors are not yet known. The chemical decoration of exopolysaccharide by various acyl groups requires activated forms of those groups to be generated by cellular metabolism. Acetyl CoA is required for the addition of acetyl groups in several systems (Leitao and Sa-Correia, 1993; Bossio et al., 1996), and it is likely that acyl CoA derivatives are required for more complicated acyl groups.

Most exopolysaccharides synthesized at the cell membrane are assumed to require a lipid acceptor for assembly. This is likely to be the bactoprenol involved in peptidoglycan and O-antigen synthesis, which has also been shown to be important in the synthesis of the capsular polysaccharide of group B streptococci (Rubens et al., 1993) and of serogroup B *Neisseria meningitidis* (Masson and Holbein, 1985). Phosphate groups may be added to the exopolysaccharide by a standard enzymatic phosphorylation reaction derived from ATP, but it is likely that the phosphate group is derived from the sugar nucleotide. The addition of sulfate groups and amino acids has been documented but not well characterized.

COMMON SYNTHETIC PATHWAYS. The most common pathway for exopolysaccharide synthesis occurs at the cell membrane or in the periplasm. A general scheme of exopolysaccharide synthesis is given in Fig. 1. The metabolic precursors discussed above are generated in the cytoplasm where they are likely used for other processes as well. The repeating sugar units of the exopolysaccharides are added to the growing polymer, likely linked to the membrane via undecaprenyl phosphate as a lipid carrier. Acyl modifications of the exopolysaccharide also likely happen at the cytoplasmic face of the membrane (or in the periplasm), prior to translocation of the polymer through the membrane. The mechanism of periplasmic synthesis is best

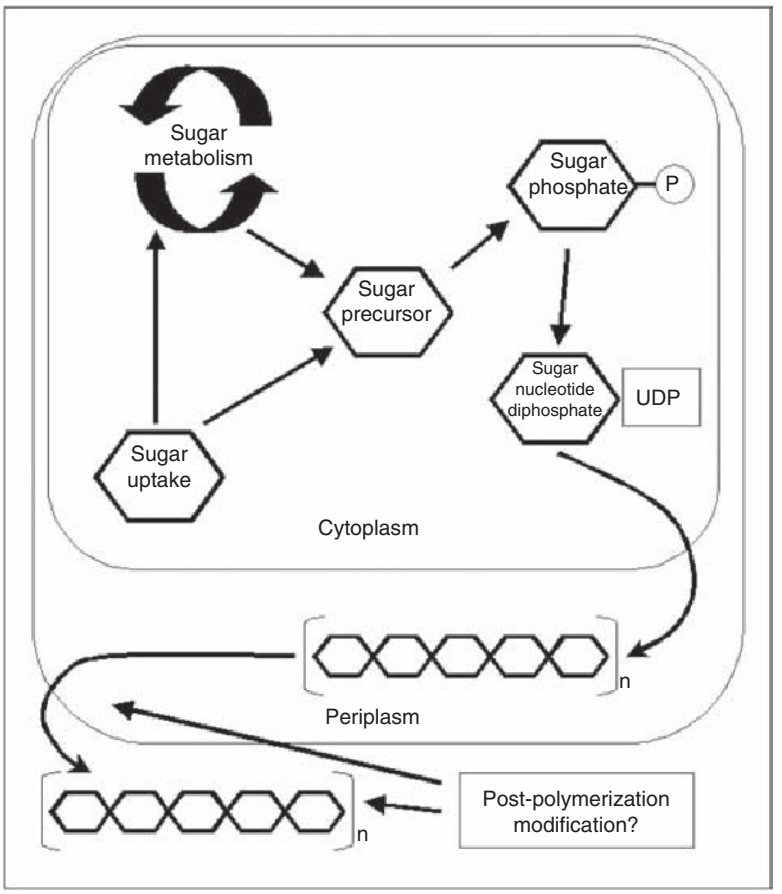

Fig. 1. General exopolysaccharide synthetic mechanism.

described for the *E. coli* K1 capsular polysaccharide (Bliss and Silver, 1997). While the exact mechanism of translocation of the exopolysaccharide is not entirely understood, several genes have been demonstrated to be required for the process (Vojnov et al., 1998; Drummelsmith, 2000; Smith, 2003).

In contrast to the complex of cytoplasmic and/or periplasmic synthesis of many exopolysaccharides, the extracellular synthesis of the levans and dextrans is relatively simple. The dextransucrase enzyme catalyzes the cleavage of the sucrose molecule, the energy source for the reaction, resulting in the addition of the glycosyl group to the growing dextran chain and the release of free fructose. Levansucrase similarly adds fructose to the growing chain of levan, releasing glucose from the sucrose substrate. Dextran synthesis has been demonstrated in nonhomologous cultures transformed with the dextransucrase gene (Neubauer et al., 2003). Levan has also been synthesized by recombinant organisms (Hettwer, 1998).

Bacterial alginate is unique among bacterial exopolysaccharides in that it is modified by a post-polymerization epimerization. Alginate is synthesized as a homopolymer of poly-D-mannuronic acid and subsequently modified by an extracellular epimerase, which converts a number of the mannose residues to L-guluronic acid. The epimerase has been identified in *Pseudomonas* (Gimmestad, 2003) and *Azotobacter* (Rehm et al., 1996).

REGULATION OF ENZYMATIC POLYMERIZATION. The first level of regulation of exopolysaccharide production is the chromosomal organization of the genes required for their synthesis. In most systems studied to date, the genes encoding the synthetic enzymes for a particular exopolysaccharide are contained in a single operon (Table 2). This organization into one or a few loci presumably allows activation of the entire synthetic machinery through one signal, as has been demonstrated in *Lactobacillus* (Lamothe et al., 2002). More complex genetic regulatory mechanisms have been described as well, including exopolysaccharide production dependent on quorum sensing in the alfalfa plant symbiont *Sinorhizobium meliloti* (Marketon et al., 2003) and cellulose production activated by cyclic diguanylic acid in *Acetobacter xylinum* (Weinhouse et al., 1997).

Table 2. Operon structure of synthetic enzymes responsible for exopolysaccharide production.

Species	Exopolysaccharide	No. of loci	Chromosomal or plasmid	Size of operon (kb)	Number of ORFs	Reference
Acetobacter xylinum	Cellulose	1	Chromosomal	9.5	4	Wong et al., 1990
Acetobacter xylinum ATCC 53582	Cellulose	1	Chromosomal	9.5	3	Saxena et al., 1994
Acinetobacter lwoffii RAG-1	Emulsan	1	Chromosomal	27	20	Nakar and Gutnick, 2001
Agrobacterium sp. ATCC 31749	Curdlan	2	Chromosomal	2.5 and Unk.	4	Stasinopoulos et al., 1999
Azotobacter vinelandii	Alginate	1	Chromosomal	~7	7	Vazquez et al., 1999
Rhizobium meliloti	Succinoglycan	1	Plasmid	16	8	Glucksmann et al., 1993
Rhizobium meliloti	EPS 139A	1	Plasmid	32	25	Becker et al., 1997
Streptococci, Group A	Hyaluronic acid	1	Chromosomal	~4	3	Dougherty and van de Rijn,1993
Streptomyces sp. 139	Galactose/glucuronic acid EPS	1	Plasmid	31.3	22	Wang et al., 2003
Xanthomonas campestris	Xanthan	1	Chromosomal	16	12	Katzen et al., 1996

Abbreviations: EPS, exopolysaccharide; and ORFs, open reading frames.

Precursor availability, whether it is the sugars or other necessary components, can clearly affect exopolysaccharide production (Mozzi et al., 2001). This is a complex area, as most of the metabolic precursors for exopolysaccharide production are also required for other cellular processes. This requires a great deal of study to fully understand how modification or disruption of a particular pathway might not only affect exopolysaccharide production, but also cellular metabolism and viability in general.

General Polysaccharide Structures

Microbial exopolysaccharides can most broadly be divided into the categories of hetero- and homopolysaccharides indicating the nature of the monomer repeat structure of the polymer chain. These polymers may be found attached to the outer suface of the cells or extruded into the extracellular space as soluble and insoluble materials. The nature and composition of branching affects many aspects of exopolymer behavior. Non-sugar components may also be present, usually in the form of amino acids and ester- and acyl-like organic acids.

While the universe of known microbial polysaccharide sugar residues is in excess of one hundred unique monomers (Kenne and Lindberg, 1983) and the number of branching structures unknown, in practice, most heteropolysaccharides of industrial interest tend to be regular structures with 2–8 repeating units composed of 2–4 distinct monomers (Sutherland, 2001). Most often, these monomers are the pyranose forms of the simple sugars D-glucose, D-galactose, and D-mannose with L-fucose and L-rhamnose also frequently present. Pentose sugars, while commonly found in eukaryotic organisms, are less frequently encountered in prokaryotic species (Sutherland, 1990). In contrast to the generally regular repeat unit structures, acyl substitutions in varying degrees are often observed in a variety of microbial poysaccharides. Decoration of chains may be regular or random, with acetate esters and pyruvate ketals most commonly observed. Alginates are the notable exception to these generalizations, having a random block repeat structure of D-mannuronic and L-guluronic acid residues in the main chain and being heavily acetylated on the D-mannuronic molecules (Sutherland, 2001).

The combination of different sugar monomers, their linkage, chain branching, and organic modification make for a wide variety of possible conformations. Each hexose can be α- or β-linked at one of the 2,3,4 or 6 positions to the proximal residue (Sutherland, 1990). Extensive compendia of polysaccharide structures are available (Kenne and Lindberg, 1983).

Common Analytic Techniques

Analysis of polysaccharide composition generally requires the reduction of the polymer to monomer via hydrolysis. Typically this involves the digestion of the polymer by acid hydrolysis; however, recombinantly prepared enzyme preparations are increasingly available (Mischnick, 1998). The resultant mixture of monomers may be analyzed by a variety of methods including thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC), with GLC methods usually necessitating additional derivatization to ensure adequate volatility (Pierce, 1968). Naturally, much information regarding primary structure and the *in vivo* modification of monomers can be lost in the process of hydrolysis, and care must be taken to not chemically alter the monomeric sugar resides themselves. Specific removal or replacement or both of anticipated side chain substituents may be completed as necessary by chemical or enzymatic treatment. Such side groups, like the polymers themselves, are routinely sized using gel permeation chromatography (GPC), light scattering, and viscometric analysis.The determination of primary structure for an unknown polysaccharide molecule is a difficult analytic task. Methanolysis followed by hydrolysis has been the primary method used to determine the locations of linkages and side chains (Chaplin, 1982). Periodate oxidation is also commonly employed to deduce information regarding glycosidic linkages, since analysis is conducted by spectrophotometric means. Enzymatic digestion by residue specific hydrolyases, with subsequent analysis of the size and identity of resulting fragments, or stepwise digests may be used as well. Determination of the glycosidic linkage conformation can be made by optical rotation. ^{13}C and cross polarization (c.p.)/magic angle spinning (m.a.s.) nuclear magnetic resonance (NMR) have also been employed to characterize both composition and structural features in native and modified polymers (Duus et al., 2000). Matrix-assisted laser desorption/ionization mass spectromety (MALDI) is also increasingly used in the direct analysis of carbohydrate content and linkages, particularly in the area of glycoprotein analysis (Harvey, 1999).

Solution Properties

Many microbial polysaccharides form thick hydrocolloidal suspensions and gels in solution. Such gels, generally the result of extended hydrophilic macromolecular networks, are the principal reason for industrial interest (Nishinari et al., 2000). These properties are modulated in microbial polysaccharides by various chemical modifi-

cations (e.g., acetylation often observed in alginate and xanthan and chain branching) and by solvated cations that assist in the coordination of interactions between chains and gelation of generally anionic carbohydrate chains (Sutherland, 1994). Not surprisingly, temperature and pH are also critical factors, owing to the influence exerted on charge and chain flexibility.

The conformation of associated polymer chains in solution is often indirectly measured in terms of rheological properties, as these are often among the most important physical parameters in most applications of microbial exopolysaccharides. Viscometry and dynamic light scattering may be utilized to estimate effective molecular weight and particle size by various models (i.e., Mark Houwink, Flory, etc.). Atomic force microscopy (AFM) is also often used to investigate the chain conformations and secondary structural features of samples in hydrated and dehydrated states. The use of predictive molecular modeling of solution properties based on putative structure for small oligosaccharides in conjunction with NMR and x-ray data for more complex and flexible polysaccharides has also been reported (Bush et al., 1999; Brisson and Jennings, 2001; Burchard, 2001; Faber et al., 2002).

Solid State Properties

As most exopolysaccharides of industrial interest are utilized in water-based systems, the analysis of dried solids is less often reported in the literature. However, since eukaryotic structural carbohydrates such as cellulose, chitin, and chitosan have been extensively characterized, microbially derived materials have become studied by many of the same techniques (Bohn et al., 2000). Solid state structural analysis is most often performed using wide and small angle x-ray scattering (WAXS and SAXS), Fourier transform infrared spectroscopy (FTIR), scanning probe microscopy (SPM), scanning electron microscopy (SEM), and atomic force microscopy (AFM; Brant, 1999). Crystallinity and self-assembly of monomeric constituents into highly organized liquid crystalline phases and then often insoluble tertiary structures are of particular interest, since such behavior should further a predictive understanding of structural and biochemical influences on bulk material properties and give a means for the control of these behaviors.

Commercial Applications

Homopolysaccharides

DEXTRANS. A class of homopolymers primarily composed of α-1,4 linked D-glucopyranosyl repeating units, dextrans are produced by several species of bacteria. In some species, 1-2, 1-3, and 1-4 linkages as well as significant percentages of side chain branching are also observed. Polymers may be comprised of one type of linkage or of several of those listed. These linkages result in a family of polymers with varying solution properties of generally high molecular weight and neutral charge. Industrial production occurs mainly using *Leuconstoc mesenteroides*, which produces dextrans (relative molecular weight, 4–5 × 10^7 Da) extracellularly via the enzymatic polymerization of sucrose by dextransucrase with approximately 95% 1-6 and 5% 1-3 linkages (Sutherland, 1990). Polymerization is known to occur in one-residue increments, although the exact mechanism of chain elongation remains unclear.

The initial application of microbial dextran was as a blood plasma extender. Since then, a variety of dextrans and post-processed materials have been developed. Sephadex, a commercial separations medium marketed by Amersham Biosciences, utilizes a crosslinked form of this polymer. Dextran sulfate is also sometimes employed as an anti-coagulant and iron transporter (Monsan et al., 2001). Crosslinked dextran gels have been investigated as a biodegradable material for the controlled release of pharmaceuticals (Gumagalieva et al., 1998; Cascone et al., 2001). Dextran conjugates may also be useful in the modulation of the pharmacokinetics and targeting of some injectable pharmaceutical therapies (Mehvar, 2000).

Mutan and alternan are two additional forms of carbohydrate via two additional glucansucrase enzymes produced by some strains of *L. mesenteroides*. Both are insoluble polymers mainly comprised of 1-3 and 1-6 linkages, and while mutan has been identified as a component of bacterial adhesion in dental plaque, no commercial applications of either currently exist (Monsan et al., 2001).

CURDLAN. A β-1,3 linked D-glucopyranosyl homopolymer, curdlan is produced by a number of bacterial species; however, production is generally from *Alcaligenes faecalis*. Curdlan tends to be a rather simple homopolymer of low molecular weight (<100 kDa) and low water solubility which gels above 55°C, forms a firm resilient gel at 100°C, and is a thermally irreversible gel above 120°C owing, in part, to triple helical rearrangement of the polymer (Nishinari et al., 2000; Zhang et al., 2002). Acetyl derivatives of curdlan have been patented for separations applications, and sulfate derivatives have been tested for antiviral and anticoagulant applications (Nakashima et al., 1987; Hatanaka et al., 1991; Sutherland, 1998). Curdlan is also used as a food additive (i.e., coating and texturizer) in Japan.

CELLULOSE. A linear homopolymer of β-1,4 linked D-glucose, cellulose is made in abundance in plants and in several genera of bacteria including *Acetobacter*, *Achromobacter*, *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Pseudomonas*, *Rhizobium*, *Sarcina* and *Zoogloea*. The principal microbial source of this polymer is the organism *Gluconacetobacter xylinum*, which produces copious amounts of polymer in static culture on a variety of carbon sources. The polymerization of glucose proceeds as cellobiosyl subunits in a membrane associated process. Bacterial cellulose is extruded from the cell as water insoluble fibrils that rapidly assemble into highly structured helical fibers. Mats of these fibers, which form at the gas liquid interface of cultures, are exceptionally hydrophilic and also have higher surface area and tensile strength than plant-derived cellulose has (Yamanaka et al., 1989).

Bacterial cellulose has found medical application as a wound healing skin substitute and dental anti-adhesion material and industrial use in acoustic diaphragms and as inert filler, paper additive, thickener, and insulator material. In addition, bacterial cellulose is the basis of the food Nata de Coco, which enjoys some popularity in Asia (Jonas and Farah, 1998; Vandamme et al., 1998). Chemically modified bacterial cellulose derivatives have been investigated, but the cost differential between bacterial and plant derived polymer is currently prohibitive to wide commercial application.

MISCELLANEOUS: LEVAN AND POLYGALACTOSAMINE. A large number of novel polysaccharide components of exopolysaccharide matrix from a variety of organisms has been identified and studied. Only a few other homopolymers have received industrial attention.

Levan is a neutral β-2,6 linked D-fructofuranosyl homopolymer made extracellularly by a variety of bacterial species through the action of levansucrase on sucrose. It has no established commercial application, but investigations into its production have been conducted and recombinant levansucrase has been successfully demonstrated (Belghith et al., 1996; Song et al., 1996).

Polygalactosamine is a cationic α-1,4 linked polygalactosamine homopolymer. It is generally water insoluble and is sometimes acetylated. Its cationic nature has led to investigations of its suitability as a potential adjuvant coating for targeted DNA uptake in gene therapy and as a conjugate for targeted drug delivery (Ouchi et al., 1994; Sato et al., 1996).

Heteropolysaccharides

XANTHAN. An anionic heteropolymer based on the β-1,4-D-glucopyanosyl backbone of cellulose, xanthan has a five-glucose repeat unit and every other residue contains a regular side chain of two D-mannose and D-glucuronic acid residues, with the glucuronic acid residue in the center of the side chain. The terminal mannose sugars are modified with pyruvic ketal groups at a frequency of approximately 50% and the mannose residue nearest the main chain is acetylated at C-6 with similar regularity. Typical molecular weights are between 10^6 and 10^7 Da (Garcia-Ochoa et al., 2000). Xanthan is synthesized by *Xanthamonas campestris* in stirred fermentations on a variety of carbon sources and using a number of mutants and cultivation techniques which have a significant effect on the side chain density, degree of pyruvation and acetylation and, thus, on the solution properties of the gel.

Xanthan is arguably the most important of the microbial polysaccharides from an industrial perspective, perhaps because it was among the first commercialized, but also because of its wide cost-effective versatility as a viscosifier and texturizer in food and health care applications and consequently its large market. Although developed at the United States Department of Agriculture (USDA) in the 1950s, xanthan continues to be extensively developed and marketed by Kelco. In addition, a notably broad academic literature on production optimization, metabolic engineering, and reactor modeling exists for xanthan fermentations. In fact, many of the principles developed for xanthan processes have been applied to many exopolysaccharide and other fermented products. Xanthan is approved for use as a food additive by the FDA, having obtained generally recognized as safe (GRAS) status, and is used in industrial processes such as ceramic glaze viscosification and applications such as emulsifier in enhanced petroleum recovery (Garcia-Ochoa et al., 2000).

GELLAN, WELAN AND RHAMSAN. A linear polymer of tetrasaccharide repeats, gellan is produced from *Sphingomonas paucimobilis*. The linear polymer of approximately 500 kDa has O-acetyl and glyceryl substituents. The degree of acylation affects the physical properties of gels formed from this exopolysaccharide. Gellan forms thermoreversible gels and has been approved for use in food as a gelling agent. Welan and rhamsan are related polymers with differing side chains.

ALGINATE. Synthesized initially as a polymannuronic acid homopolymer, bacterial alginate is converted by an extracellular epimerase to a heteropolymer of D-mannuronic acid and L-guluronic acid. Alginates currently used in commercial applications are derived from algal sources. However, the potential for significant control over polymer production in a bacterial system has led to significant research of alginate

genetics and production in *Azotobacter* (Rehm et al., 1996; Vazquez et al., 1999) and *Pseudomonas* (Leitao et al., 1993; Gimmestad et al., 2003). While the alginate from *Pseudomonas aeruginosa* has been of interest owing to its association with infection in cystic fibrosis patients, the *Azotobacter* alginate has more potential commercially because of its relatively stable output. Alginate has been studied for a wide array of potential applications including cell encapsulation, drug delivery, wound healing, and tissue engineering.

HYALURONIC ACID. Bacterial hyaluronic acid is apparently identical to that produced by eukaryotic cells. It is composed of 1,4-β-linked disaccharides of D-glucuronosyl-1,3-β-*N*-acetyl-D-glucosamine. Bacterial hyaluronic acid has been commercialized for several applications including cosmetics and a coating for prostheses in veterinary medicine. Future applications may include tissue engineering and drug delivery.

SUCCINOGLYCAN. An anionic polysaccharide, succinoglycan is composed of an octasaccharide repeat unit with three acyl groups (acetate, succinate esters, and pyruvate ketals). The exopolysaccharide produced by *Rhizobium* species contains a 7 : 1 molar ratio of D-glucose to D-galactose and a stoichiometric amount of the pyruvate, which gives it its anionic properties. The succinoglycan produced in *Rhizobium* species is vital for its relationship with its plant symbiont.

EMULSAN. An anionic exopolysaccharide, emulsan is produced from a variety of hydrocarbon sources including fatty acids and ethanol by the Gram-negative bacterium, *A. calcoaceticus* strain RAG-1. Emulsan forms an extracellular cell-associated capsule, which is subsequently released into the fermentation broth. The polysaccharide main chain contains three aminosugars, D-galactosamine, D-galactosaminouronic acid, and a dideoxydiamino hexose, in the ratio 1 : 1 : 1 (Belsky et al., 1979; Gorkovenko et al., 1997). The polymer has O-acyl and N-acyl bound side chain fatty acids ranging in chain length from C10 to C22. These fatty acid substituents constitute 5–23% (wt/wt) of the polymer (Gorkovenko et al., 1997). The emulsan amino groups are either acetylated or covalently linked by an amide bond to 3-hydroxybutyric acid. The combination of the hydrophilic anionic sugar main chain repeat units along with the hydrophobic side groups leads to the amphipathic behavior of emulsan and, therefore, its ability to form stable oil-in-water emulsions. The polymer has been used successfully in environmental cleanup and degradation studies to disperse polluting oils because of its amphipathic structure (Gutnick and Rosenberg, 1977). Emulsions formed with emulsan have been shown to be stable for months and can be broken when desired by enzymatic degradation or at elevated temperatures (Shoham and Rosenberg, 1983; Gutnick and Shabtai, 1987a; Gutnick and Shabtai, 1987b). Aside from its potential industrial applications, more recently its potential use in biomedical applications has been the focus of research (Panilaitis et al., 2002).

Areas of Research and Development

Research and development in the arena of microbial polysaccharide production proceeds in two major categories. The first is the ongoing optimization of cultivation conditions for the efficient production of polymers that are often quite expensive in comparison to natural fibers and synthetic thermopolymers. While traditional fermentation optimization literature predominates, recombinant enzyme and cell free systems are also areas of focus. The second area of work is the screening and identification of novel polysaccharides and, in the search for additional polymers of interest, the genetic modification of coenzymes. Predictability and control in this effort are slowed by a limited understanding of structure-function relationships, because interactions among systems of polymers with varying degrees of polymerization and modification are complex. Structure-function aspects and solution dynamics of polymers in general are widely studied and this literature provides some roadmaps for the biotechnologist.

Fermentation Methods and Issues

The recovery of microbial polysaccharides from fermentation broth is a highly variable process dependent on chain length, surface charge, and end-use purity specification of the polymeric product. Typically, cellular materials are removed by centifugation or filtration. Polymer is then purified by a variety of means including ion exchange chromatography (IEC) and alcohol precipitation.

Genetic Modification and Customization

After years of optimizing culture conditions and selecting for high-level producers, the natural limits of polymer production are often reached. With the significant advances in molecular biology, the focus now turns to modifying the important synthetic and regulatory pathways of exopolysaccharide synthesis at the genetic level. The method of mutant generation is commonly transposon insertion, although more traditional

methods are also utilized. In some specific cases, the expression level of specific enzymes has been altered to enhance the levels of necessary metabolic precursors (Levander et al., 2002; Boels et al., 2003). Modifications have targeted not only the yield of exopolysaccharides, but also specific structural modification. Emulsan structural analogs have been produced from transposon-mutagenized *Acinetobacter calcoaceticus* RAG-1 that vary not only in yield, but also in the modification of the fatty acid acyl chains that decorate the sugar backbone (Johri et al., 2002). It is likely that future improvements in exopolysaccharide yield and functionality will be due to such specific genetic modifications rather than gross mutagenesis.

Prospects

Important future advancements in polysaccharide yields and industrialization will build upon the foundation of genetics touched on above as well as on more rapid and specific screening approaches to identify novel structures suited for selective functional attributes. Perhaps in the long term, an ultimate aim will be template-based synthesis of polysaccharides to explore the limits of the relationship between chain length and distribution and function. Additionally, novel approaches employing traditional polysaccharides that are chemically or enzymatically functionalized in vitro will also become interesting directions in the field. In all of the above scenarios, industrial production will depend on novelty of function coupled with costs of production. In the broadest perspective, the exploitation of polysaccharides to fulfill a wide range of materials science and engineering needs has lagged in comparison to that of proteins. With rapid advancements in genetics, analytical tools, and screening methods, this gap should begin to close and new avenues in the field should continue to expand.

Literature Cited

Becker A., S. Ruberg, H. Kuster, A. A. Roxlau, M. Keller, T. Ivashina, H. P. Cheng, G. C. Walker, and A. Puhler. 1997. The 32-kilobase exp gene cluster of Rhizobium meliloti directing the biosynthesis of galactoglucan: genetic organization and properties of the encoded gene products. J. Bacteriol. 179(4):1375–1384.

Belghith, H., K. B. Song, C. H. Kim, S. K. Rhee. 1996. Optimal conditions for levan formation by an overexpressed recombinant levansucrase. Biotechnol. Lett. 18(4):467–472.

Belsky, I., D. L. Gutnick, and E. Rosenberg. 1979. Emulsifier of Arthrobacter RAG-1: Determination of emulsifier-bound fatty acids. FEBS Lett. 101:175–178.

Bliss, J. M., R. P. Silver. 1997. Evidence that KpsT, the ATP-binding component of an ATP-binding cassette transporter, is exposed to the periplasm and associates with polymer during translocation of the polysialic acid capsule of Escherichia coli K1. J. Bacteriol. 179(4):1400–1403.

Boels I. C., M. Kleerebezem, W. M. de Vos. 2003. Engineering of carbon distribution between glycolysis and sugar nucleotide biosynthesis in Lactococcus lactis. Appl. Environ. Microbiol. 69(2):1129–1135.

Bohn, A., H-P. Fink, J. Ganster, M. Pinnow. 2000. X-ray texture investigations of bacterial cellulose. Macromolec. Chem. Phys. 201(15):1913–1921.

Bossio, J. C., C. E. Semino, N. Inon de Iannino, M. A. Dankert. 1996. The in vitro biosynthesis of the exopolysaccharide produced by Rhizobium leguminosarum bv. trifolii, strain NA 30. Cell. Molec. Biol. 42(5):737–758.

Brant, D. A. 1999. Novel approaches to the analysis of polysaccharide structures. Curr. Opin. Struct. Biol. 9(5):556–562.

Brisson, J. R., and H. J. Jennings. 2001. NMR and molecular modeling of complex carbohydrates and carbohydrate-protein interactions: Applications to anti-bacteria vaccines. Molec. Immunol. Complex Carbohydr. 2 491:543–550.

Burchard, W. 2001. Structure formation by polysaccharides in concentrated solution. Biomacromolecules 2(2):342–353.

Bush, C. A., M. Martin-Pastor, A. Imberty. 1999. Structure and conformation of complex carbohydrates of glycoproteins, glycolipids, and bacterial polysaccharides. Ann. Rev. Biophys. Biomolec. Struct. 28:269–293.

Cascone, M. G., N. Barbani, C. Cristallini, P. Giusti, G. Ciardelli, L. Lazzeri. 2001. Bioartificial polymeric materials based on polysaccharides. J. Biomater. Sci. Polymer Ed. 12(3):267–281.

Chaplin, M. F. 1982. A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. Analyt. Biochem. 123(2):336–341.

Dougherty, B. A., and I. van de Rijn. 1993. Molecular characterization of hasB from an operon required for hyaluronic acid synthesis in group A streptococci: Demonstration of UDP-glucose dehydrogenase activity. J. Biol. Chem. 268(10):7118–7124.

Drummelsmith, J. W. C. 2000. Translocation of group 1 capsular polysaccharide to the surface of Escherichia coli requires a multimeric complex in the outer membrane. EMBO J. 19(1):57–66.

Duus, J. O., C. H. Gotfredsen, K. Bock. 2000. Carbohydrate structural determination by NMR spectroscopy: Modern methods and limitations. Chem. Rev. 100(12):4589–4614.

Faber, E. J., J. A. van Kuik, J. P. Kamerling, J.F. Vliegenthart. 2002. Modeling of the structure in aqueous solution of the exopolysaccharide produced by Lactobacillus helveticus 766. Biopolymers 63(1):66–76.

Fernandez-Prada, C. M., E. Zelazowska, M. Nikolich, T. L. Hadfield, R. M. Roop 2nd, G. L. Robertson, D. L. Hoover. 2003. Interactions between Brucella melitensis and human phagocytes: Bacterial surface O-polysaccharide inhibits phagocytosis, bacterial killing, and subsequent host cell apoptosis. Infect. Immun. 71(4):2110–2119.

Fraysse, N., F. Couderc, V. Poinsot. 2003. Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. Eur. J. Biochem. 270(7):1365–1380.

García-Ochoa, F., V. E. Santos, J. A. Casas, and E. Gómez. 2000. Xanthan gum: Production, recovery, and properties. Biotechnol. Adv. 18(7):549–579.

Giavasis, I., L. M. Harvey, B. McNeil. 2000. Gellan gum. Crit. Rev. Biotechnol. 20(3):177–211.

Gimmestad, M., H. Sletta, H. Ertesvag, K. Bakkevig, S. Jain, S. J. Suh, G. Skjak-Braek, T. E. Ellingsen, D. E. Ohman, S. Valla. 2003. The Pseudomonas fluorescens AlgG protein, but not its mannuronan c-5-epimerase activity, is needed for alginate polymer formation. J. Bacteriol. 185(12):3515–3523.

Glucksmann, M. A., T. L. Reuber, and G. C. Walker. 1993. Genes needed for the modification, polymerization, export, and processing of succinoglycan by Rhizobium meliloti: A model for succinoglycan biosynthesis. J. Bacteriol. 175(21):7045–7055.

Gorkovenko, A., J. Zhang, R. A. Gross, A. L. Allen, and D. L. Kaplan. 1997. Bioengineering of emulsifier structure: Emulsan analogs. Can. J. Microbiol. 43:384–390.

Gumargalieva, K. Z., O. V. Shipunova, G. E. Zaikov, B. A. Jubanov, and S. A. Moshkevitch. 1998. About biodegradation of polymer compounds based on crosslinked dextrans. Polymer. Med. Nova Science Publishing. Commack, NY. 111–125.

Gutnick, D. L., and E. Rosenberg. 1977. Oil tankers and pollution: A microbiological approach. Ann. Rev. Microbiol. 31:379–396.

Gutnick, D. L., and Y. Shabtai. 1987a. Exopolysaccharide bioemulsifiers in biosurfactants and biotechnology. *In:* N. Kosaric, W. L. Cairns, and N. C. C. Gray (Eds.) Exopolysaccharide Bioemulsifiers. Marcel Dekker. New York, NY. 25:211–246.

Gutnick, D. L., and Y. Shabtai. 1987b. Exopolysaccharide bioemulsifiers in biosurfactants and biotechnology. *In:* N. Kosaric, W. L. Cairns, and N. C. C. Gray (Eds.) Exopolysaccharide Bioemulsifiers. Marcel Dekker. New York, NY. 25:232.

Harvey, D. J. 1999. Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates. Mass Spect. Rev. 18(6):349–450.

Hatanaka, K., I. Nakajima, T. Yoshida, T. Uryu, O. Yoshida, N. Yamamoto, T. Mimura, and Y. Kaneko. 1991. Effect of degree of sulfation on anti-HIV activity of synthetic (1–>5)-alpha-D-ribofuranan sulfate. J. Carbohydr. Chem. 10(4):681–690.

Hettwer, U., F. Jaeckel, J. Boch, M. Meyer, K. Rudolph, M. S. Ullrich. 1998. Cloning, nucleotide sequence, and expression in Escherichia coli of levansucrase genes from the plant pathogens Pseudomonas syringae pv. glycinea and P. syringae pv. phaseolicola. Appl. Environ. Microbiol. 64(9):3180–3187.

Johri, A. K., W. Blank, D. Kaplan. 2002. Bioengineered emulsans from Acinetobacter calcoaceticus RAG-1 transposon mutants. Appl. Microbiol. Biotechnol. 59(2–3):217–223.

Jonas, R., and L. F. Farah. 1998. Production and application of microbial cellulose. Polym. Degrad. Stabil. 59(1–3): 101–106.

Kaplan, J. B., M. F. Meyenhofer, D. H. Fine. 2003. Biofilm growth and detachment of Actinobacillus actinomycetemcomitans. J. Bacteriol. 185(4):1399–1404.

Katzen, F., A. Becker, A. Zorreguieta, A. Puhler, and L. Ielpi. 1996. Promoter analysis of the Xanthomonas campestris pv. campestris gum operon directing biosynthesis of the xanthan polysaccharide. J. Bacteriol. 178(14):4313–4318.

Kenne, L., and B. Lindberg. 1983. Bacterial Polysaccharides. *In:* G. Aspinall (Ed.) The Polysaccharides. Academic Press. Orlando, FL. 2:287–363.

Lamothe, G. T., L. Jolly, B. Mollet, F. Stingele. 2002. Genetic and biochemical characterization of exopolysaccharide biosynthesis by Lactobacillus delbrueckii subsp. bulgaricus. Arch. Microbiol. 178(3):218–228.

Leitao, J. H., I. Sa-Correia. 1993. Manipulation of Pseudomonas aeruginosa alginate pathway by varying the level of biosynthetic enzymes and growth temperature. J. Appl. Bacteriol. 74(4):454–459.

Levander, F., M. Svensson, P. Radstrom. 2002. Enhanced exopolysaccharide production by metabolic engineering of Streptococcus thermophilus. Appl. Environ. Microbiol. 68(2):784–790.

Magnani, D. 1998. Study RC-186R. *In:* Polysaccharides. Business Communications Company. Norwalk, CT.

Marketon, M. M., S. Glenn, A. Eberhard, J. E. Gonzalez. 2003. Quorum sensing controls exopolysaccharide production in Sinorhizobium meliloti. J. Bacteriol. 185(1):325–331.

Masson, L., B. Holbein. 1985. Role of lipid intermediate(s) in the synthesis of serogroup B Neisseria meningitidis capsular polysaccharide. J. Bacteriol. 161(3):861–867.

Mehvar, R. 2000. Dextrans for targeted and sustained delivery of therapeutic and imaging agents. J. Control. Rel. 69(1):1–25.

Mischnick, P. 1998. Determination of the substituent distribution of O-(2-hydroxypropyl)inulin by methylation analysis. Starch/Staerke 50(1):33–36.

Monsan, P., S. Bozonnet, C. Albenne, G. Joucla, R. Willemot, and M. Remaud-Siméon. 2001. Homopolysaccharides from lactic acid bacteria. Int. Dairy J. 11(9):675–685.

Mozzi, F., G. Rollan, G. S. de Giori, G. Font de Valdez. 2001. Effect of galactose and glucose on the exopolysaccharide production and the activities of biosynthetic enzymes in Lactobacillus casei CRL 87. J. Appl. Microbiol. 91(1):160–167.

Nakar, D., and D. L. Gutnick. 2001. Analysis of the wee gene cluster responsible for the biosynthesis of the polymeric bioemulsifier from the oil-degrading strain Acinetobacter lwoffii RAG-1. Microbiology 147(Pt. 7):1937–1946.

Nakashima, H., O. Yoshida, T. S. Tochikura, T. Yoshida, T. Mimura, Y. Kido, Y. Motoki, Y. Kaneko, T. Uryu, N. Yamamoto. 1987. Sulfation of polysaccharides generates potent and selective inhibitors of Human Immunodeficiency Virus: Infection and replication in vitro. Jpn. J. Cancer Res. 78(11):1164–1168.

Neu, T. R., T. Dengler, B. Jann, K. Poralla. 1992. Structural studies of an emulsion-stabilizing exopolysaccharide produced by an adhesive, hydrophobic Rhodococcus strain. J. Gen. Microbiol. 138 (Pt. 12):2531–2537.

Neubauer, H., A. Bauche, B. Mollet. 2003. Molecular characterization and expression analysis of the dextransucrase DsrD of Leuconostoc mesenteroides Lcc4 in homologous and heterologous Lactococcus lactis cultures. Microbiology 149 (Pt. 4):973–982.

Nishinari, K., H. Zhang, S. Ikeda. 2000. Hydrocolloid gels of polysaccharides and proteins. Curr. Opin. Colloid Interf. Sci. 5(3–4):195–201.

Olson, M. E., H. Ceri, D. W. Morck, A. G. Buret, R. R. Read. 2002. Biofilm bacteria: Formation and comparative susceptibility to antibiotics. Can. J. Vet. Res. 66(2):86–92.

Ouchi, T., K. Inosaka et al. 1994. Use of alpha-1,4-polygalactosamine as a carrier of macromolecular prodrug of 5-fluorouracil. Polymer. Drugs Drug Admin. 545:204–213.

Panilaitis, B., A. Johri, W. Blank, D. Kaplan, J. Fuhrman. 2002. Adjuvant activity of emulsan, a secreted lipopolysaccharide from Acinetobacter calcoaceticus. Clin. Diagn. Lab. Immunol. 9(6):1240–1247.

Pierce, A. E. 1968. Silylation of Organic Compounds: A Technique for Gas-phase Analysis. Pierce Chemical Company. Rockford, IL.

Rehm, B. H., H. Ertesvag, S. Valla. 1996. A new Azotobacter vinelandii mannuronan C-5-epimerase gene (algG) is part of an alg gene cluster physically organized in a manner similar to that in Pseudomonas aeruginosa. J. Bacteriol. 178(20):5884–5889.

Rubens, C. E., L. Heggen, R. F. Haft, M. R. Wessels. 1993. Identification of cpsD, a gene essential for type III capsule expression in group B streptococci. Molec. Microbiol. 8(5):843–855.

Sato, T., N. Shirakawa et al. 1996. Formation of a DNA/polygalactosamine complex and its interaction with cells. Chem. Lett. 9:725–726.

Saxena, I. M., K. Kudlicka, K. Okuda, and R. M. Brown Jr. 1994. Characterization of genes in the cellulose-synthesizing operon (acs operon) of Acetobacter xylinum: Implications for cellulose crystallization. J. Bacteriol. 176(18):5735–5752.

Schnider-Keel, U., K. B. Lejbolle, E. Baehler, D. Haas, C. Keel. 2001. The sigma factor AlgU (AlgT) controls exopolysaccharide production and tolerance towards desiccation and osmotic stress in the biocontrol agent Pseudomonas fluorescens CHA0. Appl. Environ. Microbiol. 67(12):5683–5693.

Shirkey, B., D. Kovarcik, D. J. Wright, G. Wilmoth, T. F. Prickett, R. F. Helm, E. M. Gregory, M. Potts. 2000. Active Fe-containing superoxide dismutase and abundant sodF mRNA in Nostoc commune (Cyanobacteria) after years of desiccation. J. Bacteriol. 182(1):189–197.

Shoham, Y., and E. Rosenberg. 1983. Enzymatic depolymerization of emulsan. J. Bacteriol. 156:161–167.

Smith, C. S., A. Hinz, D. Bodenmiller, D. E. Larson, Y. V. Brun. 2003. Identification of genes required for synthesis of the adhesive holdfast in Caulobacter crescentus. J. Bacteriol. 185(4):1432–1442.

Song, K. B., H. Belghith, S. K. Rhee. 1996. Production of levan, a fructose polymer, using an overexpressed recombinant levansucrase. Enz. Engin. XIII 799:601–607.

Stasinopoulos, S. J., P. R. Fisher, B. A. Stone, and V. A. Stanisich. 1999. Detection of two loci involved in (1–>3)-[beta]-glucan (curdlan) biosynthesis by Agrobacterium sp. ATCC31749, and comparative sequence analysis of the putative curdlan synthase gene. Glycobiology 9(1):31–41.

Steenbergen, S. M., E. R. Vimr. 2003. functional relationships of the sialyltransferases involved in expression of the polysialic acid capsules of Escherichia coli K1 and K92 and Neisseria meningitidis Groups B or C. J. Biol. Chem. 278(17):15349–15359.

Sutherland, I. W. 1990. Biotechnology of Microbial Exopolysaccharides. Cambridge University Press. Cambridge, UK.

Sutherland, I. W. 1994. Structure-function-relationships in microbial exopolysaccharides. Biotechnol. Adv. 12(2):393–448.

Sutherland, I. W. 1998. Novel and established applications of microbial polysaccharides. Trends Biotechnol. 16(1):41–46.

Sutherland, I. W. 2001. Exopolysaccharides in biofilms, flocs and related structures. Water Sci. Technol. 43(6):77–86.

Vandamme, E. J., S. De Baets, A. Vanbaelen, K. Joris, P. De Wulf. 1998. Improved production of bacterial cellulose and its application potential. Polym Degrad. Stabil. 59(1–3):93–99.

Vazquez, A., S. Moreno, J. Guzman, A. Alvarado, and G. Espin. 1999. Transcriptional organization of the Azotobacter vinelandii algGXLVIFA genes: Characterization of algF mutants. Gene 232(2):217–222.

Vojnov, A. A., A. Zorreguieta, J. M. Dow, M. J. Daniels, M. A. Dankert. 1998. Evidence for a role for the gumB and gumC gene products in the formation of xanthan from its pentasaccharide repeating unit by Xanthomonas campestris. Microbiology 144(Pt. 6):1487–1493.

von Bismarck, P., R. Schneppenheim, U. Schumacher. 2001. Successful treatment of Pseudomonas aeruginosa respiratory tract infection with a sugar solution: A case report on a lectin based therapeutic principle. Klin. Pädiatr. 213(5):285–287.

Wang, L., S. Li, and Y. Li. 2003. Identification and characterization of a new exopolysaccharide biosynthesis gene cluster from Streptomyces. FEMS Microbiol. Lett. 220(1):21–27.

Weinhouse, H., S. Sapir, D. Amikam, Y. Shilo, G. Volman, P. Ohana, M. Benziman. 1997. c-di-GMP-binding protein, a new factor regulating cellulose synthesis in Acetobacter xylinum. FEBS Lett. 416(20):207–211.

Whitfield, C. 1988. Bacterial Extracellular Polysaccharides. Can. J. Microbiol. 34(4):415–420.

Wong, H. C., A. L. Fear, R. D. Calhoon, G. H. Eichinger, R. Mayer, D. Amikan, M. Benziman, D. H. Gelfand, J. H. Meade, A. W. Emerick, R. Bruner, A. Ben-Bassat, and R. Tal. 1990. Genetic organization of the cellulose synthase operon in Acetobacter xylinum. Proc. Natl. Acad. Sci. USA 87(20):8130–8134.

Yamanaka, S., K. Watanabe, N. Kitamura, M. Iguchi, S. Mitsuhashi, Y. Nishi, M. Uryu. 1989. The Structure and Mechanical-Properties of Sheets Prepared from Bacterial Cellulose. J. Mat. Sci. 24(9):3141–3145.

Zhang, J., R. Wang, P. Jiang, Z. Liu. 2002. Production of an exopolysaccharide bioflocculant by Sorangium cellulosum. Lett. Appl. Microbiol. 34(3):178–181.

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CHAPTER 3.4

Bacterial Enzymes

WIM J. QUAX

Introduction

In the living world, each chemical reaction is catalyzed by its own enzyme. Enzymes exhibit a high specificity, as they are able to discriminate between slightly different substrate molecules. Furthermore, they have the ability to operate at moderate temperature, pressure and pH, which makes them attractive catalysts for industrial and household conversion processes. The first reports on the industrial use of enzyme products go back to the beginning of the last century. It was the German scientist Röhm who introduced the use of bovine pancreas extracts for the removal of stains in dirty clothing (Röhm, 1915; Fig. 1). Around the same time, the Laboratoire Amylo in France experimented with the use of extract from *Bacillus* for conversion of starch into sugars (Fig. 2). As a result, the company Rapidase (Seclin, France), which is now part of the life science division of DSM, was formed. With the development of microbial fermentations in the second half of the last century, the number of industrial processes performed by enzymes and the amount of enzymes produced have increased sharply. At present, a renewed worldwide research effort has been directed to identifying more sustainable and environmentally friendly biocatalytic processes. The availability of highly specific and cheap enzymes resulting from genetic and protein engineering has been very instrumental in reviving interest in the industrial application of enzymes.

Most classically used industrial enzymes are hydrolytic (proteolytic, amylolytic, or lipolytic). Hydrolytic enzymes hardly require any cofactors, which allows their application in a great variety of conditions. These enzymes are usually separated from the cell broth after fermentation and formulated in more or less high concentrations. Recently, more specialized bioconversions have been developed in which enzymatic activity is maintained only by special cofactors that must be regenerated or, even worse, by living cells. In this chapter, the emphasis will be on bacterial enzymes that can be used in isolated form.

The organization of this chapter has focused on application. Owing to the versatility and stability of hydrolytic enzymes, the same enzyme may be used in totally different parts of industrial processes. Table 1 summarizes the current use of enzymes in various industry and household applications. The data are compiled from information provided by enzyme producers, customers, and industry organizations and from information acquired as a result of my involvement with industrial enzyme production for many years. As it relates to products of commercial importance, access to the data is not always possible, and relating the biochemical and genetic characteristics of production strains, which are usually proprietary, to better described strains in the literature is sometimes difficult. Nevertheless, this manuscript should provide an overview of the importance of bacterial enzymes for sustainable and efficient conversion in industrial processes. Of the $1.8 billion annual world sales of industrial enzymes, about 50% are sales of bacterial enzymes and most of the remaining 50% are sales of fungal enzymes.

Scientific Background: the Source of Enzymes

Historically, the selection of microorganisms that produce enzymes has been empirical, starting with samples from very diverse natural sources. Cultures enriched by growth on substrates were used to inoculate fermentations. In a later stage, pure bacterial strains were selected. Intellectual property protections associated with bioprocesses have hampered the taxonomical characterization of industrially used strains. For the same reason, it is not always possible to trace the origin and history of currently used organisms. Once it became possible to protect man-made bacterial strains by a patent (Chakrabarty, 1981), the taxonomy of the bacterial strains became a key element in the development of industrial enzymes. Ever since, the 16S rRNA sequence has been routinely determined for every bacterial

KAISERLICHES PATENTAMT.

AUSGEGEBEN DEN 4. MAI 1915.

PATENTSCHRIFT

— № 283923 —

KLASSE 8i. GRUPPE 5.

DR. OTTO RÖHM IN DARMSTADT.

Verfahren zum Reinigen von Wäschestücken aller Art.

Patentiert im Deutschen Reiche vom 12. Dezember 1913 ab.

Die tryptischen Enzyme haben bekanntlich die Eigenschaft, Eiweiß und Fett abzubauen. Von der Erwägung ausgehend, daß der Schmutz der menschlichen Kleidungsstücke aller Art zu einem großen Teil aus Fett- und Eiweißresten besteht, werden der Waschbrühe tryptische Enzyme zugesetzt. Es zeigte sich, daß die Wäsche viel rascher, mit viel geringerer Kraftanstrengung und bei einer weit unter dem Siedepunkt des Wassers liegenden Temperatur rein wurde und ein viel schöneres Aussehen erhielt, als ohne Zusatz der Enzyme. Auch kommt man mit weniger Seife aus. Der Hauptvorteil der Verwendung von Enzymen gegenüber anderen, namentlich alkalischen Zusätzen beruht darin, daß sie das Gewebe nicht im allermindesten angreifen und auch für die Hände der Wäscherinnen vollkommen unschädlich sind.

Die benötigten Mengen Enzym sind äußerst gering. Für 100 l Waschbrühe genügen z. B. 2 g Pankreatin.

Weiter wurde gefunden, daß die tryptischen Enzyme auch äußerst wichtige Toilettenmittel sind. Denn bekanntlich scheidet der Körper durch die Hauptporen alle möglichen Eiweiß- und Fettreste ab, die sich teilweise in den Hauptporen festsetzen. Die tryptischen Enzyme sind nun hervorragend geeignet, diese Stoffe löslich zu machen. Zusatz von Enzymen zum Waschwasser macht das Wasser weich und übt auf die Haut einen außerordentlich wohltätigen Einfluß aus. Die Haut wird rein und auffallend weich und zart, und obendrein ist der Seifenverbrauch geringer.

Als Zusatz zum Wasch- und Badewasser genügt 0,5 bis 1 g Pankreatin auf 100 l Wasser.

Da derart kleine Mengen schlecht zu handhaben sind, empfiehlt es sich, die Enzyme für den praktischen Gebrauch, sei es als Wasch- oder Toilettenmittel, entsprechend zu verdünnen. Geeignet hierzu ist jedes indifferente, leicht lösliche Mittel, z. B. Kochsalz u. dgl.

PATENT-ANSPRÜCHE:

1. Verfahren zum Reinigen von Wäschestücken aller Art, gekennzeichnet durch den Zusatz tryptischer Enzyme, wie Pankreatin, zur Waschbrühe.

2. Anwendung tryptischer Enzyme, wie Pankreatin, zur Herstellung von Wasch- und Toilettenmitteln.

BERLIN. GEDRUCKT IN DER REICHSDRUCKEREI.

Fig. 1. A copy of the original patent by Röhm which describes for the first time the use of proteases as a cleaning aid.

strain producing an enzyme with interesting properties (Jones et al., 1998b). Later, the DNA sequence of the enzyme-encoding gene and its corresponding amino acid sequence became the key subjects for patent protection (Yamagata and Udaka, 1994; Outtrup et al., 1998; van Solingen et al., 2001) because genetic engineering eliminated restrictions on enzyme production (i.e., the enzymes could be produced by both the original host bacterium and specialized expression hosts).

In general, early important criteria for evaluating enzyme technology included the ease of fermentation and recovery, lack of adverse side-products, yield, and finally the properties of the enzyme (see Table 2). It is no surprise that this emphasis on easily recovered enzymes has resulted in industrial production organisms that are predominantly secreting organisms. Gram-positive species with only a single membrane are highly represented among enzyme host cells. Especially bacilli known for their high secretory capability are often used. Nevertheless, in the absence of good alternatives, some interesting enzymes such as glucose isomerases expressed in *Streptomyces* (Jorgensen et al., 1988) are recovered from the cytoplasm of bacteria. Other products are secreted from Gram-negative organisms such as lipases from *Pseudomonas* (Gerritse et al., 1998a).

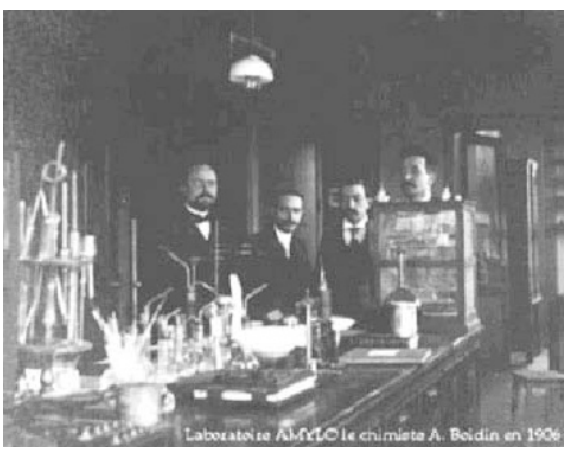

Fig. 2. The founders of the Rapidase Company, the official website of the city of Seclin (France) (source: http://www.ville-seclin.fr/, where the history of industrial activity including the "Usine Rapidase" is described).

Commercial Applications

Starch

Starch, the primary storage polymer in higher plants, consists of a mixture of amylose (15–30% w/w) and amylopectin (70–85% w/w). Amylose is composed of α-1,4-linked glucose units linked in linear chains of molecular weight ca. 60,000–800,000. Amylopectin is a branched polymer containing α-1,6 branch points every 24–30 glucose units (Fig. 3); its molecular weight may be as high as 100 million (Buleon et al., 1998). Corn (maize) starch represents 75% of the world starch production. Virtually all of the 20 million

Table 2. Critical parameters for selecting an industrial enzyme.

- Enzyme activity
  - ○ Specific activity (units/mg)
  - ○ Application dosage
- Stability during
  - ○ storage
  - ○ application
  - ○ immobilization for multiple use
- pH range for activity and stability
  - ○ broad range of pH
  - ○ broad range of process conditions
- Safety (allergenicity)
  - ○ Non-toxic to men and environment
  - ○ Non allergenic
- High yield production
  - ○ High yield expression in bacterial host
  - ○ Secretion for high yield and for easy purification

Table 1. Bacterial enzymes and their field of application.

	Starch	Detergents	Food	Textile	Fine chemicals	Brewing and juices	Paper and pulp	Feed
Amylases	+++++	++	++	++	–	–	–	–
Proteases	–	+++++	++	+	–	+	–	–
Lipase	–	++	+	–	++++	–	–	–
Esterase	–	–	–		+++	–	–	–
Cellullase	–	++	+	+++	–	–	+	–
Glucanase	–	–	+	–	–	+++	–	–
Xylanase	–	–	+	–	–	++	++	+
Glucose isomerase	++++	–	–	–	–	–	–	–
β-Lactam acylase	–	–	–	–	++++	–	–	–
Phytases	–	–	–	–	–	–	–	++

Abbreviations: +++++ to +, the importance of the enzyme class to the specific use is graded on the basis of the amount of enzyme produced and its economic value; and –, enzyme of no importance to this use.

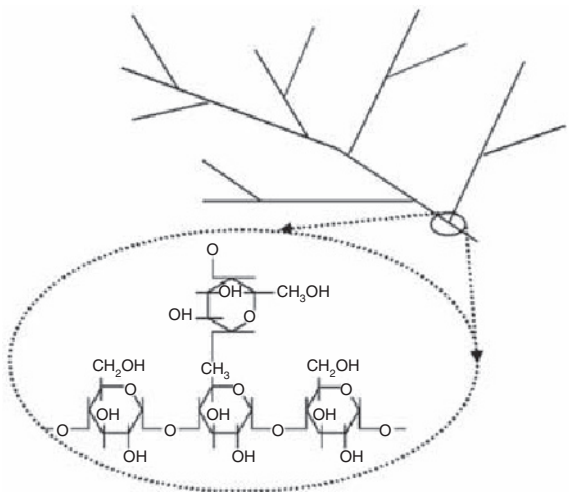

Fig. 3. Starch and actions of amylases on amylopectin. The 1,4 bonds (horizontal) are cleaved by α-amylases and the 1,6 bonds (vertical), which are formed every 24–30 glucose units, can be cleaved by pullulanase (debranching enzyme).

ton world production of corn starch (Economic Research Service, personal communication) is converted into glucose by a two-step enzyme catalyzed process involving 1) liquefaction of solid starch with an endoamylase into dextrins consisting of 7–10 molecules of glucose and 2) saccharification of the resulting liquefied starch with an exoamylase (glucoamylase) into single glucose monomers. The industrially most important endoamylases are α-amylases isolated from bacilli (Welker and Campbell, 1967a; Aiba et al., 1983; Yuuki et al., 1985). The glucoamylase with the best industrial specifications is produced from the fungus *Aspergillus niger* (Reilly, 1979). The produced glucose is used in more or less equal shares for the production of concentrated dextrose syrups, fuel ethanol, and high-fructose corn syrup.

Amylases

α-Amylase (EC 3.2.1.1) hydrolyzes starch, glycogen, and related polysaccharides by cleaving internal α-1,4-glucosidic bonds at random. The reports on the industrial use of bacterial amylase go back to the early 1920s, with a product trade-named "Rapidase," marketed by a European company with the same name. This enzyme, introduced to replace the acid hydrolysis process, which suffered from large salt loads and extreme yield losses, has long been classified as a product of *Bacillus subtilis*. Taxonomic data of the 1970s have revealed, however, that the production organism is a related but distinct species nowadays known as *Bacillus amyloliquefaciens* (Welker and Campbell, 1967b). Notably the amylase (AmyE) from *Bacillus subtilis* 168 has no liquefying activity at all and is in fact unrelated. In the classical process, starch is first heated in a jet cooking treatment that serves to open up the starch granules for gelatinization, and after cooling the mixture to 60°C, the α-amylase is added to the starch.

In the early 1980s, a major change was introduced in the industry. Now the enzyme is added during the first step of the starch degradation process, and gelatinization occurs at high temperature (up to 110°C), allowing the liquefaction during the steam explosion step. This has speeded up hydrolysis rates and decreased conversion costs significantly. The introduction of the more thermostable α-amylase from *Bacillus licheniformis* has been crucial for this improvement (Outtrup and Aunstrup, 1975; Chandra et al., 1980; Edman et al., 1999). Next to the amylase from *Bacillus licheniformis*, the enzyme from *Bacillus stearothermophilus* has been introduced for industrial use. This enzyme, with stability slightly higher than that of the *Bacillus licheniformis* amylase, however, has never been widely used, since it generates maltodextrins in a size distribution that is unfavorable for the subsequent glucoamylase treatment. In an effort to combine the best properties of these two amylases, chimeric enzymes formed of the NH_2-terminal portion of *Bacillus stearothermophilus* α-amylase and the COOH-terminal portion of *Bacillus licheniformis* α-amylase have been made (Gray et al., 1986). The hybrid enzyme molecules, however, were shown to be less stable than each of the parent wildtype α-amylases. Finally, an enzyme mixture composed of the amylases from *Bacillus licheniformis* and *Bacillus stearothermophilus* was introduced with more success. Nowadays most commercial amylases are produced from a small subgroup of *Bacillus* species such as *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus licheniformis*, or *Bacillus stearothermophilus*. These enzymes show a high degree of homology and similarity (Yuuki et al., 1985; Nakajima et al., 1986).

Thermostability, pattern of breakdown into dextrins, ease of production, and activity at low pH (<6) are important criteria used industrially for choosing amylases. In recent years, the amylases in commercial use have been optimized by protein engineering and directed evolution. Enzyme properties such as heat stability, substrate specificity, or performance at different pH have been altered (Quax et al., 1991a; see also Future Prospects in this Chapter). The generation of engineered variants and the availability of the corresponding cloned genes have inspired the development of host strains genetically engineered to optimize expression of amylases. To optimize yields in fermentation processes, classical mutagenesis was used to develop industrial strains for many decades, and much effort was

made to develop transformation protocols and genetically stable multicopy systems for industrial host strains such as *Bacillus licheniformis* (Sanders et al., 1985). For efficiency and regulatory reasons, host strains of the same species or genus from which the α-amylase is derived are preferred (Jorgensen and Jorgensen, 1993). Particularly for the production of mutant amylases, a *Bacillus licheniformis* strain without a wildtype α-amylase gene and preferably a strain without other enzymatic activities such as proteases is used (Quax et al., 1991b). The α-amylases are produced throughout fermentation as a precursor with a signal sequence that is cleaved off during secretion, and secretion facilitates recovery. As a matter of fact, the secretion of amylase is so efficient that a potent expression/secretion system based on *Bacillus licheniformis* strain T9 and the amylase expression signals has been developed. This host strain has been at the basis of the PlugBug® concept that was introduced by Gist-brocades in the late 1980s (Quax et al., 1993). This system has been used to produce high amounts of both mutant α-amylases and human interleukin-3 (Van Leen et al., 1991).

Apart from the use of α-amylases for the production of sweeteners, the enzyme has also been applied in fuel ethanol production from liquefied starch (Kosaric et al., 1983). Though the demand for fuel ethanol is fluctuating, fuel ethanol has the potential to become a major product of corn starch and concomitant growth of the amylase supply will be required.

Isomerases A major part of the glucose produced from starch liquefaction and saccharification is processed further into high fructose corn syrup (HFCS). Eight million tons are produced worldwide (Economic Research Service, personal communication). Glucose isomerases (EC 5.3.1.5) catalyze the reversible isomerization of glucose to fructose. Fructose is now commonly used as a sugar substitute because it is sweeter than sucrose or glucose. Many microorganisms are known to produce glucose isomerase; see for example the review article by Wen-Pin Chen (1980), which lists a large number of microorganisms capable of producing glucose isomerase. The best producers of industrial glucose isomerases are from the Actinomyces group including *Streptomyces rubiginosis*, *Actinoplanes missouriensis* and *Ampullariella* spp. (Quax et al., 1991b; Wong et al., 1991; Saari et al., 1997).

Activity on glucose (these enzymes are in fact xylose isomerases), no need for heavy metal cofactors (e.g., cobalt), amenability to immobilization, thermal stability (process conditions are at 55°C), and ease of production are the most important features of glucose isomerases. Generally, the naturally occurring glucose isomerases also show a high affinity for sugars other than glucose, such as D-xylose, D-ribose and L-arabinose. As a matter of fact, the K_m values for xylose are generally significantly lower and the V_{max} values usually higher than those for glucose, which is reflected in the official name of the enzyme (D-xylose ketol isomerase; EC 5.3.1.5). The enzyme causes glucose isomerization to fructose until about a 1:1 equilibrium mixture (the ratio present in natural sucrose) is formed, and the product has the same sweetness as sucrose. Because the enzyme is not secreted, its cost of production is relatively high. Therefore glucose isomerase is immobilized in column reactors, allowing prolonged use of one batch of enzyme. Typically the reactors operate for 60–100 days of continuous conversion at 55–60°C.

Glucose isomerase requires a bivalent cation such as Mg^{+2}, Co^{+2}, or Mn^{+2} for its catalytic activity. Determination of three-dimensional (3D) structures of different glucose isomerases has revealed the presence of two metal ions in the monomeric unit (Kreft et al., 1983; Farber et al., 1987; Henrick et al., 1987). Apart from a role in the catalytic mechanism, bivalent cations are also reported to increase the thermostability of some glucose isomerases (Callens et al., 1988). Although the pH optimum of glucose isomerases is usually 7.0–9.0, use of glucose isomerase at lower pH is beneficial for the following reasons: 1) under alkaline conditions, the formation of colored byproducts and a nonmetabolizable sugar (D-psicose) is a problem, and 2) the process step preceding the isomerization is performed at pH 4.5 (Roels and Tilburg, 1979). Despite an extensive screening of many microorganisms by industry researchers for a glucose isomerase with a higher activity at lower pH (Van Straten et al., 1997), no novel commercial glucose isomerase has been found.

Protein engineering has been used with more success to obtain glucose isomerases with a lower pH optimum (Drummond et al., 1989; Luiten et al., 1990; Zhu et al., 2000). The mutation of lysine253 into arginine253 of the isomerase from *Actinoplanes missouriensis* has almost doubled the operation time of the immobilized product under industrial conditions (Quax et al., 1991b; Fig. 4). In addition, technical optimizations such as an improved immobilization technique have enhanced the performance of traditional glucose isomerases such as that produced from *Streptomyces murinus* (Jorgensen et al., 1988). The mutants by definition are produced in genetically modified host strains. Also the classical nonmodified versions of the enzymes are nowadays being produced efficiently in nonsecreted form in genetically modified *Streptomyces* host cells. However, the exact nature of the strains and the

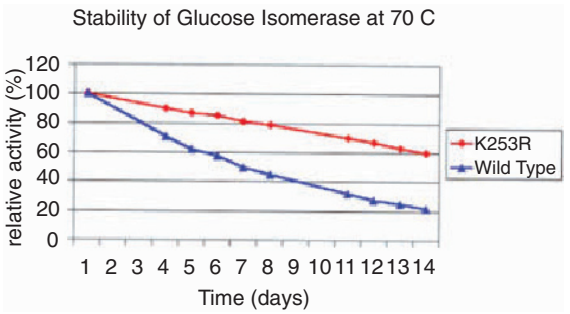

Fig. 4. The application test of protein engineered, thermostable glucose isomerase. The activity of immobilized enzyme is plotted as a function of time. The stability at 70°C indicates how the enzymes will behave under industrial conditions. The variant Lys253Arg of *Actinoplanes missouriensis* glucose isomerase has been shown to have (also under industrial conditions) a doubled half-life.

genetic constructions used by industry for these purposes are poorly documented.

PULLULANASES AND CYCLODEXTRIN-GLUCANOTRANSFERASES The endoamylases cyclomaltodextrinase (CGTase, EC 3.2.1.54), maltogenic amylase (EC 3. 2.1.133), and neopullulanase (EC 3.2.1.135) are minor enzymes capable of hydrolyzing two or three of the following: cyclomaltodextrins, pullulan, and starch. These enzymes hydrolyze cyclomaltodextrins and starch to maltose, and pullulan to panose, by cleavage of α-1,4 glycosidic bonds (see Fig. 3), whereas α-amylases are essentially inactive on cyclomaltodextrins and pullulan. Uniquely, pullulanases are also able to cleave the α-1,6 bonds (see Fig. 3), which makes them especially important for completely converting starch into glucose monomers. Pullulanases have been described from many species, but the enzyme from *Bacillus acidopullolyticus* seems to be specially suited for use in the starch processing industry (Kelly et al., 1994).

The cyclodextrins produced from glucose have applications ranging from the formulation of pharmaceuticals to surfactants (solubility enhancers; Hesselink et al., 1989; Albers and Muller, 1995). The right endoamylase for cyclodextrin production should act quickly and yield the desired product spectrum at high temperatures and low pH. The enzymes from *Bacillus coagulans* and *Bacillus circulans* are well known in the market (Kitahata et al., 1983; Hofmann et al., 1989). Recently, variants of cyclodextrin-glucanotransferases (CGTases) with an altered cyclodextrin product spectrum have been engineered by mutagenesis of specific residues (Norris et al., 1983; Wind et al., 1998).

Detergents

PROTEASES Subtilisins (EC 3.4.21.62), a large class of microbial serine proteases, are responsible for the breakthrough in industrial enzyme development. As early as 1959, the Swiss company Gebränder Schnyder AG marketed the first detergent powder with a protease produced from a *Bacillus* strain under the name Bio 40. Schweizerische Ferment AG in Basel delivered the protease. The name of the enzyme, subtilisin, refers to the producing organism *Bacillus subtilis*. In 1963, the Dutch company Kortmann and Schulte marketed the first bacterial-enzyme cleaning product (Biotex® with Alcalase®) and it became a big success. Alcalase®, the major extracellular serine protease from *Bacillus licheniformis*, was manufactured by the Danish company Novo (now Novozymes). Between 1965 and 1966, the big soap producers (Procter and Gamble, Unilever, Colgate, and Henkel) realized the potential of the hydrolytic action of bacterial protease in removing protein-based stains and they began adding Alcalase® and a similar product, Maxatase®, to their major detergent brands. This has led to the creation of a worldwide industrial enzyme market based on *Bacillus licheniformis* fermentation. Proteases hydrolyze the peptide bonds of proteins staining fabric, releasing smaller polypeptides and individual amino acid units. In 1969, a major drawback (fatal allergic reactions of employees exposed to dust set free during enzyme production) became apparent. Thanks to improved dust-free formulations, the enzyme industry was able to recover. To satisfy the desire to lower the temperature and concomitantly increase the alkalinity of laundry processes, extreme alkaline proteases (Maxacal® originating from *Bacillus alcalophilus* [Van Eekelen et al., 1988; Van der Laan et al., 1991] by Gist-brocades and Savinase® from *Bacillus lentus* [Betzel et al., 1988] by Novo-Nordisk) were introduced into the market in the early 1980s. Interestingly, the gene sequences showed that these proteases differed by only a single amino acid. Recently, the strain producing Savinase® has been reclassified as *Bacillus clausii* (Christiansen et al., 2002). The gene for the Alcalase® serine protease, also known as Carlsberg subtilisin, was cloned in 1985 (Jacobs et al., 1985). The availability of the cloned genes and detailed 3D structures of various subtilisin molecules (Drenth et al., 1972; McPhalen and James, 1988; Van der Laan et al., 1992) has facilitated protein-engineered improvements in enzymes and their adaptation to the detergent matrix. More stable variants and especially more bleach-stable variants, which were obtained by substituting the methionine residue next to the active

site serine, are dominating the marketplace today (Estell et al., 1985; Van Eekelen et al., 1989). For liquid detergent application, the more neutral subtilisin BPN-P originating from *Bacillus amyloliquefaciens* has been the product of choice for many years. In the United States, about 50% of liquid detergents and 25% of powder detergents contain proteases for improved cleaning. In Europe where powder detergents are more popular, virtually all brands have protease additives.

Lipases After the successful introduction of proteases for the removal of proteinaceous stains in laundry detergents, the next challenge was the development of lipases for the removal of greasy stains. The search for suitable lipases however turned out to be far more difficult than the introduction of proteases.

Detergent lipases were selected according to the following criteria: a) broad activity on a variety of fats and lipids, b) stability in alkaline detergent formulations, c) sufficient solubility in water to soak into fabrics, d) compatibility with proteases present in detergent formulations, and e) ease of production. The first lipase introduced in detergent powder is a lipase of fungal origin that fits well with criteria c) and e). However, owing to the acidophilic nature of fungi, the compatibility of their lipases with the alkaline conditions in detergents is poor. Therefore bacterial lipases (EC 3.1.1.3) originating from *Pseudomonas* species have received much attention. Especially the lipase from *Pseudomonas alcaligenes* has an excellent activity in the pH range compatible with detergent conditions. Criterion e) is however far more problematic for fungal lipase production. The expression in heterologous host strains such as *Bacillus* or *Escherichia coli* turned out to be impossible because a lipase-specific chaperone Lif (El-Khattabi et al., 1999) or LipB was required. Furthermore the lipase is secreted via the terminal branch of the general secretion pathway (Xcp-machinery), which involves very specific interactions (for a review, see Filloux et al., 1998; Fig. 5). Apart from the expression yield also the recovery of *Pseudomonas* lipases from the fermentation broth requires special processes owing to the hydrophobic nature of lipases and the presence of lipopolysaccharides. Despite these obstacles, the lipase from *Pseudomonas alcaligenes* was introduced as a detergent additive in 1995 by Gist-brocades under the trade name Lipomax® (Gerritse et al., 1998b; Cox et al., 2001). As a result of a stepwise improvement of the production strain and fermentation process, commercially viable yields of lipase were obtained (Gerritse et al., 1998a, 1998b; Cox et al., 2001).

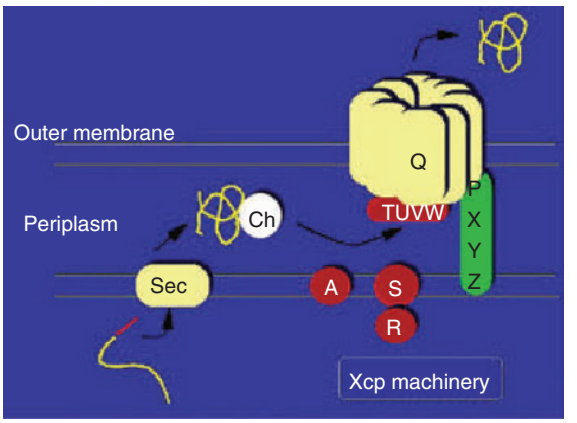

Fig. 5. The secretion machinery of *Pseudomonas alcaligenes*, the producer of Lipomax®, an alkaline lipase for detergent compositions. The XcpQ protein forms a multimeric ring in the outer membrane. The Xcp T, U, V and W proteins are thought to be involved in the gating of the pore, and proteins P, X, Y and Z form a connection between the outer membrane pore and the inner membrane. XcpA, S and P play a role in the processing of other Xcp proteins. Sec is innermembrane translocase; Ch is the periplasmic chaperone.

As most lipases do not meet all the above-mentioned criteria, the first protein engineering of lipases was based on amino acid sequence information only (e.g., the study on lipase from *Pseudomonas mendocina*; Gray et al., 1995). When the first 3D-structures became available in the late 1980s, protein engineering efforts increased dramatically. A European-wide funded project focused on solving new lipase structures, and now more than 15 X-ray structures of lipases are available in the protein database (PDB), of which 12 are microbial and 5 are of prokaryotic origin. The X-ray structure of lipase containing a phosphonate inhibitor covalently bound to its active site serine revealed that a lid was displaced from the active site by a hinge bending movement, creating an increased hydrophobic surface. Many of the lipase structures are solved in both a closed and an open conformation, i.e., with the lid or lids displaced from the active site. A list of selected solved bacterial lipase structures is given in Table 3. The overall structure of the triacylglycerol lipases has a central L-sheet with the active serine placed in a loop termed the "catalytic elbow." Above the serine, a hydrophobic cleft is present or formed after activation of the lipases. Molecular modeling of these structures has been used to construct models of lipase homologues (e.g., the engineering of the *Pseudomonas alcaligenes* lipase; Aehle et al., 1995). The use of lipases for the generation of enzymatic peroxide bleach in detergents has been pioneered but not yet applied.

Table 3. Three-dimensional structures of prokaryotic lipases.

Species	Molecular weight (kDa)	Structure[a]	Reference
Burkholderia glumae (*Pseudomonas glumae*, *Chromobacter viscosum*)	23	PDB1QGE	Noble et al., 1994
Pseudomonas aeruginosa	30	PDB1EX9	Nardini et al., 2000
Bacillus subtilis	19	PDB1I6W	van Pouderoyen et al., 2001
Burkholderia cepacia (*Pseudomonas cepacia*)	33	PDB2LIP	Schrag et al., 1997
Bacillus stearothermophilus	43	PDB1KU0	Jeong et al., 2002

[a]Code name for the corresponding file in the Protein Database Bank [www.rcsb.org/pdb] (PDB code).

Cellulases Cellulases are enzymes capable of hydrolyzing the 1,4 β-D-glucosidic linkages in cellulose. Cellulolytic enzymes have been traditionally divided into three major classes: endoglucanases, exoglucanases (or cellobiohydrolases), and β-glucosidases (Knowles et al., 1987). A large number of bacteria, yeasts and fungi is known to produce this group of enzymes. Initially, cellulolytic enzymes have been developed for application in converting wood and cellulose pulp into sugars for bio-ethanol production. Later on, it was discovered that cellulases can be used for the treatment of textiles. For example, repeated washing of cotton-containing fabrics results in a grayish cast to the fabric, which is believed to be due to fibrils disrupted and disordered by mechanical action. This grayish cast, sometimes called "pills," is particularly noticeable on colored fabrics. The ability of cellulase to remove the disordered top layer of the fiber and thus improve the overall appearance of the fabric has been used to recondition used fabrics to make their colors more vibrant.

Despite the availability of fungal cellulases having some of the above properties, new cellulases that are more compatible with the alkaline detergent formulations have been sought. Alkalophilic *Bacillus* species have been found to express cellulases (EC 3.2.1.4) with excellent properties for detergent conditions, and one of these cellulases is now expressed from *Bacillus subtilis* and marketed under the trade name Puradax® (Jones and Quax, 1998a). Also, cellulases from *Thermomonospora fusca* have been found to be of interest (Irwin et al., 1998) to textile decorators. Some of these cellulases can be abundantly expressed in a *Streptomyces lividans* host cell (Jung et al., 1993).

Amylases The thermostable α-amylase (EC 3.2.1.1) from *Bacillus licheniformis* is perfectly compatible with detergent conditions, and nowadays small amounts of this enzyme are widely added to detergent powder formulations for the removal of starch stains. A protein engineered variant, Purastar® Ox, has been developed specifically for inclusion in bleach-containing detergent formulations (Genencor, 2001). This brings the number of different enzyme systems added to modern detergent powders up to four: proteases, lipases, cellulases and amylases.

Food Processing

Microorganisms play a major role in the processing of dairy products, beer, wine and many other food products. Isolated enzymes are also being used in specialized processes, although in much smaller amounts than are used in the immense starch processing industry, which will be discussed in a separate chapter.

The baking of bread is one of the oldest biotechnological processes known to man. Yeast enzymes and endogenous flour enzymes are the primary modifiers and metabolizers of flour sugars and proteins. However, the levels of endogenous enzymes vary considerably depending on wheat growth, harvest and storage conditions. Correction and supplementation of the flour with bacterial enzymes result in more tasteful and better quality bread. *Bacillus amyloliquefaciens* α-amylase (EC 3.2.1.1) is used to obtain an improved loaf volume and crumb structure (Lin and Lineback, 1990). In addition, α-amylase contributes to anti-staling by mildly hydrolyzing starch polymers, which prevents their crystallization and thereby hardening of bread. The neutral protease of the same bacterium is used for improving the rheological properties of biscuit and cracker dough (Lyons, 1982). This protease fragments the gluten protein in wheat flour, which gives the dough its elastic properties. As a result, the dough requires a reduced fermentation time and the resulting biscuits have a prolonged freshness.

Dairy products and beverages are processed under mildly acidic conditions favoring the use of enzymes of fungal origin. However, in the processing of beer the enzymes from selected *Bacillus* strains play an essential role. The α-amylase from *Bacillus amyloliquefaciens* is used to improve the enzymatic liquefaction potential of the malt. A β-glucanase from the same bacterium (Hofemeister et al., 1986) is used to reduce the viscosity of the wort, which improves the filtration of the beer.

Textiles

Amylases Woven fabrics from natural plant and animal fibers represent the oldest forms of textile. The introduction of mechanical processes in the nineteenth century prompted the introduction of protective agents to prevent warp-end breaks. Starch added as a sizing agent strengthens fibers and makes the yarn more resistant to high mechanical stress during the weaving process. Traditionally, malt extracts and animal derived preparations have been used to remove starch-based thickeners in the desizing operation. However, as early as 1917, a high temperature stable bacterial enzyme preparation obtained by dedicated fermentation was introduced (Wallerstein, 1939). Today we know that the bacterium used was *Bacillus amyloliquefaciens* (Welker and Campbell, 1967a). At present both *Bacillus amyloliquefaciens* and *Bacillus licheniformis* α-amylases are being used for this process.

Cellulases In various treatments of cotton fibers, these enzymes have resulted in better wash-down effects, resistance to pilling, softening, and better dye uptake. Later it was discovered that the enzymatic treatment of textiles could result in decorative effects on clothing similar to the stone washing of denim (Gusakov et al., 2000). This has resulted in a large market for cellulases in providing a worn look to jeans. The enzymatic production of stone-washed denim products (no need for pumice) has become a fast growing market with more than $40 million in sales per year. A variety of cellulase products (many of fungal origin) is marketed for this purpose. Recently, enzymes from the actinomycete *Thermomonospora fusca* have been developed (Spezio et al., 1993). The cellulase (EC 3.2.1.4) can be efficiently produced from a genetically engineered *Streptomyces lividans* (Jung et al., 1993). Care needs to be taken to prevent loss of fiber strength from cellulase treatment that is too lengthy or intense.

Proteases These enzymes (e.g., subtilisin [EC 3.4.21.62]) are used to treat protein fabrics such as wool and silk. By breaking down the fibrils on the surface, the look and feel of the fabric can be softened.

Fine Chemicals

In nature, a huge repertoire of chemical transformations is catalyzed by many thousands of enzymes. Its precise 3D architecture allows each enzyme to exhibit a remarkable specificity for the conversion of a particular set of substrates. The introduction of these enzymes as biocatalysts in the industrial production of fine chemicals probably represents the uppermost innovation in the enzyme field in recent years. Since a company produces in-house many of the biocatalysts used within industrial processes (i.e., production for captive use or captive consumption), the information on the actual scale and commercial impact of many of these biocatalytic processes is often limited. Nevertheless, from the scarce publications on industrial use of biocatalysts it can be concluded that numerous energy intensive chemical processes involving a high output of pollutants have now been replaced by environmentally friendly enzymatic processes (Schmid et al., 2001).

Amidases

β-Lactam Acylases Penicillin G acylase (benzylpenicillin amidohydrolase, also named "penicillin amidase;" EC 3.5.1.11) is an enzyme used commercially to produce 6-aminopenicillanic acid (6-APA), the most important intermediate for the industrial production of semisynthetic penicillins. This is achieved by the hydrolysis of penicillin G (for review, see Bruggink et al., 1998; Fig. 6, left column).

Numerous bacterial species have been described in the literature as penicillin G acylase-producing strains, but only certain strains of the species *E. coli*, *Kluyvera citrophila* and *Alcaligenes faecalis* were found to produce an enzyme compatible with the requirements of industrial deacylation (Balasingham et al., 1972; Barbero et al., 1986; Verhaert et al., 1997). Driven by environmental legislation in the past decade, all chemical deacylation processes in industry have been replaced by the less polluting enzymatic cleavage process. Recombinant DNA methods have been applied not only to increase the yields of commercially used penicillin G acylases (Bruns et al., 1985) but also to decipher the complex processing of these enzymes (Schumacher et al., 1986). The penicillin G acylase of *E. coli* ATCC11105 was found to be produced as a large precursor protein, which is secreted into the periplasm and further processed to the mature protein constituting a small (α) and a large (β) subunit. Cloning and sequencing has revealed a close homology (90% identity) to the *Kluyvera citrophila* and a distant homology (50% identity) to the *Alcaligenes faecalis* acylase gene. The heterodimeric structure, however, is evolutionarily preserved not only among penicillin acylases but also within the much larger family of β-lactam acylases.

Whereas the conversion of penicillin-G requires an enzyme with a specificity for the aromatic phenyl acetate side chain, the processing of the second largest β-lactam fermentation

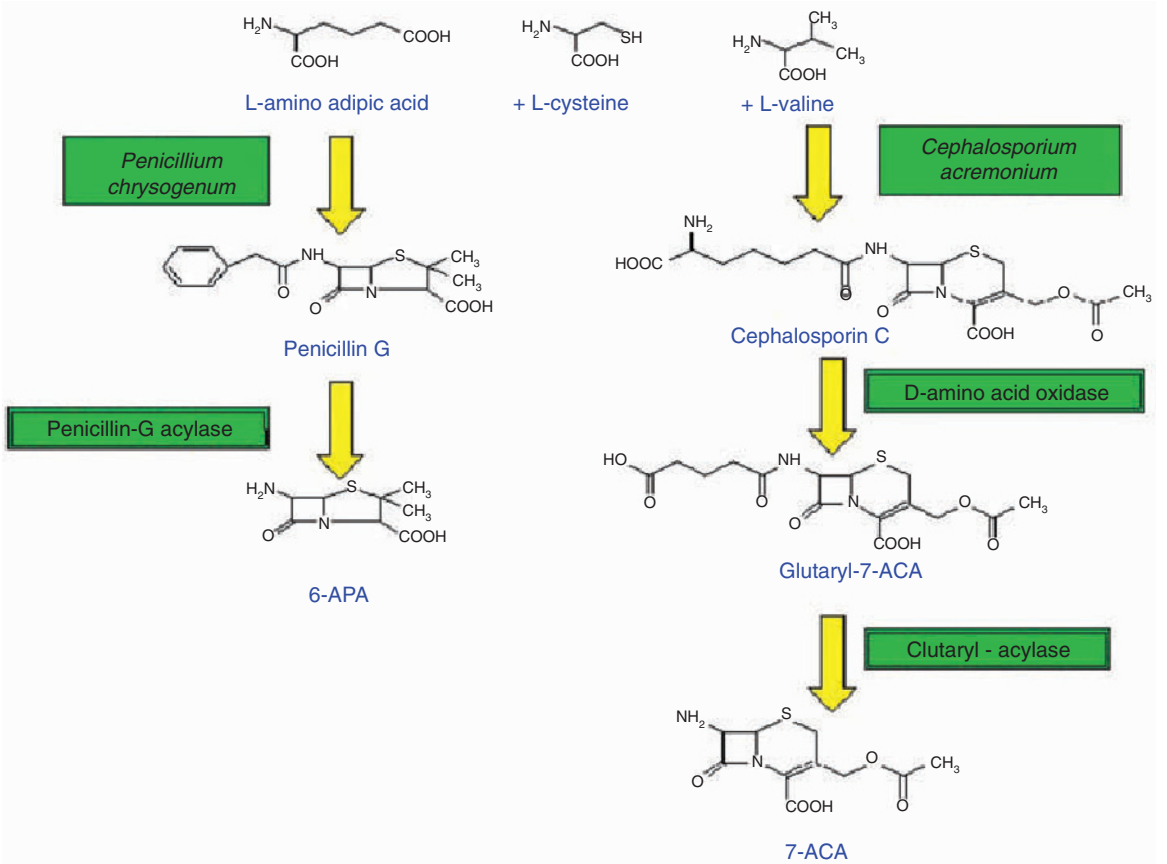

Fig. 6. The role of β-lactam acylases in the manufacturing of semisynthetic cephalosporins and penicillins. In the left pathway, the production of 6-amino penicillanic acid (6-APA) from the fermentation product penicillin-G is shown. In the right pathway, the production of 7-aminocephalosporanic acid (7-ACA) from the fermentation product cephalosporin-C is depicted.

product, cephalosporin-C, requires the cleavage of aminoadipyl, an aliphatic side chain, from the β-lactam nucleus. Since a one-step enzymatic deacylation (Aramori et al., 1991a) was not feasible, a combination of two enzyme-mediated reactions has been introduced to produce 7-aminocephalosporanic acid (ACA). In this process, D-amino acid oxidase and a glutaryl acylase perform an enzymatic deacylation of cephalosporin-C (see Fig. 6, right column). This glutaryl acylase (EC 3.5.1.-) can be obtained from several *Pseudomonas* species (Shibuya et al., 1981; Matsuda et al., 1987; Aramori et al., 1991a; Ishiye and Niwa, 1992; Ishii et al., 1994; Li et al., 1998) or from a *Bacillus* species (Aramori et al., 1991b). Research towards a one-step cephalosporin-C deacylating enzyme so far has been unsuccessful.

A third important intermediate, 7-aminodesacetoxycephalosporanic acid (7-ADCA), is produced from penicillin G by an expensive chemical ring expansion reaction. Subsequent deacylation of cephalosporin G can be achieved enzymatically by a penicillin-G acylase such as the enzyme from *Alcaligenes faecalis*; Fig. 7, left column). The latest development in the field is the use of a genetically modified *Penicillium chrysogenum* equipped with an expandase gene from *Streptomyces clavuligerus* to produce adipyl-7-ADCA upon fermentation with adipate feed (Crawford et al., 1995; Fig. 7, right column). Deacylation of adipyl-7-ADCA cannot be done with penicillin acylases, but requires an enzyme with affinity for the adipate side chain (Schroen et al., 2000; Xie et al., 2001). Some of the aforementioned glutaryl acylase enzymes have a low activity on this substrate. Recently by directed evolution, several mutants of *Pseudomonas* SY-77 acylase (EC 3.5.1) with a high activity on adipyl-7-ADCA have been isolated (Otten et al., 2002; Sio et al., 2002; Fig. 7).

Semisynthetic cephalosporins and penicillins are industrially produced from intermediates depicted in Figs. 6 and 7. As β-lactam acylases are hydrolytic enzymes, in theory the reaction can be reversed under conditions of low water

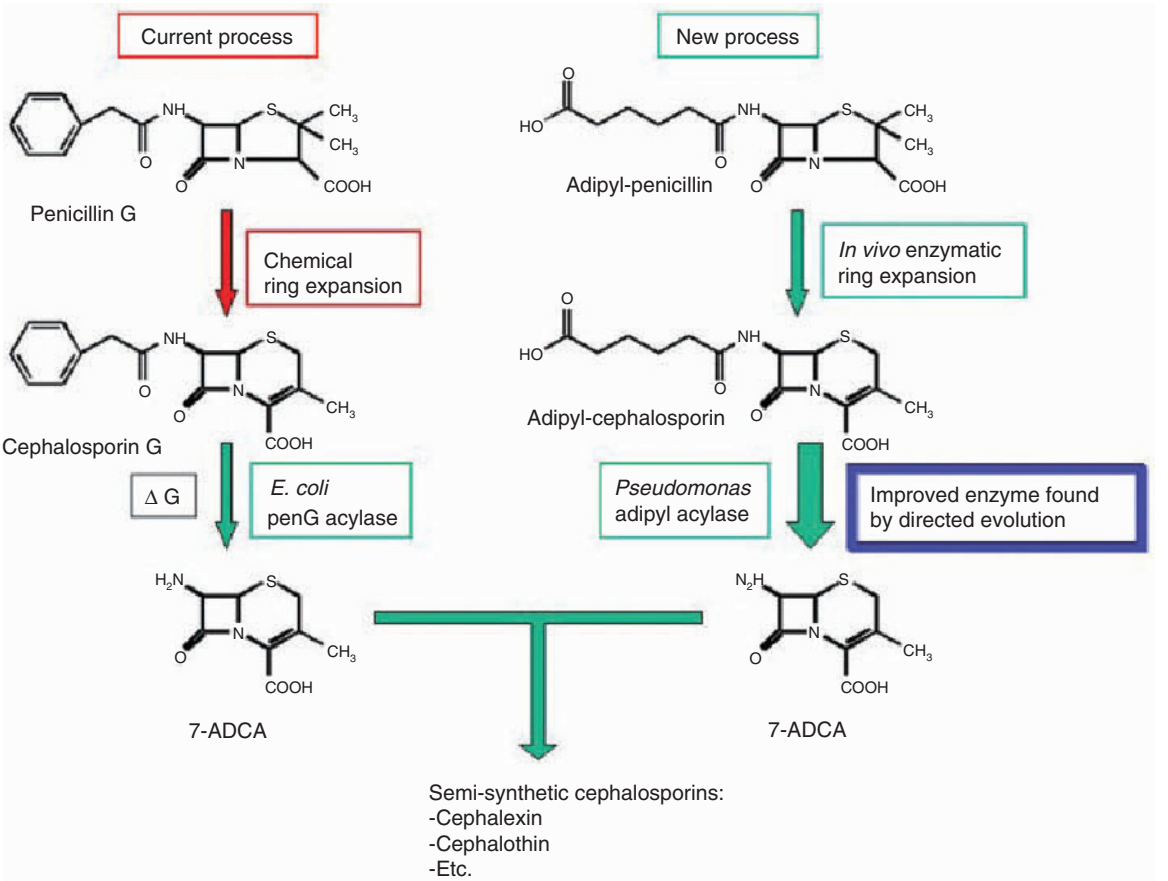

Fig. 7. In the left panel, the classical process for obtaining 7-ADCA is shown. A novel biosynthetic pathway for adipyl-cephalosporin using *Penicillium* is depicted in the right column. The final conversion towards 7-ADCA is done with an adipyl cephalosporin acylase. Using directed evolution, the glutaryl acylase of *Pseudomonas* SY77 has been converted into an adipyl acylase (Otten et al., 2002; Sio et al., 2002).

concentration. Precisely this property of β-lactam acylases is being used for the selective coupling of specific side-chains to form pharmaceutically valuable β-lactams such as ampicillin, cephalexin (Boesten and Moody, 1995), and loracarbef (Koeller and Wong, 2001). Directed evolution will undoubtedly result in the isolation of variants with novel synthetic properties (Alkema et al., 2000).

Other Amidases Aspartame is a dipeptide with an immense sweet taste. The synthesis of this low-calorie sweetener is performed with the neutral protease (EC 3.4.24.27) from *Bacillus thermoproteolyticus* also known as "thermolysin." Applied as a reversal of the hydrolytic reaction, the enzyme shows a remarkable specificity in the coupling of N-protected-L-aspartic acid and D/L phenylalanine methyl ester. Owing its extreme thermostability, the enzyme is very stable in the high solvent conditions used for the reaction. As an alternative to thermolysin, a highly stable variant of *Bacillus stearothermophilus* neutral protease obtained by protein engineering is now available (Mansfeld et al., 1997; Van den Burg et al., 1998).

Amidases are also applied for the chiral resolution of racemic amino-acid amides to allow the biocatalytic synthesis of non-natural L-amino acids, which are important building blocks for pharmaceuticals. An amidase (EC 3.5.1.4) from *Pseudomonas putida* has been developed for the kinetic resolution of a wide range of amino acid amides (Schmid et al., 2001).

Lipases and Esterases Lipases from *Pseudomonas aeruginosa*, *Pseudomonas cepacia* and *Pseudomonas fluorescence* (EC 3.1.1.3) are being used for a large number of different synthetic reactions in organic chemistry with special emphasis on kinetic resolution of chiral compounds (Coffen, 1997). As lipases are active in organic solvents, not only hydrolytic but also transesterification reactions can be performed.

For racemic alcohols, this may be an enantioselective transformation with acyl donors such as vinyl esters, anhydrides or diketenes (Koeller and Wong, 2001; Schmid et al., 2001). Using this process, enantiomerically pure alcohols are produced on a several hundred ton scale by Badische Anilin und Soda Fabriken (BASF). *Pseudomonas* lipase can also react with amines as nucleophiles, allowing the resolution of optically active amines such as *S*-methoxyisopropylamine, an important building block for the herbicide "FrontierX2."

The enzymatic activity of lipases is very comparable to that of esterases, with the main difference being the chain length and hydrophobicity of the acid moiety of the substrate. Therefore in fine chemical applications, lipases and esterases are being used as alternatives for several conversions. For instance, for the kinetic resolution of 2-aryl propionic acids such as naproxen and ibuprofen, both a lipase and an esterase have been found that can perform a stereoselective hydrolysis yielding the pharmaceutically preferred enantiomer *S*-naproxen (Bertola et al., 1992; Hedstrom et al., 1993). High activity and ease of production have made the carboxylesterase from *Bacillus subtilis* Thai I-8 the prime choice of industry (Quax and Broekhuizen, 1994).

The markets for fine chemicals that can be made from esterss are very important and diverse. Thus, the ability to perform ester hydrolysis or esterification reactions in a manner that ensures high specificity and high stereoselectivity is of great importance. Therefore, the use of genomics information to search for new esterases is of great interest (Robertson et al., 1999; Droge et al., 2001).

Areas of Research

Feed

Animal feed is mainly composed of polymeric structures that have to be digested in the gut. Any pretreatment of the agricultural stock may lead to an improved digestibility and hence yield of feedstuffs. It is therefore no surprise that most of the hydrolytic enzymes including the pancreatic extracts used as the first enzyme preparation in the 1920s have been tested in one way or another for the processing of animal feed. Only after an increased understanding of the digestive physiology did realistic applications come within reach. The examples described below are the result of expert advice and evaluation of feed industry experiences.

Phytases Phosphorus is an important component of feed, as it is crucial for bone and skeleton formation. About 70% of phosphorus in vegetable feed ingredients is present in the form of phytate, an inositol-bound organic form of phosphorus that has a low bioavailability in monogastric animals. For this reason, the diet for monogastric animals like pigs and chickens is supplemented with significant amounts of inorganic phosphate that causes eutrophication in regions of the world with a dense monogastric animal population, such as the Netherlands. The addition of microbial phytases (EC 3.1.3.26) has resulted in a doubling of the bioavailability of phytate, obviating the need for addition of inorganic phosphate (Simons et al., 1990). This has led to lowering phosphate in manure to unprecedented levels in the Netherlands and to phosphate pollution reductions that are more significant than the reductions from the detergent phosphate ban in the mid-1970s.

Phytase from fungi has been shown to be extremely compatible with the low pH conditions of the animal gastric tract (Jongbloed et al., 1992), but also phytases from bacteria, such as *Bacillus subtilis*, are being developed for use as a feed additive (Kerovuo et al., 2000b; Park et al., 1999; Kerovuo and Tynkkynen, 2000a).

Xylanases Pentosans present in wheat and rye diets are often poorly metabolized. Especially arabinoxylans negatively influence the digestion and absorption of nutrients in the foregut of animals. When a xylanase (EC 3.2.1.8) treated arabinoxylan fraction was used, the nutritional parameters were similar to those when an arabinose and xylan monomeric mixture was used, indicating that xylanases are a valuable feed additive. Especially sought are enzymes with endo-1,4-β-xylanase activity that are stable in the digestive tract of poultry (Mondou et al., 1986).

Paper and Pulp

In the pulp and paper manufacturing process, elemental chlorine is applied for the bleaching of the pulp. As a byproduct of this process toxic chlorinated phenols as well as polychlorinated biphenyls are formed. Next to alternative bleaching chemicals such as ozone, the use of enzymes has gained more interest. Especially the removal of residual lignin results in a lower required amount of bleaching chemicals, allowing the replacement of elementary chlorine by the less polluting chlorine dioxide. The removal of lignin can be facilitated by a pretreatment of the pulp with xylanases or by laccases. This xylanase pretreatment cleaves the hemicellulose fraction that links the lignin to the cellulose. The laccase treatment results in a direct oxidative degradation of the lignin. The search for sufficiently active laccase systems is still in its infancy,

but xylanases have been developed for commercial use.

The pulping process in a paper mill is performed at temperatures of 65–80°C at pH 9–12. Xylanases (EC 3.2.1.32; endo-1,3-β-xylanase) from some thermophilic bacilli were found to be compliant with these conditions (Gat et al., 1994), and the xylanase from *Bacillus stearothermophilus* T6 was developed and tested on a large scale (Lundgren et al., 1994). This enzyme shows activity at high temperature (60–70°C) and high pH (7–9). The enzyme can be expressed and purified in high yields from *Bacillus subtilis* (Lapidot et al., 1996). The search for even more thermostable and more alkaline-stable xylanases has been targeted towards extremophiles (Saul et al., 1995; Outtrup et al., 1998).

General Expression Hosts

Bacteria are attractive for large scale manufacturing of commercially relevant proteins owing to their fast growth rate and their high protein synthesis capacity. Enhanced levels of gene expression, however, often result in the intracellular accumulation of inactive protein aggregates also known as inclusion bodies. For most enzyme manufacturing processes, the recovery of active protein from these aggregates is uneconomical. The only enzyme process that has been in use for many years has been the manufacturing of bovine chymosin (rennin), with the Gram-negative bacterium *E. coli* as a host (Nishimori et al., 1981; Emtage et al., 1983).

Export of overexpressed heterologous enzymes from the cytoplasm has been explored as a solution to prevent inclusion body formation and to produce functional proteins in an easily recoverable form. With the identification of some periplasmic chaperone and foldase functions in Gram-negative bacteria, the concept of using the periplasm as a "construction compartment" in which chaperones aid the folding and functional assembly of proteins has come within reach. The ultimate goal from the viewpoint of industrial scale recovery—accumulation of proteins on a gram per liter scale in the extracellular medium—requires however the passage through two membranes. Recently described have been some nonpathogenic species such as *Pseudomonas alcaligenes* that have the capacity to secrete commercially important enzymes (lipases, proteases, cellulases and phospholipases) in significant amounts into the extracellular medium (Gerritse et al., 1998a). The outer membrane secretion machinery is crucial for the export of proteins from the periplasm. At high expression levels, the outer membrane can become a barrier as exemplified by the effect on *Pseudomonas alcaligenes* lipase overexpression of selecting the Xcp gene cluster using the phenotype enhancement method (Gerritse et al., 1998b). The *xcp* gene cluster encodes the type II secretion pathway in Gram-negative bacteria, also referred to as the main terminal branch (MTB) of the general secretion pathway (GSP). Proteins secreted via the GSP pass the cell envelope in two separate steps. First, they are translocated across the inner membrane into the periplasm, a process mediated by the Sec machinery. Subsequently, the periplasmic intermediates are translocated across the outer membrane as fully folded proteins (Fig. 5). Several nonspecific chaperones function in the periplasm of *E. coli*. The peptidyl-prolyl-*cis-trans*-isomerases (PPI) catalyze the *cis-trans* isomerization of X-proline peptide bonds, which was found to be rate limiting upon high level production of functional single chain Fv (scFV) fragments in the periplasm of *E. coli* (Jager and Pluckthun, 1997). A second class of nonspecific chaperones, the thiol-disulfide oxidoreductases (Dsb) that catalyze the formation of disulfide bonds, has been shown to play a crucial role in the formation of disulfide bonds in heterologous proteins expressed in *E. coli* (Joly and Swartz, 1997; Joly et al., 1998). Recently homologues of *dsb* genes have been found in *Pseudomonas aeruginosa* to be involved lipase folding (Reetz and Jaeger, 1998).

In addition to nonspecific chaperones, the folding of a variety of extracellular proteins requires the action of specific chaperones. For example, the correct folding of lipases is mediated by the lipase-specific foldases (Lif). It has been shown that folding of the lipase of *Pseudomonas aeruginosa*, when expressed in *E. coli*, is dependent on the coexpression of the *Pseudomonas aeruginosa lif* gene (El-Khattabi et al., 1999). Interestingly, it was found that the amount of Lif can become limiting in an industrial *Pseudomonas alcaligenes* strain upon overexpression of the endogenous lipase gene (Gerritse et al., 1998a).

Bacillus species have always been the paradigm hosts for the production of bacterial enzymes and around 50% of the total worldwide enzyme production is by bacilli. Nevertheless, the protein secretion machinery of *Bacillus* has certain limitations, and in a systematic analysis, members of the European *Bacillus* Secretion Group (EBSG) over the past years have identified bottlenecks in the secretion pathway of *Bacillus subtilis* that relate to different stages in the secretion process. Different proteins can run into different limiting factors (Bolhuis et al., 1999). During transport over the membrane, signal peptidases can become limiting factors in pre-protein processing. For example, overproduction of signal peptidase was shown to be beneficial for the secretion of heterologous β-

lactamase from *Bacillus subtilis* (Van Dijl et al., 1992). Alternatively, signal peptidases can interfere with efficient pre-protein processing under conditions of high-level overproduction of secretory proteins. This is illustrated by the observation that the disruption of the *sipS* gene, encoding one of the five signal peptidases of *Bacillus subtilis*, resulted in highly increased rates of processing of an α-amylase precursor (Tjalsma et al., 1997).

Finally, late stages in the secretion process, including the folding of mature proteins and cell wall passage, can become secretion bottlenecks. It was found that the lipoprotein PrsA becomes limiting under conditions of high-level secretion of α-amylases, as it is required for the folding into a protease-resistant conformation upon translocation (Kontinen and Sarvas, 1993). In another experiment, it was found that the cell wall, which is relatively thick (10–50 nm) and contains a high concentration of immobilized negative charge (e.g., teichoic or teichuronic acids), can act as a barrier in translocation (Saunders and Guyer, 1986; Stephenson et al., 1998b). Thus, proteins with a net positive charge might be retained in the wall. Furthermore, it was shown that the wall-bound serine protease CWBP52, encoded by the *wprA* gene, can degrade slowly folding enzymes at the site of preprotein translocation. Hence, CWBP52 depletion has resulted in an increased yield of secreted α-amylase (Stephenson and Harwood, 1998a).

More successful approaches to remove bottlenecks in the production of proteins from *Bacillus* involve the elimination of detrimental factors, such as extracellular proteases. In a stepwise approach, strains with an increasing number of protease gene deletions have been constructed, resulting in a sevenfold protease negative strain that shows significant higher yields of susceptible bacterial enzymes (Ye et al., 1999).

Patents and Regulatory Systems

Regulations and Enzymes

Bacterial enzymes for food applications must comply with the regulations put forward by the United States Food and Drug Administration (FDA) or comparable bodies in other countries. Most enzymes are considered as food processing aids and usually do not end up in the final consumer end product. Nevertheless all products undergo a strict testing program including toxicity and efficacy testing. Finally the industrial production process has to comply with the regulations stipulated by the Environmental Protection Agency (EPA). These documents may be accessed through at the Office of Pollution Prevention and Toxics' Biotechnology Program homepage (http://www.epa.gov/opptintr/biotech/). Alternatively, the documents are available from the {EPA homepage} (http://www.epa.gov/fedrgstr/) at the Environmental Sub Set entry for this document under "Regulations."

The industrial and household enzyme products not used for food applications must comply with the regulations of the EPA and general product safety regulations. Especially with respect to preventing allergenicity, there are strict specifications for formulating enzymes and preventing dust formation. The production host strains must be nontoxic and preferably with a record of safe use. Most of the enzyme products have Generally Recognized as Safe (GRAS) status.

Patents and Taxonomy

Purified enzyme products can be covered by a broad substance patent claim as long as the disclosure complies with the three elements of a patent application: the substance should be novel, the disclosure should involve an inventive step, and the substance should have a use. The aspect of novelty can be readily checked since the amino acid sequence of a newly described enzyme can be easily compared to a protein or DNA database. As the number of described amino acid and DNA sequences has exploded in the past years and since patent examiners tend to use the criterion of 70% amino acid sequence identity to specify homologous enzymes, it is clear that broad substance patent claims will be difficult to obtain in future. Rather, patent protection will be sought more for specific methods and applications of certain enzymes. Enzymes that have been obtained by protein engineering or directed evolution represent a special group of patent claims. As the sequence identity to existing enzymes will generally be very high (>99%), the variant will need to have a property that distinguishes it from wildtype enzymes to become patentable. Patent claims in those cases have mostly been restricted to the specific examples shown in the description.

With the granting of patent claims on living organisms (Chakrabarty, 1981), a new dimension was added to the intellectual property protection. Patent claims on the bacteria themselves were initially rejected because living things were not considered patentable. Finally the United States Supreme Court reversed the initial decision, making the argument that a genetically engineered microorganism is not a product of nature, but rather a product of a person's work and is thus patentable under the United States law. This decision has added a new element to the patenting of bacterial enzymes and the host cells pro-

ducing them. Now also the bacterial strains as isolated from natural sources could be patented (Collins et al., 1998a; Collins et al., 1998b; Outtrup et al., 1998). A detailed description in the form of a correct taxonomic determination of the strain is now essential to obtain good patent protection. This has led to the development of modern tools for the description of claimed species, such as the 16S RNA identification.

Prospects

Extremophiles

Enzymes isolated from microorganisms living under harsh conditions are adapted to those extreme conditions. For example, an amylase and a protease that are fully stable and active at 95°C have been isolated from *Pyrococcus furiosus*, a hyperthermophile living in a 90°C hotspring (Brown et al., 1990; Eggen et al., 1990). Especially the progress in research on archaea and the ability to culture these strains in the laboratory have generated a lot of enthusiasm for household and industrial uses of enzymes from extremophiles. As the growth conditions for these extremophiles are difficult to create on an industrial scale, the goal is to express the genetic material encoding these enzymes in mesophilic hosts. Numerous novel genes encoding thermostable (Koch et al., 1990; Hakamada et al., 2000), alkali stable (Shendye and Rao, 1993; Kobayashi et al., 1995; Saeki et al., 2000), and acid stable (Tamuri et al., 1997) enzymes have been characterized in recent years. This can result in not only enzymes better suited to existing applications (such as detergents [alkaline] and starch [high temperature]) but also completely new applications such as the enzymatic bleaching of pulp, a process requiring both high temperature and very alkaline conditions. The yields in production of enzymes from extremophiles, however, are generally low because compatibility of these proteins with the folding and secretion machinery of mesophilic hosts is low. The impact of these novel enzymes on the household and industrial enzyme market therefore remains to be seen and "expressibility" must be considered when selecting extremophilic enzymes with desired properties (Van Solingen et al., 2001). The best results have been obtained with enzymes from extremophilic eubacterial origin, such as the thermostable xylanase (produced on a large scale for enzymatic pulp treatment) from *Bacillus stearothermophilus* (Lundgren et al., 1994). In research and diagnostic laboratories, the thermostable DNA polymerases (such as the Taq polymerase from *Thermus aquaticus* and Pfu polymerase from *Pyrococcus furiosis*) have shown their tremendous value already (Peterson, 1988; Picard et al., 1994). The diagnostic enzymes including the huge diversity of restriction enzymes and polymerases are, however, beyond the scope of this chapter.

Directed Evolution

In the past two decades, the technique of protein engineering has allowed investigators to create new enzymes and proteins. Interestingly, some of the most striking commercial successes have not been the result of rational design based on a 3D structure, but merely the payoff of smart combinations of random mutagenesis and screening. The power of this combination resides in the fact that many variants with subtle differences can be probed quickly. In practice, however, major weaknesses are still encountered, as most screening assays for enzymatic activity are rather limited in throughput. A major improvement can be made if a selection instead of a screening can be introduced. This combination of gene pool diversification and selection for function (collectively termed "directed evolution") is now considered as one of the most successful protein engineering strategies. Two processes play a key role in evolution: mutation and selection. Gene mutation methods have been expanded enormously with the advent of the polymerase chain reaction (PCR) techniques (error prone PCR and PCR with spiked oligonucleotide primers and staggered extension process) and DNA shuffling (Crameri et al., 1997; Zhao et al., 1998; Matsumura et al., 1999). However, the selection for function is less obvious as the majority of industrial enzymes are secreted into the extracellular medium, which interferes with growth selection. Most of the newly described directed evolution studies have been on intracellular enzymes with in vivo selectable functions, such as β-lactamase, which can be selected for by increasing the antibiotic concentrations (Stemmer, 1994). Attempts have been made to use display techniques involving coupling of the phenotype of an extracellular enzyme with the genotype. As demonstrated with the industrially important enzyme α-amylase from *Bacillus licheniformis*, it is possible to use phage display for the selection of enzymes with improved substrate binding properties (Verhaert et al., 2002). Binding to substrate transition state analogues has been used to select for enzymes with altered catalytic properties. Although binding of phages to transition state analogues is feasible, the use of this technique to select for industrially relevant catalytic properties remains to be established (Legendre et al., 2000). More success has been obtained with the compartmentalization of bacteria that are secret-

ing mutant enzymes. By fixing the mutant bacterial cells in a solid matrix, the diffusion of the secreted mutant protease was delayed. This provides a way of coupling the phenotype to the genotype, inasmuch as the converted growth substrate remains in the same compartment as the bacterial cell (Tawfik and Griffiths, 1998; Griffiths and Tawfik, 2003). Finally, a novel dimension has been given to evolution techniques by the use of genes isolated directly from soil samples (without culturing the donor organism). In a large experiment, genes encoding amylases were cloned directly from soil and identified by expression on starch plates, and the resulting genes have been "evolved" using DNA shuffling. This has resulted in a very thermostable α-amylase (Richardson et al., 2002). This shows that isolating enzyme encoding genes from extremophiles combined with directed evolution in the laboratory can be a path forward for enzyme engineering.

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Literature Cited

Aehle, W., G. Gerritse, and H. B. Lenting. 1995. Lipases with Improved Surfactant Resistance. Patent WO 95/30744.

Aiba, S., K. Kitai, and T. Imanaka. 1983. Cloning and expression of thermostable alpha-amylase gene from Bacillus stearothermophilus in Bacillus stearothermophilus and Bacillus subtilis. Appl. Environ. Microbiol. 46:1059–1065.

Albers, E., and B. W. Muller. 1995. Cyclodextrin derivatives. Pharmaceut. Crit Rev. Ther. Drug Carrier Syst. 12:311–337.

Alkema, W. B., C. M. Hensgens, E. H. Kroezinga, E. De Vries, R. Floris, J. M. Van der Laan, B. W. Dijkstra, and D. B. Janssen. 2000. Characterization of the beta-lactam binding site of penicillin acylase of Escherichia coli by structural and site-directed mutagenesis studies. Protein Engin. 13:857–863.

Genencor Cleaning Enzymes Product List. 2001. Genencor Cleaning Enzymes Product List. Genencor International. Rochester, NY.

Aramori, I., M. Fukagawa, M. Tsumura, M. Iwami, T. Isogai, H. Ono, Y. Ishitani, H. Kojo, M. Kohsaka, Y. Ueda, and H. Imanaka. 1991a. Cloning and nucleotide sequencing of new glutaryl 7-aca and cephalosporin c acylase genes from pseudomonas strains. J. Ferment. Bioengin. 72(4):232–243.

Aramori, I., M. Fukagawa, M. Tsumura, M. Iwami, H. Ono, H. Kojo, M. Kohsaka, Y. Ueda, and H. Imanaka. 1991b. Cloning and nucleotide sequencing of a novel 7 beta-(4-carboxybutanamido)cephalosporanic acid acylase gene of Bacillus laterosporus and its expression in Escherichia coli and Bacillus subtilis. J. Bacteriol. 173:7848–7855.

Balasingham, K., D. Warburton, P. Dunnill, and M. D. Lilly. 1972. The isolation and kinetics of penicillin amidase from Escherichia coli. Biochim. Biophys. Acta 276:250–256.

Barbero, J. L., J. M. Buesa, G. Gonzalez De Buitrago, E. Mendez, A. Perez-Aranda, and J. L. Garcia. 1986. Complete nucleotide sequence of penicillin acylase gene from Kluyvera citrophila. Gene 49:69–80.

Bertola, M. A., W. J. Quax, B. W. Robertson, A. F. Marx, and C. J. van der Laken. 1992. Microbial Esterases and Process for the Preparation of 2-arylpropionic Acids. Patent EP 233656-B.

Betzel, C., K. S. Wilson, and S. Branner. 1988. Crystallization and preliminary X-ray diffraction studies of an alkaline protease from Bacillus lentus. J. Molec. Biol. 204:803–804.

Boesten, W. H. J., and H. M. Moody. 1995. Process for the Enzymatic Preparation of a Beta-lactam Derivative. Patent WO 9503420.

Bolhuis, A., H. Tjalsma, H. E. Smith, A. De Jong, R. Meima, G. Venema, S. Bron, and J. M. van Dijl. 1999. Evaluation of bottlenecks in the late stages of protein secretion in Bacillus subtilis. Appl. Environ. Microbiol. 65:2934–2941.

Brown, S. H., H. R. Costantino, and R. M. Kelly. 1990. Characterization of amylolytic enzyme activities associated with the hyperthermophilic archaebacterium Pyrococcus furiosus. Appl. Environ. Microbiol. 56:1985–1991.

Bruggink, A., E. C. Roos, and E. Devroom. 1998. Penicillin acylase in the industrial production of beta-lactam antibiotics. Organ. Proc. Res. Devel. 2:128–133.

Bruns, W., Hoppe, J., Tsai, H., Bruning, H. J., Maywald, F., Collins, J., Mayer, H. 1985. Structure of the penicillin acylase gene from Escherichia coli: a periplasmic enzyme that undergoes multiple proteolytic processing. J. Mol. Appl. Genet. 3(1):36–44.

Buleon, A., P. Colonna, V. Planchot, and S. Ball. 1998. Starch granules: Structure and biosynthesis. Int. J. Biol. Macromol. 23:85–112.

Callens, M., H. Kersters-Hilderson, W. Vangrysperre, and C. K. De Bruyne. 1988. D-xylose isomerase from Streptomyces violaceoruber: Structural and catalytic roles of bivalent metal ions. Enzyme Microb. Technol. 10:695–700.

Chakrabarty, A. M. 1981. Microorganisms Having Multiple Compatible Degradative Energy-generating Plasmids and Preparation Thereof. US Patent 4,259,444.

Chandra, A. K., S. Medda, and A. K. Bhadra. 1980. Production Of extracellular thermostable alpha-amylase by Bacillus licheniformis. J. Ferment. Technol. 58:1–10.

Chen, W.-P. 1980. Glucose isomerase [review]. Proc. Biochem. 15:36–41.

Christiansen, T., B. Christensen, and J. Nielsen. 2002. Metabolic network analysis of Bacillus clausii on minimal and Semirich medium using (13)C-labeled glucose. Metab. Engin. 4:159–169.

Coffen, D. L. 1997. Enzyme-catalyzed reactions. *In:* S. E. Ahuja (Ed.) Chiral Separations: Applications and Tech-

nology. American Chemical Society. Washington, DC. 59–91.

Collins, N. C., W. D. Grant, and B. E. Jones. 1998a. Gram-negative Alkaliphilic Microorganisms. US Patent 5,733,767.

Collins, N. C., W. D. Grant, and B. E. Jones. 1998b. Gram-positive Alkaliphilic Microorganisms. US Patent 5,707,851.

Cox, M., G. Gerritse, L. Dankmeyer, and W. J. Quax. 2001. Characterization of the promoter and upstream activating sequence from the Pseudomonas alcaligenes lipase gene. J. Biotechnol. 86:9–17.

Crameri, A., G. Dawes, E. Rodriguez Jr., S. Silver, and W. P. Stemmer. 1997. Molecular evolution of an arsenate detoxification pathway by DNA shuffling. Nature Biotechnol. 15:436–438.

Crawford, L., A. M. Stepan, P. C. Mcada, J. A. Rambosek, M. J. Conder,V. A. Vinci, and C. D. Reeves. 1995. Production of cephalosporin intermediates by feeding adipic acid to recombinant Penicillium chrysogenum strains expressing ring expansion activity. Biotechnology NY 13:58–62.

Drenth, J., W. G. J. Hol, J. N. Jansonius, and R. Koekoek. 1972. Subtilisin novo: The three-dimensional structure and its comparison with subtilisin Bpn. Eur. J. Biochem. 26:177–181.

Droge, M. J., R. Bos, and W. J. Quax. 2001. Paralogous gene analysis reveals a highly enantioselective 1,2-o-isopropylideneglycerol caprylate esterase of Bacillus subtilis. Eur. J. Biochem. 268:3332–3338.

Drummond, R. J., W. Bloch, B. W. Matthews, P. L. Toy, and H. H. Nicholson. 1989. Procaryotic xylose isomerase muteins and method to increase protein stability. Patent WO 89/01520.

Edman, M., T. Jarhede, M. Sjostrom, and A. Wieslander. 1999. Different sequence patterns in signal peptides from mycoplasmas, other Gram-positive bacteria, and Escherichia coli: A multivariate data analysis. Prot. Struct. Funct. Genet. 35:195–205.

Eggen, R., A. Geerling, J. Watts, and W. M. Devos. 1990. Characterization of pyrolysin, a hyperthermoactive serine protease from the archaebacterium Pyrococcus furiosus. FEMS Microbiol. Lett. 71:17–20.

El-Khattabi, M., C. Ockhuijsen, W. Bitter, K. E. Jaeger, and J. Tommassen. 1999. Specificity of the lipase-specific foldases of Gram-negative bacteria and the role of the membrane anchor. Molec. Gen. Genet. 261:770–776.

Emtage, J. S., S. Angal, M. T. Doel, T. J. R. Harris, B. Lowe, P. A. Jenkins, and G. Lilley. 1983. Synthesis of calf prochymosin (prorennin) in Escherichia coli (synthetic oligonucleotide/gene expression/industrial enzyme). Proc. Natl. Acad. Sci. USA 80:3671–3675.

Estell, D. A., T. P. Graycar, and J. A. Wells. 1985. Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. J. Biol. Chem. 260:6518–6521.

Farber, G. K., G. A. Petsko, and D. Ringe. 1987. The 3.0 A crystal structure of xylose isomerase from Streptomyces olivochromogenes. Protein Engin. 1:459–466.

Filloux, A., G. Michel, and M. Bally. 1998. GSP-dependent protein secretion in Gram-negative bacteria: The Xcp system of Pseudomonas aeruginosa. FEMS Microbiol. Rev. 22:177–198.

Gat, O., A. Lapidot, I. Alchanati, C. Regueros, and Y. Shoham. 1994. Cloning and dna sequence of the gene coding for Bacillus stearothermophilus T-6 xylanase. Appl. Environ. Microbiol. 60:1889–1896.

Gerritse, G., R. W. Hommes, and W. J. Quax. 1998a. Development of a lipase fermentation process that uses a recombinant Pseudomonas alcaligenes strain. Appl. Environ. Microbiol. 64:2644–2651.

Gerritse, G., R. Ure, F. Bizoullier, and W. J. Quax. 1998b. The phenotype enhancement method identifies the Xcp outer membrane secretion machinery from Pseudomonas alcaligenes as a bottleneck for lipase production. J. Biotechnol. 64:23–38.

Gray, G. L., S. E. Mainzer, M. W. Rey, M. H. Lamsa, K. L. Kindle, C. Carmona, and C. Requadt. 1986. Structural genes encoding the thermophilic alpha-amylases of Bacillus stearothermophilus and Bacillus licheniformis. J. Bacteriol. 166:635–643.

Gray, G. L., S. D. Power, and A. J. Poulouse. 1995. Lipase from Pseudomonas Mendocina Having Cutinase Activity. US Patent 5,389,536.

Griffiths, A. D., and D. S. Tawfik. 2003. Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization. EMBO J. 22:24–35.

Gusakov, A. V., A. P. Sinitsyn, A. G. Berlin, A. V. Markov, and N. V. Ankudimova. 2000. Surface hydrophobic amino acid residues in cellulase molecules as a structural factor responsible for their high denim-washing performance. Enz. Microb. Technol. 27:664–671.

Hakamada, Y., Y. Hatada, K. Koike, T. Yoshimatsu, S. Kawai, T. Kobayashi, and S. Ito. 2000. Deduced amino acid sequence and possible catalytic residues of a thermostable, alkaline cellulase from an alkaliphilic bacillus strain. Biosci. Biotechnol. Biochem. 64:2281–2289.

Hedstrom, G., M. Backlund, and J. P. Slotte. 1993. Enantioselective synthesis of ibuprofen esters in Aot isooctane microemulsions by Candida cylindracea lipase. Biotechnol. Bioengin. 42:618–624.

Henrick, K., D. M. Blow, H. L. Carrell, and J. P. Glusker. 1987. Comparison of backbone structures of glucose isomerase from streptomyces and arthrobacter. Protein Engin. 1:467–469.

Hesselink, P. G. M., S. van Vliet, H. De Vries, and B. Witholt. 1989. Optimization of steroid side chain cleavage by Mycobacterium sp. in the presence of cyclodextrins. Enz. Microb. Technol. 11:398–404.

Hofemeister, J., A. Kurtz, R. Borriss, and J. Knowles. 1986. The beta-glucanase gene from Bacillus amyloliquefaciens shows extensive homology with that of Bacillus subtilis. Gene 49:177–187.

Hofmann, B. E., H. Bender, and G. E. Schulz. 1989. Three-dimensional structure of cyclodextrin glycosyltransferase from Bacillus circulans at 3.4 A resolution. J. Molec. Biol. 209:793–800.

Irwin, D., D. H. Shin, S. Zhang, B. K. Barr, J. Sakon, P. A. Karplus, and D. B. Wilson. 1998. Roles of the catalytic domain and two cellulose binding domains of Thermomonospora fusca E4 in cellulose hydrolysis. J. Bacteriol. 180:1709–1714.

Ishii, Y., Y. Saito, T. Fujimura, T. Isogai, H. Kojo, M. Yamashita, M. Niwa, and M. Kohsaka. 1994. A novel 7-beta-(4-carboxybutanamido)-cephalosporanic acid acylase isolated from Pseudomonas strain C427 and its high-level production in Escherichia coli. J. Ferment. Bioengin. 77:591–597.

Ishiye, M., and M. Niwa. 1992. Nucleotide sequence and expression in Escherichia coli of the cephalosporin acylase gene of a Pseudomonas strain. Biochim. Biophys. Acta 1132:233–239.

Jacobs, M., M. Eliasson, M. Uhlen, and J. I. Flock. 1985. Cloning, sequencing and expression of subtilisin Carlsberg from Bacillus lichenformis. Nucleic Acids Res. 13:8913–8927.

Jager, M., and A. Pluckthun. 1997. The rate-limiting steps for the folding of an antibody Scfv fragment. FEBS Lett. 418:106–110.

Jeong, S. T., H. K. Kim, S. J. Kim, S. W. Chi, J. G. Pan, T. K. Oh, and S. E. Ryu. 2002. Novel zinc-binding center and a temperature switch in the Bacillus stearothermophilus L1 lipase. J. Biol. Chem. 277:17041–17047.

Joly, J. C., and J. R. Swartz. 1997. In vitro and in vivo redox states of the Escherichia coli periplasmic oxidoreductases Dsba and Dsbc. Biochemistry 36:10067–10072.

Joly, J. C., W. S. Leung, and J. R. Swartz. 1998. Overexpression of Escherichia coli oxidoreductases increases recombinant insulin-like growth factor-I accumulation. Proc. Natl. Acad. Sci. USA 95:2773–2777.

Jones, B., and W. Quax. 1998a. Alzheimer tau test and detergent cellulase made by genetic engineering [no. 9 in a series of articles to promote a better understanding of the use of genetic engineering]. J. Biotechnol. 66:229–233.

Jones, B. E., W. D. Grant, A. W. Duckworth, and G. G. Owenson. 1998b. Microbial diversity of soda lakes. Extremophiles 2:191–200.

Jongbloed, A. W., Z. Mroz, and P. A. Kemme. 1992. The effect of supplementary Aspergillus niger phytase in diets for pigs on concentration and apparent digestability of dry matter, total phosphorus and phytic acid in different sections of the animentary tract. J. Anim. Sci. 70:1168.

Jorgensen, O. B., L. G. Karlsen, N. B. Nielsen, S. Pedersen, and S. Rugh. 1988. A new immobolized glucose isomerase with high productivity produced by a strain of Streptomyces murinus. Starch/Starke 40:307–313.

Jorgensen, S. T., and P. L. Jorgensen. 1993. A Process for Expressing Genes in Bacillus Licheniformis. Patent WO 93/10248.

Jung, E. D., G. Lao, D. Irwin, B. K. Barr, A. Benjamin, and D. B. Wilson. 1993. DNA sequences and expression in Streptomyces lividans of an exoglucanase gene and an endoglucanase gene from Thermomonospora fusca. Appl. Environ. Microbiol. 59:3032–3043.

Kelly, A. P., B. Diderichsen, S. Jorgensen, and D. J. McConnell. 1994. Molecular genetic analysis of the pullulanase b gene of Bacillus acidopullulyticus. FEMS Microbiol. Lett. 115:97–105.

Kerovuo, J., and S. Tynkkynen. 2000a. Expression of Bacillus subtilis phytase in Lactobacillus plantarum 755. Lett. Appl. Microbiol. 30:325–329.

Kerovuo, J., J. Rouvinen, and F. Hatzack. 2000b. Analysis of myo-inositol hexakisphosphate hydrolysis by bacillus phytase: Indication of a novel reaction mechanism. Biochem. J. 352:623–628.

Kitahata, S., M. Taniguchi, S. D. Beltran, T. Sugimoto, and S. Okada. 1983. Purification and some properties of cyclodextrinase from Bacillus coagulans. Agric. Biol. Chem. 47:1441–1447.

Knowles, J., P. Lehtovaara, M. Penttila, T. Teeri, A. Harkki, and I. Salovuori. 1987. The cellulase genes of Trichoderma. Ant. v. Leeuwenhoek 53:335–341.

Kobayashi, T., Y. Hakamada, S. Adachi, J. Hitomi, T. Yoshimatsu, K. Koike, S. Kawai, and S. Ito. 1995. Purification and properties of an alkaline protease from alkalophilic Bacillus sp. KSM-K16. Appl. Microbiol. Biotechnol. 43:473–481.

Koch, R., P. Zablowski, A. Spreinat, and G. Antranikian. 1990. Extremely thermostable amylolytic enzyme from the archaebacterium Pyrococcus furiosus. FEMS Microbiol. Lett. 71:21–26.

Koeller, K. M., and C. H. Wong. 2001. Enzymes for chemical synthesis. Nature 409:232–240.

Kontinen, V. P., and M. Sarvas. 1993. The PrsA lipoprotein is essential for protein secretion in Bacillus subtilis and sets a limit for high-level secretion. Molec. Microbiol. 8:727–737.

Kosaric, N., A. Wieczorek, G. P. Cosentino, R. J. Magee, and J. E. Prenosil. 1983. Ethanol fermentation. Biotechnology 3:258–385.

Kreft, J., H. Berger, M. Haertlein, B. Mueller, G. Goebel, and W. Weidinger. 1983. Cloning and expression in E coli and Bacillus subtilis of the hemolysin determinant from Bacillus cereus. J. Bacteriol. 155:681–689.

Lapidot, A., A. Mechaly, and Y. Shoham. 1996. Overexpression and single-step purification of a thermostable xylanase from Bacillus stearothermophilus T-6. J. Biotechnol. 51:259–264.

Legendre, D., N. Laraki, T. Graslund, M. E. Bjornvad, M. Bouchet, P. A. Nygren, T. V. Borchert, and J. Fastrez. 2000. Display of active subtilisin 309 on phage: Analysis of parameters influencing the selection of subtilisin variants with changed substrate specificity from libraries using phosphonylating inhibitors. J. Molec. Biol. 296:87–102.

Li, Y., W. Jiang, Y. Yang, G. Zhao, and E. Wang. 1998. Overproduction and purification of glutaryl 7-amino cephalosporanic acid acylase. Protein Expr. Purif. 12:233–238.

Lin, W., and D. R. Lineback. 1990. Changes in carbohydrate fractions in enzyme-supplemented bread and the potential relationship to staling. Starch 42:385–394.

Luiten, R. G. M., W. J. Quax, P. W. Schuurhuizen, and N. Mrabet. 1990. Novel Glucose Isomerase Enzymes and Their Use. Patent EP 0351029-A.

Lundgren, K. R., L. Berkvist, S. Hogman, H. Joves, G. Eriksson, T. Bartfai, J. van der Laan, E. Rosenberg, and Y. Shoham. 1994. Tcf mill trial on softwood pulp with korsnas thermostable and alkaline stable xylanase T6. FEMS Microbiol. Rev. 13:365–368.

Lyons, T. P. 1982. Proteinase enzymes relevant to the baking industry. Biochem. Soc. Trans. 10:287–290.

Mansfeld, J., G. Vriend, B. W. Dijkstra, O. R. Veltman, B. B. van Den, G. Venema, R. Ulbrich-Hofmann, and V. G. Eijsink. 1997. Extreme stabilization of a thermolysin-like protease by an engineered disulfide bond. J. Biol. Chem. 272:11152–11156.

Matsuda, A., K. Matsuyama, K. Yamamoto, S. Ichikawa, and K. Komatsu. 1987. Cloning and characterization of the genes for two distinct cephalosporin acylases from a Pseudomonas strain. J. Bacteriol. 169:5815–5820.

Matsumura, I., J. B. Wallingford, N. K. Surana, P. D. Vize, and A. D. Ellington. 1999. Directed evolution of the surface chemistry of the reporter enzyme beta-glucuronidase. Nature Biotechnol. 17:696–701.

McPhalen, C. A., and M. N. G. James. 1988. Structural comparison of two serine proteinase-protein inhibitor complexes: Eglin-C-Subtilisin carlsberg and Ci-2-subtilisin novo. Biochemistry 27:6582–6598.

Mondou, F., F. Shareck, R. Morosoli, and D. Kluepfel. 1986. Cloning of the xylanase gene of Streptomyces lividans. Gene 49:323–329.

Nakajima, R., T. Imanaka, and S. Aiba. 1986. Comparison of animo acid sequences of eleven different alpha-amylases. Appl. Microbiol. Biotechnol. 23:355–360.

Nardini, M., D. A. Lang, K. Liebeton, K. E. Jaeger, and B. W. Dijkstra. 2000. Crystal structure of Pseudomonas aeruginosa lipase in the open conformation: The prototype for Family I.1 of bacterial lipases. J. Biol. Chem. 275:31219–31225.

Nishimori, K., Y. Kawaguchi, M. Hidaka, T. Uozumi, and T. Beppu. 1981. Communication: Cloning in Escherichia coli of the structural gene of prorennin, the precursor of calf milk-clotting enzyme rennin. J. Biochem. 90:901–904.

Noble, M. E., A. Cleasby, L. N. Johnson, M. R. Egmond, and L. G. Frenken. 1994. Analysis of the structure of Pseudomonas glumae lipase. Protein Engin. 7:559–562.

Norris, L., F. Norris, L. Christiansen, and N. Fiil. 1983. Efficient site-directed mutagenesis by simultaneous use of two primers. Nucleic Acids Res. 11:5103–5112.

Saari. G. C., Kumar, A. A., Kawasaki, G. H., Insley, M. Y., O'Hara, PJ. 1987. Sequence of the Ampullariella sp. strain 3876 gene coding for xylose isomerase. J. Bacteriol. 169(2):612–618.

Otten, L. G., C. F. Sio, J. Vrielink, R. H. Cool, and W. J. Quax. 2002. Altering the substrate specificity of cephalosporin acylase by directed evolution of the beta-subunit. J. Biol. Chem. 277:42121–42127.

Outtrup, H., and K. Aunstrup. 1975. Production and application of a thermostable alpha-amylase from Bacillus licheniformis. Proceedings of the First Intersectional Congress of the Association of Microbiological Societies IAMS (IUMS) 5:205–210.

Outtrup, H., C. Dambmann, H. Bisgaard-Frantzen, A. A. Olsen, and M. Schuelein. 1998. Alkalophilic Bacillus Sp. Ac13 and Protease, Xylanase, Cellulase Obtainable Therefrom. US Patent 3,723,250.

Park, S. C., Y. W. Choi, and T. K. Oh. 1999. Comparative enzymatic hydrolysis of phytate in various animal feedstuff with two different phytases. J. Vet. Med. Sci. 61:1257–1259.

Peterson, M. G. 1988. DNA sequencing using Taq polymerase. Nucleic Acids Res. 16:10915.

Picard, V., E. Ersdalbadju, A. Q. Lu, and S. C. Bock. 1994. A rapid and efficient one-tube PCR-based mutagenesis technique using PFU DNA polymerase. Nucleic Acids Res. 22:2587–2591.

Quax, W. J., Y. Laroche, A. W. H. Vollebregt, P. Stanssens, and M. Lauwereys. 1991a. Mutant Microbial Alpha-amylases with Increased Thermal, Acid and/or Alkaline Stability. Patent WO 91/00353.

Quax, W. J., N. T. Mrabet, R. G. Luiten, P. W. Schuurhuizen, P. Stanssens, and I. Lasters. 1991b. Enhancing the thermostability of glucose isomerase by protein engineering. Biotechnology NY 9:738–742.

Quax, W. J., A. F. Bonekamp, and M. van Tilborg. 1993. Correct secretion of heterologous proteins from Bacillus licheniformis. *In:* H. S. Baltz (Ed.) Industrial Microorganisms: Basic and Applied Molecular Genetics. ASM Press. Washington, DC. 143–150.

Quax, W. J., and C. P. Broekhuizen. 1994. Development of a new bacillus carboxyl esterase for use in the resolution of chiral drugs. Appl. Microbiol. Biotechnol. 41:425–431.

Reetz, M. T., and K. E. Jaeger. 1998. Overexpression, immobilization and biotechnological application of Pseudomonas lipases. Chem. Phys. Lipids 93:3–14.

Reilly, P. J. 1979. Starch hydrolysis with soluble and immobilized glucoamylase. Appl. Biochem. Bioengin. 2:185–207.

Richardson, T. H., X. Tan, G. Frey, W. Callen, M. Cabell, D. Lam, J. Macomber, J. M. Short, D. E. Robertson, and C. Miller. 2002. A novel, high performance enzyme for starch liquefaction: Discovery and optimization of a low pH, thermostable alpha-amylase. J. Biol. Chem. 277:26501–26507.

Robertson, D. E., D. Murphy, J. Reid, M. M. Antony, S. Link, R. V. Swanson, P. V. Warren, and A. Kosmotka. 1999. Esterases. US Patent 5,942,430.

Roels, J., A., and R. van Tilburg. 1979. Temperature dependence of the stability and the activity of immobilized glucose isomerase. ACS Symp. Series 106:147–172.

Röhm, O. 1915. Verfahren zum Reinigen von Wäschestucken aller Art. Patent DE 283923.

Saeki, K., M. Okuda, Y. Hatada, T. Kobayashi, S. Ito, H. Takami, and K. Horikoshi. 2000. Novel oxidatively stable subtilisin-like serine proteases from alkaliphilic bacillus spp.: Enzymatic properties, sequences, and evolutionary relationships. Biochem. Biophys. Res. Commun. 279:313–319.

Sanders, J. P. M., J. A. van den Berg, P. M. Andreoli, Y. J. Vos, J. H. van Ee, and L. J. S. M. Mulleners. 1985. Molecular Cloning and Expression in Industrial Microorganism Species. Patent EP 0134048.

Saul, D. J., L. C. Williams, R. A. Reeves, M. D. Gibbs, and P. L. Bergquist. 1995. Sequence and expression of a xylanase gene from the hyperthermophile Thermotoga sp. strain Fjss3-B.1 and characterization of the recombinant enzyme and its activity on kraft pulp. Appl. Environ. Microbiol. 61:4110–4113.

Saunders, C. W., and M. S. Guyer. 1986. The Production of Human Serum Albumin in Bacillus: New Gene Fusions, Plasmids and Bacillus Strains Useful in Production of Human Serum Albumin Economically by Cultivation. Patent EP 0229712a2 13.

Schmid, A., J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, and B. Witholt. 2001. Industrial biocatalysis today and tomorrow. Nature 409:258–268.

Schrag, J. D., Y. Li, M. Cygler, D. Lang, T. Burgdorf, H. J. Hecht, R. Schmid, D. Schomburg, T. J. Rydel, J. D. Oliver, L. C. Strickland, C. M. Dunaway, S. B. Larson, J. Day, and A. McPherson. 1997. The open conformation of a pseudomonas lipase. Structure 5:187–202.

Schroen, C. G., S. Vandewiel, P. J. Kroon, E. Devroom, A. E. Janssen, and J. Tramper. 2000. Equilibrium position, kinetics, and reactor concepts for the adipyl-7-Adca-hydrolysis process [in process citation]. Biotechnol. Bioengin. 70:654–661.

Schumacher, G., D. Sizmann, H. Haug, P. Buckel, and A. Boeck. 1986. Penicillin acylase from E. coli: Unique gene-protein relation. Nucleic Acids Res. 14:5713–5727.

Shendye, A., and M. Rao. 1993. Cloning and extracellular expression in Escherichia coli of xylanases from an alkaliphilic thermophilic bacillus sp Ncim-59. FEMS Microbiol. Lett. 108:297–302.

Shibuya, Y., K. Matsumoto, and T. Fujii. 1981. Isolation and Properties of 7β-(4-carboxybutanamido) cephalosporanic acid acylase-producing bacteria. Agric. Biol. Chem. 45:1561–1567.

Simons, P. C. M., H. A. J. Versteegh, A. V. Jongbloed, P. A. Kemme, P. Skump, K. D. Bos, M. G. E. Wolters, R. F. Beudeker, and G. Verschoor. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr. 64:525–540.

Sio, C. F., A. M. Riemens, J. M. van der Laan, R. M. Verhaert, and W. J. Quax. 2002. Directed evolution of a glutaryl acylase into an adipyl acylase. Eur. J. Biochem. 269: 4495–4504.

Spezio, M., D. B. Wilson, and P. A. Karplus. 1993. Crystal structure of the catalytic domain of a thermophilic endocellulase. Biochemistry 32:9906–9916.

Stemmer, W. P. 1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature 370:389–391.

Stephenson, K., and C. R. Harwood. 1998a. Influence of a cell-wall-associated protease on production of alpha-amylase by Bacillus subtilis. Appl. Environ. Microbiol. 64:2875–2881.

Stephenson, K., N. M. Carter, C. R. Harwood, M. F. Petitglatron, and R. G. Chambert. 1998b. The influence of protein folding on late stages of the secretion of alpha-amylases from Bacillus subtilis. FEBS Lett. 430:385–389.

Tamuri, M., M. Kanno, and Y. Ishii. 1997. Heat and Acid-stable Alpha-amylase Enzymes and Processes for Producing the Same. US Patent 4283722.

Tawfik, D. S., and A. D. Griffiths. 1998. Man-made cell-like compartments for molecular evolution. Nat. Biotechnol. 16:652–656.

Tjalsma, H., M. A. Noback, S. Bron, G. Venema, K. Yamane, and J. M. van Dijl. 1997. Bacillus subtilis contains four closely related Type I signal peptidases with overlapping substrate specificities: Constitutive and temporally controlled expression of different Sip genes. J. Biol. Chem. 272:25983–25992.

Van den Burg, B., G. Vriend, O. R. Veltman, G. Venema, and V. G. Eijsink. 1998. Engineering an enzyme to resist boiling. Proc. Natl. Acad. Sci. USA 95:2056–2060.

van der Laan, J. C., Gerritse, G., Mulleners, L. J., van der Hoek, R. A., Quax, W. J. 1991. Cloning, characterization, and multiple chromosomal integration of a Bacillus alkaline protease gene. Appl. Environ. Microbiol. 57(4):901–909.

Van der Laan, J. M., A.V. Teplyakov, H. Kelders, K. H. Kalk, O. Misset, L. S. J. M. Mulleners, and B. W. Dijkstra. 1992. Crystal structure of the high-alkaline serine protease-Pb92 from Bacillus alcalophilus. Protein Engin. 5:405–411.

Van Dijl, J. M., A. Dejong, J. Vehmaanpera, G. Venema, and S. Bron. 1992. Signal peptidase-I of Bacillus subtilis: Patterns of conserved amino acids in prokaryotic and eukaryotic Type-I signal peptidases. EMBO J. 11:2819–2282.

Van Eekelen, C. A. G., J. C. van der Laan, and L. J. S. Mulleners. 1988. Molecular Cloning and Expression of Genes Encoding Proteolytic Enzymes. Patent EP 0283075.

Van Eekelen, C. A. G., L. J. S. Mulleners, J. C. van der Laan, O. Misset, R. A. Cuperus, and J. H. Alensink. 1989. Novel Proteolytic Enzymes and Their Use in Detergents. Patent EP 0328229.

van Leen. R. W., Bakhuis, J. G., van Beckhoven, R. F., Burger, H., Dorssers, L. C., Hommes, R. W., Lemson, P. J., Noordam, B., Persoon, N. L., Wagemaker, G. 1991. Production of human interleukin-3 using industrial microorganisms. Biotechnology 9:47–52.

Van Pouderoyen,G., T. Eggert, K. E. Jaeger, and B. W. Dijkstra. 2001. The crystal structure of Bacillus subtilis lipase: A minimal alpha/beta hydrolase fold enzyme. J. Molec. Biol. 309:215–226.

Van Solingen, P., D. Meijer, W. A. van der Kleij, C. Barnett, R. Bolle, S. D. Power, and B. E. Jones. 2001. Cloning and expression of an endocellulase gene from a novel streptomycete isolated from an East African soda lake. Extremophiles 5:333–341.

Van Straten, N. C. R., H. I. Duynstee, E. Devroom, G. A. Van der Marel, and J. H. van Boom. 1997. Enzymatic cleavage of N-phenylacetyl-protected ethanolamine phosphates. Liebigs Annalen 0 (6):1215–1220.

Verhaert, R. M., A. M. Riemens, J. M. van der Laan, J. van Duin, and W. J. Quax. 1997. Molecular cloning and analysis of the gene encoding the thermostable penicillin g acylase from Alcaligenes faecalis. Appl. Environ. Microbiol. 63:3412–3418.

Verhaert, R. M., J. Beekwilder, R. Olsthoorn, J. van Duin, and W. J. Quax. 2002. Phage display selects for amylases with improved low pH starch-binding. J. Biotechnol. 96:103–118.

Wallerstein, L. 1939. Enzyme preparation from microorganisms. Indust. Engin. Chem. 31:1218–1224.

Welker, N. E., and L. L. Campbell. 1967a. Comparison of the alpha-amylase of Bacillus subtilis and Bacillus amyloliquefaciens. J. Bacteriol. 94:1131–1135.

Welker, N. E., and L. L. Campbell. 1967b. Unrelatedness of Bacillus amyloliquefaciens and Bacillus subtilis. J. Bacteriol. 94:1124–1130.

Wind, R. D., J. C. Uitdehaag, R. M. Buitelaar, B. W. Dijkstra, and L. Dijkhuizen. 1998. Engineering of cyclodextrin product specificity and pH optima of the thermostable cyclodextrin glycosyltransferase from Thermoanaerobacterium thermosulfurigenes Em1. J. Biol. Chem. 273:5771–5779.

Wong, H. C., T. Ting, H.-C. Lin, F. Reichert, K. Myambo, K. W. K. Watt, P. L. Toy, and R. J. Drummond. 1991. Genetic organization and regulation of the xylose degradation genes in Streptomyces rubiginosum. J. Bacteriol. 173:6849–6858.

Xie, Y., S. E. van De, T. De Weerd, and N. H. Wang. 2001. Purification of adipoyl-7-amino-3-deacetoxycephalosporanic acid from fermentation broth using stepwise elution with a synergistically adsorbed modulator. J. Chromatogr. A908:273–291.

Yamagata, H., and S. Udaka. 1994. Starch-processing enzymes produced by recombinant bacteria. Bioprocess. Technol. 19:325–340.

Ye, R. Q., J. H. Kim, B. G. Kim, S. Szarka, E. Sihota, and S. L. Wong. 1999. High-level secretory production of intact, biologically active staphylokinase from Bacillus subtilis. Biotechnol. Bioengin. 62:87–96.

Yuuki, T., T. Nomura, H. Tezuka, A. Tsuboi, H. Tsukagoshi, N. Yamagata, and S. Udaka. 1985. Complete nucleotide sequence of gene coding for heat- and pH-stable alpha-amylase of Bacillus licheniformis: Comparison of the amino acid sequence of 3 bacterial liquefying alpha-amylases deduced from the DNA. J. Biochem. 98:1147–1156.

Zhao, H. M., L. Giver, Z. X. Shao, J. A. Affholter, and F. H. Arnold. 1998. Molecular evolution by STaggered Extension Process (STEP) in vitro recombination. Nature Biotechnol. 16:258–261.

Zhu, G. P., D. Luo, Y. F. Cai, X. Y. Zhu, M. K. Teng, and Y. Z. Wang. 2000. Mutations of Q20l and G247d improved the specific-activity and optimum pH of glucose isomerase. Sheng Wu Gong. Cheng Xue. Bao. 16:469–473.

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CHAPTER 3.5

Bacteria in Food and Beverage Production

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Introduction

Foods are plants and animals (or their products) and each often contain many types of microorganisms. These microbes from natural and external sources contaminate foods by contact, which can occur anytime between production and consumption. Microbial contamination of foods can have many undesirable consequences ranging from spoilage to foodborne illness. However, some microorganisms possess properties that can benefit food production or conversion. Many food-grade microorganisms are used to produce a variety of fermented foods from raw animal and plant materials. The acidic, and in part organolytic, properties of fermented products result from the fermentative activities of these microorganisms. Foods such as ripened cheeses, fermented sausages, sauerkraut and pickles have not only a greatly extended shelf life compared to the raw materials from which they are derived, but also aroma and flavor characteristics contributed directly or indirectly by the fermenting organisms. Consumption of fermented food product has increased dramatically during the last two decades and will continue to increase. The production and availability of fermentative microorganisms (starter cultures) used in food conversion have advanced to meet this demand. This includes the development of novel and better strains through genetic engineering.

Lactic acid bacteria (LAB) are among the most important groups of microorganisms used in food fermentations. LAB contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances (bacteriocins) and large amounts of lactic acid. Many LAB benefit human and animal health, whereas others spoil beer, wine, and processed meats. They can be isolated from the respiratory, intestinal, and genital tracts of humans and animals and from plants.

Fermented dairy products have been made for thousands of years, but only within the last century has the microbiological basis of these fermentations been elucidated. And LAB are the principal organisms involved in fermenting dairy products. Prior to the availability of starter cultures, milk fermentations relied on the LAB naturally present in raw milk. The first commercial starter cultures, unknown mixes of strains most likely from raw milk, were prepared in Denmark just before the end of the 19th century. In the 1930s and 40s, the concept of pure single-strain starter cultures evolved, and a bacteriophage was identified as an important agent of slow fermentation in cheese. With their development in the 1960s and 70s, concentrated (often frozen or freeze-dried) starter cultures could be inoculated directly into the bulk starter vessel without the need for prior preparation in the creamery. In the last two decades, major advances on dairy starter technology have included improved culture selection procedures that enhance bacteriophage resistance. Molecular technology has been applied to map the genetic constructs of starter culture organisms and, by using plasmid/gene transfer mechanisms, to improve starter culture performance.

Sausage is one of the oldest processed meat products. The writings of ancient Egyptians described the preservation of meat by salting and sun drying. The ancient Babylonians, Greeks, and Romans used sausage as a food source during times of war. Microorganisms were recognized as being important to the production of sausages not until about 1921. In the 1940s and 1950s, pure microbial starter cultures became available to processors, and in 1968 frozen culture concentrates became commercially available to the meat industry. Use of these cultures was not widespread until the early 1980s largely because producers clung to the traditional methods of making sausage (using previously fermented meat as the source of LAB) and feared they might lose the quality and consumer acceptance of their final product. Today the importance of the use of starter cultures is recognized in most countries.

The fermentation of vegetables, a practice that originated in the Orient, has been used as a means of preserving food for more than 2,000

years. In the third century B.C. during the construction of the Great Wall of China, the Chinese produced fermented vegetables (cabbages, radishes, turnips, cucumbers, etc.) on a large scale. The most common fermented vegetables available in the United States are pickles, sauerkraut, and olives. Carrots, cauliflower, celery, okra, onions, and sweet and hot peppers also are sold as fermented vegetable products.

Currently, more than 2,000 different fermented foods are consumed by humans worldwide; many are ethnic and produced in small quantities to meet the demand of a group in a particular region. Some are produced commercially, and only a few by large commercial food processors. As consumers' interest in natural and health foods increases, future consumption of fermented foods also will increase significantly worldwide.

Classification of Bacteria in Food and Beverage Products

The classical approach to bacterial classification is based on morphological and physiological features. LAB are Gram-positive, non-sporeforming cocci, coccobacilli, or rods with a DNA base composition of less than 50 mol% G+C (Stiles and Holzapfel, 1997). They generally lack catalase and ferment glucose mainly to lactic acid (homofermentative) or to lactic acid, CO_2, and ethanol or acetic acid or both (heterofermentative). The importance of LAB in the fermentation of food products (dairy, meat, vegetables, fruits, and beverages) has been used as a basis to differentiate the group, although some are also members of normal flora of the mouth, intestine, and vagina of mammals (Klein et al., 1998). Therefore, LAB associated with food are generally restricted to the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. Table 1 shows differential characteristics of LAB at the genus level (Axelsson, 1998). Bacterial taxonomic classification has been expanded recently to include cell wall composition (de Ambrosini et al., 1996), cellular fatty acids (Gilarova et al., 1994), isoprenoid quinones and other characteristics of cells (Stiles and Holzapfel, 1997). Molecular characteristics also have become important taxonomic tools, such as electrophoretic properties of the gene products, DNA:DNA hybridization studies, and structures and sequence of ribosomal RNA (rRNA; Collins-Thompson et al., 1991; Makela et al., 1992; Stackebrandt and Teuber, 1988; Vandamme et al., 1996). The 16S rRNA data for LAB suggest new groupings that cross the established taxonomic lines. Not all of the new groupings have become established in bacterial taxonomy, but recent phylogenetic considerations indicate that the lactobacilli, leuconostocs and pediococci can be reclassified as three major groups: the *Leuconostoc* group, the *Lactobacillus delbrueckii* group, and the *Lactobacillus casei-Pediococcus* group. The newly established genera *Carnobacteria*, *Tetragenococcus* (previously *Pediococcus halophilus*) and *Vagococcus* (previously motile streptococci) form a phylogenetic cluster with the genus *Enterococcus* (Vandamme et al., 1996). However, generally LAB that are important to food microbiology include only certain species of the genera *Lactobacillus*, *Lactococcus* (*Streptococcus*), *Leuconostoc* and *Pediococcus* (Stiles and Holzapfel, 1997). The genus *Lactobacillus* is by far the largest of the genera included in LAB. It is also very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties.

Based on sugar fermentation patterns, LAB can be further divided into three broad metabolic categories (Axelsson, 1998). The first category includes the group I lactobacilli and some individual species from other genera that are obligately homofermentative, meaning the sugars can only be fermented by glycolysis. The second category includes leuconostocs, group III lactobacilli, oenococci, and weissellas that are obligately heterofermentative, meaning that only the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway is available for sugar fermentation. The apparent difference on the enzyme level between these two categories is the presence or absence of the key enzymes of glycolysis and the 6-PG/PK pathway. The third category, including the remaining LAB (i.e., group II lactobacilli and most species of enterococci, lactococci, pedicocci, streptococci, tetragenococci, and vagococci), holds an intermediate position. They resemble the obligately homofermentative LAB in that they possess a constitutive fructose-1,6-diphosphate aldolase, resulting in the use of glycolysis form hexose fermentation.

Other Bacteria In addition to LAB, other bacteria also are involved in food fermentations, some of which contribute significantly to flavor development and other characteristics of fermented products. Propionibacteria are probably best known for their role as dairy starter cultures, in which they produce the characteristic eyes and flavor of Swiss-type cheeses (Cogan and Accolas, 1996). The family Propionibacteriaceae, genus *Propionibacterium* and the closely related genus *Corynebacterium*, are classified as members of the Actinomycetaceae group. Five species of dairy propionibacteria are currently

Table 1. Differential characteristics of lactic acid bacteria at the genus level, based on morphology and physiology.

	Rods		Cocci						
Characteristic	*Carnobacterium*	*Lactobacillus*	*Enterococcus*	*Lactococcus Vagococcus*	*Leuconostoc Oenococcus*	*Pediococcus*	*Streptococcus*	*Tetragenococcus*	*Weissella*[a]
Tetrad formation	–	–	–	–	–	+	–	+	–
CO_2 from glucose[b]	–[c]	±	–	–	+	–	–	–	+
Growth:									
at 10°C	+	±	+	+	+	±	–	+	+
at 45°C	–	±	+	–	–	±	±	–	–
in 6.5% NaCl	ND[d]	±	+	–	±	±	–	+	±
in 18% NaCl	–	–	–	–	–	–	–	+	–
at pH 4.4	ND	±	+	±	±	+	–	–	±
at pH 9.6	–	–	+	–	–	–	–	+	–
Lactic acid[e]	L	D, L, DL	L	L	D	L, DL	L	L	D, DL

+, positive; –, negative; ±, response varies between species; ND, not determined.
[a]may also be rods.
[b]test for homo- or heterofermentation of glucose; +, homofermentation; –, heterofermentation.
[c]small amounts of CO_2 can be produced.
[d]no growth in 8% NaCl has been reported.
[e]configuration of lactic acid produced from glucose.
Modified from Axelsson (1998).

recognized: *P. freudenreichii* subsp. *freudenreichii*, *P. freudenreichii* subsp. *shermanii*, *P. thoenii*, *P. acidipropionici*, and *P. jensenii*. In addition to being important starter organisms in dairy fermentations, propionibacteria also contribute to natural fermentations of silage and olives and can produce a variety of industrially important products (Jay, 1996).

Acetobacters are Gram-negative aerobic rods and cocci and consist of three species, *Acetobacter aceti*, *A. pasteurianus*, and *A. peroxydans* (Lee, 1996). The organisms are widely distributed in nature where they are abundant in plant materials undergoing alcoholic fermentations. They are important for their role in the production of vinegar.

Fundamental Metabolism

The essential feature of LAB metabolism is efficient carbohydrate fermentation coupled to substrate-level phosphorylation. The generated adenosine triphosphate (ATP) is subsequently used for biosynthetic purposes. LAB as a group exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is lactic acid (>50% of sugar carbon). However, LAB adapt to various conditions and change their metabolism accordingly. This may lead to significantly different end-product patterns.

Carbohydrate Metabolism

LAB, as non-respiring microorganisms, principally generate ATP by fermentation of carbohydrates coupled to substrate-level phosphorylation. Many compounds can serve as fermentable growth substrates, and many pathways for their fermentation have evolved (Kandler, 1983). These pathways have the following three general stages (Thompson, 1988): 1) Conversion of the fermentable compound to the phosphate donor for substrate phosphorylation. This stage often contains metabolic reactions in which NAD^+ is reduced to NADH. 2) Phosphorylation of ADP by the energy-rich phosphate donor. 3) Metabolic steps that bring the products of the fermentation into chemical balance with the starting materials. The most frequent requirement in the last stage is a mechanism for oxidation of NADH, generated in the first stage of fermentation, to NAD^+ so that the fermentation can proceed. The two major pathways for metabolism of hexose in lactic acid bacteria are the homofermentative (Embden-Meyerhof) and heterofermentative (phosphoketolase) pathways (Figs. 1 and 2). The transport and phosphorylation of sugars occur according to the following metabolisms: transport of free sugar and phosphorylation by an ATP-dependent glucokinase for glucose; other sugars, such as mannose and fructose, enter the major pathways at the level of glucose-6-phosphate or fructose-6-phosphate after isomerization or phosphorylation or both (Axelsson, 1998). One exception is galactose metabolism in LAB which use the phosphoenolypyruvate:sugar phosphotransferase system (PTS) for uptake of this sugar. Some species of LAB use this system for all sugars, in which phosphoenolypyruvate is the phosphoryl donor.

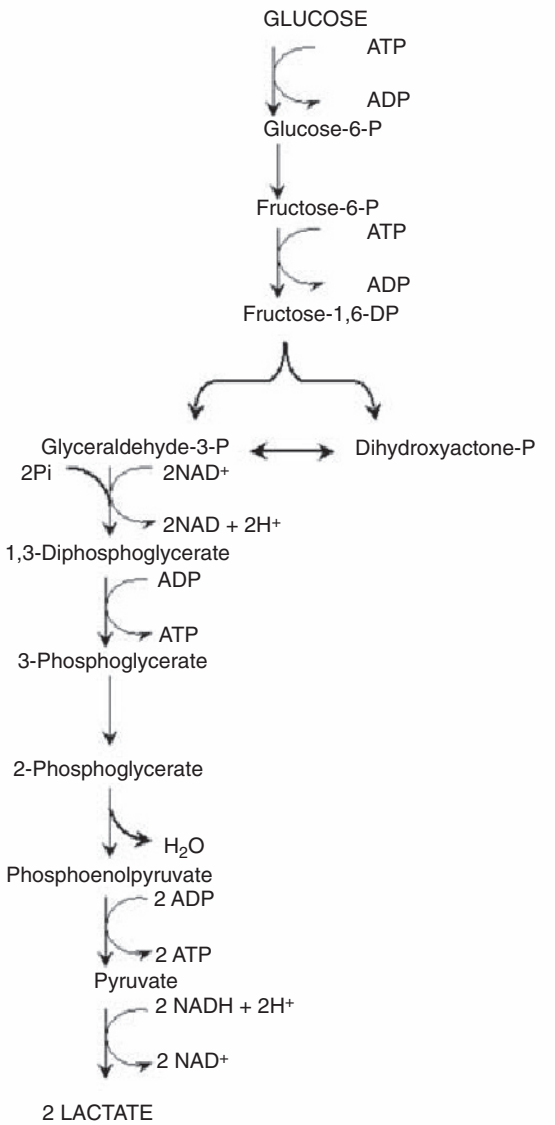

Fig. 1. Homolactic fermentation pathway of glucose (glycosis, Embden-Meyerhof pathway).

Proteolysis LAB have a very limited capacity to synthesize amino acids using inorganic nitrogen sources (Mayo, 1993). They are therefore

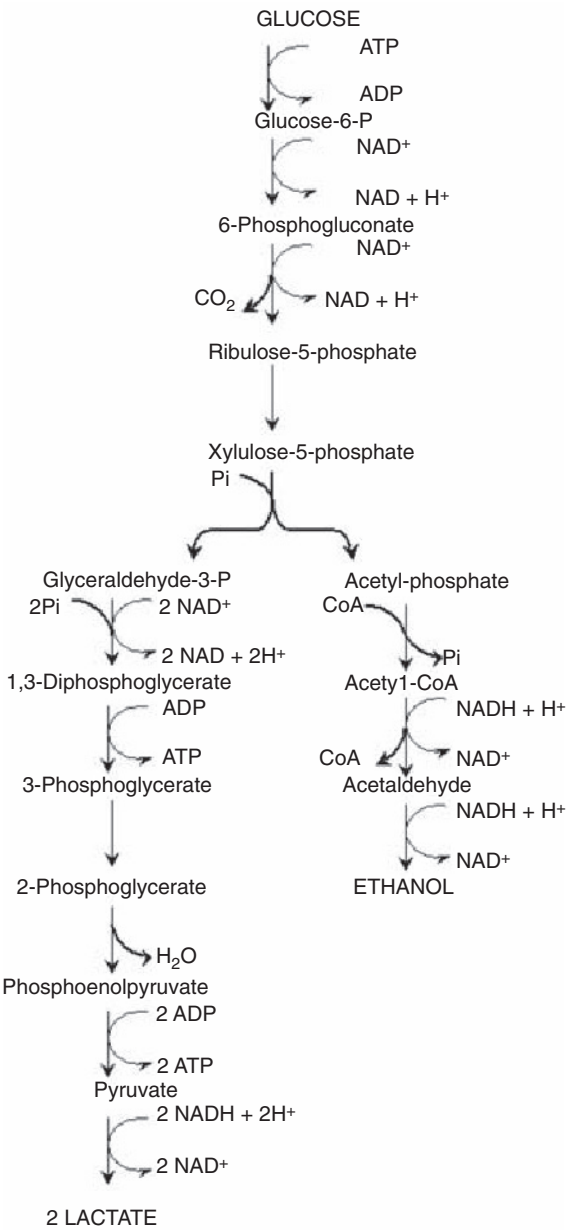

Fig. 2. Heterolactic fermentation pathway of glucose (6-phosphogluconate/phosphoketolase pathway).

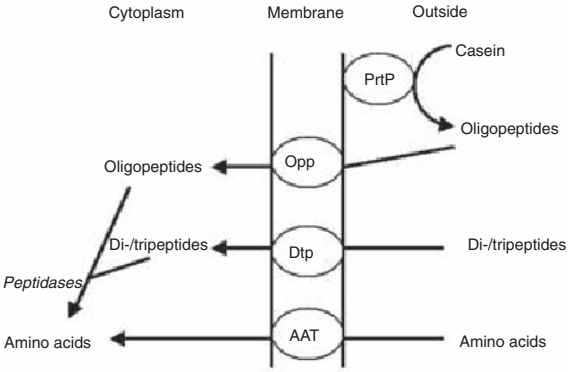

Fig. 3. Model of the lactococcal proteolytic system. Included also is transport of di- and tripeptides and free amino acids, but they contribute very little to the total growth of lactococci. PrtP, membrane-anchored proteinase; Opp, oligopeptide transport system; Dtp, di-/tripeptide transport system; AAT, amino acid transport system.

dependent on preformed amino acids being present in the growth medium as a source of nitrogen. The requirement for amino acids differs among species and strains within species (Chebbi et al., 1977). Some strains are prototrophic for most amino acids, whereas others may require 13–15 amino acids. The quantities of free amino acids present in food often are not sufficient to support the growth of bacteria to a high cell density; therefore, they require a proteolytic system capable of utilizing the peptides and proteins present in food that hydrolyzes proteins to obtain essential amino acids. All dairy lactococci used for acidification of milk (e.g., in cheese manufacture) have proteolytic activity (Poolman et al., 1995). The lactococcal proteolytic system consists of enzymes outside the cytoplasmic membrane, transport systems, and intracellular enzymes. The transport systems on the cell membrane include oligopeptide transport, di/tripeptide transport, and amino acid transport systems (Fig. 3; Pritchard and Coolbear, 1993). The transport of free amino acids contributes very little to the total growth of lactococci in milk. Two general classes of proteolytic enzymes of LAB are proteinases and peptidases. An extracellular, membrane-anchored serine proteinase (PrtP) has been identified as being essential for this activity. Once inside the cell, peptides are hydrolyzed by peptidases.

The proteolytic activity also contributes to the development of the flavor and rheological characteristics of fermented products (Mayo, 1993). For many varieties of cheeses, such as Swiss and Cheddar, desirable flavor tones are derived by proteolysis. However, proteolysis also can lead to undesirable flavors due to the accumulation of bitter peptides.

Bacteriocin Production Bacteriocins are protein antibiotics of relative high molecular weight that affect largely the same or closely related species by adsorption to receptors on the target cells. Bacteriocins can be added as a preservative (e.g., nisin), or they can be produced in the product (e.g., starter culture) or in the gastrointestinal tract (e.g., probiotic strains; De Vuyst and Vandamme, 1994; Montville and Winkowski, 1997). There are four classes of bacteriocins produced by LAB: 1) lantibiotics; 2) small hydrophobic

heat-stable peptides; 3) large heat-labile protein; and 4) complex bacteriocins (Table 2; Ouwehand, 1998).

Class I: Class I bacteriocins or lantibiotics are small peptides containing the unusual dehydroamino acids and thioether amino acids lanthionin and 3-methyllanthionine, which are synthesized by Gram-positive bacteria during posttranslational modifications. These peptides are thought to attach to the membrane of target cells and, by an as yet unknown conformational rearrangement, lead to increased permeability and disruption of the membrane potential. There are two types of lantibiotics, types A and B. The lantibiotics produced by LAB all belong to type A, which are elongated screw-shaped peptides, whereas type B lantibiotics are mainly globular. Nisin produced by *Lactococcus lactis* ssp. *lactis* has been studied extensively. It has a broad spectrum of activity against Gram-positive bacteria. The primary target is believed to be the cell membrane. Unlike some other antimicrobial peptides, nisin does not need a receptor for its interaction with the cell membrane; however, the presence of a membrane potential is required.

Class II: Class II bacteriocins contain a wide variety of small heat-stable peptides and have been subdivided into three subclasses (Table 2; Eijsink et al., 1998). Numerous class II bacteriocins are membrane-active peptides that destroy the integrity of the membrane by forming pores. Lactococcin is an example. In contrast to nisin, lactococcins require a specific receptor protein to act on target cells regardless of their membrane potential.

Classes III and IV: These bacteriocins differ markedly from those of Classes I and II. The larger (>30 kDa) heat-labile antimicrobial proteins such as lacacins A and B are classified as class III bacteriocins. Class IV bacteriocins such as leuconocin S and pediocin SJ-1 have lipid or carbohydrate moieties. The mechanisms of action and immunity of these complex bacteriocins remain unknown.

Starter Cultures

Starter cultures are food-grade microorganisms of known and stable metabolic activities and other characteristics that are used to produce fermented foods of desirable appearance, body, texture and flavor (Ray, 1996). Starter cultures in manufacture of food products were used long before it was known that bacteria were involved at all. Initial development of starter cultures resulted from the need and changes in the cheese industry. Since the early 1900s, there has been a marked worldwide increase in the industrial production of fermented food products. Process technology has progressed toward greater mechanization, larger factory size, shorter processing times, and more food processed daily in the processing plant. All of this relies on the optimization of the starter culture's activity, whereby the culture must maintain stable fermentative properties and resistance to bacteriophage (Lee, 1996). Currently, starter cultures for many types of fermented foods, including dairy products, meat products, vegetable products, and baking products, and for alcohol fermentation are commercially available.

Development of Starter Cultures Starter cultures used in dairy products can be divided into mesophilic and thermophilic cultures based on their optimum growth temperature (Table 3; Cogan and Accolas, 1996). Mesophilic cultures grow at temperatures of 10–40°C, with an optimum of ca. 30°C. Composed of acid-forming lactococci as well as flavor-producing bacteria, these cultures are used to make a variety of cheeses, fermented milk products, and ripened cream butter. Thermophilic cultures have optimum temperatures of 40–50°C and are used for yogurt and cheese varieties with high cooking temperatures. Starter cultures also have been used to produce meat products such as sausages and fermented vegetables. *Pediococcus acidilactici*, *Lactobacillus plantarum* and/or *Staphylococcus carnonsus* are often used as starter cultures

Table 2. Classes of bacteriocins produced by lactic acid bacteria.

Class	Subclass	Description	Bacteriocin producer
I		Lantibiotics	*Carnobacterium*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus*
II		Small (<10kDa), heat stable (100–121°C)	*Carnobacterium*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus*
	IIa	*Listeria*-active peptides	
	IIb	Two-peptide bacteriocins	
	IIc	Thiol-activated peptides	
III		Large (>30kDa) heat-labile proteins	*Lactobacillus*
IV		Complex bacteriocins: protein with lipid and/or carbohydrate	*Lactobacillus*, *Leuconostoc*, *Pediococcus*

Table 3. Examples of mesophilic and thermophilic starter culture organisms used by the dairy industry.

Type	Species
Mesophilic	*Lactococcus lactis* spp. *lactis*
	Lactococcus lactis spp. *cremoris*
	Lactococcus lactis spp. *lactis* var. *diacetylactis*
	Leuconostoc lactis
	Leuconostoc cremoris
Thermophilic	*Lactobacillus delbrueckii* spp. *lactis*
	Lactobacillus helveticus
	Lactobacillus delbrueckii spp. *bulgaricus*
	Lactobacillus acidophilus
	Streptococcus salivarius spp. *thermophilus*

in sausage production, whereas cabbage, cucumbers, and olives are fermented with *Lactobacillus plantarum*. Starter cultures, usually composed of different species or of several strains of a single species, are often categorized on the basis of their composition (Ray, 1996): 1) single-strain starter: one strain of a certain species; 2) multiple-strain starter (defined-strain starter): different known strains of one species; 3) multiple-mixed-strain starter: different defined strains of different species; and 4) raw mixed-strain starter: species and strains partly or all unknown.

General Requirements for Starter Cultures (Buckenhuskes, 1993)

Safety Starter cultures contain bacteria that lack virulence factors for humans and thus are free of pathogens and toxins.

Technological Effectiveness Starter culture bacteria dominate over naturally occurring microflora. The microorganisms perform the required metabolic activities. Starter culture preparations are free of bacteriophage and microorganisms that may inhibit or reduce starter culture activity.

Economic Aspects The propagation and production of starter cultures must be economically feasible. The starter culture can be preserved by freezing or freeze-drying with little practical loss of activity. Essential properties of starter cultures such as fermentative ability are stable under defined storage conditions for several months.

Frozen concentrated starter cultures were developed in the 1970s for direct inoculation into substrates to be cultured. Efforts have been made to produce freeze-dried concentrated cultures, which have less bulk and don't require transport on dry ice, thereby eliminating the problem of accidental thawing. The dried cultures can be used directly for product manufacture or to produce bulk starters (Cogan and Accolas, 1996). However, many starter culture strains do not survive well in the dried state. Hence, the care of dried cultures in large commercial operations has been limited. A recent advance has been the availability of custom-designed starter cultures to meet the specific needs of a food processor. An understanding of the genetic basis of both desirable fermentative characteristics as well as phage inhibition defenses of starter cultures has advanced the production of designer cultures.

Genetics of LAB Starter Cultures

The characterized genetic elements of LAB starter cultures include chromosomes, transposable elements, and plasmids (Gasson, 1990; Rodriguez and Vidal, 1990). Chromosomes of LAB are smaller than those of other eubacteria, ranging from 1.1 to 2.6 Mbp, depending on the species. Transposable elements, genetic elements capable of moving as discrete units from one site to another in the genome, have been identified in LAB. Insertion sequences, the simplest of transposable elements, are widely distributed in bacteria and also have been found in LAB. Their ability to mediate molecular rearrangements and affect gene regulation has had both positive and negative implications for food fermentations (Gasson, 1990). Plasmids have been identified in many LAB. Some plasmids encode many of the activities essential for food fermentations (de Vos, 1999), including lactose metabolism, proteinase activity, oligopeptide transport, bacteriophage resistance mechanisms, bacteriocin production and immunity, bacteriocin resistance, exopolysaccharide production, and citrate utilization.

Natural gene transfer systems among LAB are largely transduction and conjugation, although transformation also has been reported as a means of genetic exchanges (Rodriguez and Vidal, 1990). Transduction, the transfer of bacterial genetic material by a bacteriophage, has been demonstrated in lactococci, lactobacilli, and *Streptococcus thermophilus*. Usefulness of transduction in construction of strains for the fermentation industry is limited because of the relatively narrow host range of transducing bacteriophage. Conjugation, the transfer of genetic material from one bacterial cell to another, which requires cell-to-cell contact, has been well characterized in lactococci. Most plasmid-encoded characteristics important in the manufacture of fermented dairy products can be transferred by conjugation. Using an approach that does not require antibiotic-resistance markers, conjugation has been used to transfer plasmids that encode bacteriophage-resistance genes into commercial lactococcal strains (Verrips and van den Berg, 1996). These strains have enhanced resistance to infection by bacterioph-

age and have been used successfully in the dairy industry for years.

Starter Culture Improvement Past emphasis on starter culture improvement was largely based on screening natural isolates for traits of interest and monitoring existing strains to select for beneficial variations. More recently, advances in molecular technology have enabled us to understand more about the biology, physiology, and taxonomy of LAB and other microorganisms important to food fermentations (McKay and Baldwin, 1990). In the future, it may be possible to definitively identify and then combine the most desirable nutritional, sensory, and/or therapeutic properties of starter cultures to construct "superior" strains for food fermentations (Geisen and Holzapfel, 1996). At present, however, strain improvement focuses on eliminating problems that beset their use as starter cultures. Features such as bacteriophage infection, genetic instability, variation and unpredictability in performance, and the production of low-grade or poor-quality products all lead to economic losses. These are characteristics that have been examined extensively with interest in identifying and generating strains with superior attributes. Considerable research has been devoted to studying phage and phage-host interactions in LAB, the mechanisms by which lactose, citrate, and protein are metabolized, and the basis for instability and unpredictability in strain performance. Also because of their food-grade, nonpathogenic, generally recognized as safe (GRAS) status, LAB are considered to be ideal hosts for the production of proteins and other compounds that they do not produce naturally and that have medical or food-related applications (Kuipers et al., 1997).

Bacteriophage and Bacteriophage Resistance Food fermentations rely on actively growing LAB that either are added as starter cultures or grow spontaneously in the food matrix. The fermentation capabilities of LAB can be severely inhibited by bacteriophage infection, which has been a major commercial problem (Klaenhammer, 1991). Bacteriophages are bacterial viruses that were first identified as "filter-transmissible" agents at the beginning of 1900. Significant progress has been made toward the characterization of bacteriophages from LAB. All of the bacteriophages examined contain double-stranded linear DNA genomes with either cohesive or circularly permuted terminally redundant ends. Both lytic and temperate bacteriophages have been identified. Bacteriophage infection may lead to a decrease or complete inhibition of lactic acid production by the starter culture. This effect has had a major impact on the manufacture of fermented food products, as lactic acid synthesis is required to produce these products. In addition, slow acid production disrupts manufacturing schedules and typically results in products that are of lower economic value. Therefore, bacteriophage resistance remains one of the most important characteristics of any industrial LAB strain, whether natural or genetically engineered (Klaenhammer, 1991).

Selective environmental pressure placed on LAB by bacteriophages over thousands of years has resulted in strains that possess many bacteriophage defense mechanisms. The best-characterized bacteriophage-resistant LAB is lactococci (Dinsmore and Klaenhammer, 1995; Sanders, 1988). Phage defense mechanisms identified in lactococci include abortive infection mechanisms, the restriction-modification system, and interference with phage adsorption. Abortive phage infection is a powerful defense, acting after phage injection to decrease phage development efficiency and so reduce the number of emerging phages when the cell bursts. This mechanism effectively decreases phage replication rates and results in poor to no plaque formation on agar assays. The restriction modification system is another common phage defense mechanism that reduces plaquing efficiencies of phages on normally permissive hosts by several log cycles. Such systems operate by coordinated activities of a restriction enzyme that recognizes and cleaves foreign DNA and a modification enzyme that labels DNA as host derived. These defense loci are encoded by plasmids capable of conjugal transfer, suggesting that genetic exchange between LAB has an important role in the development of bacteriophage-resistant starter cultures (Sanders, 1988). Recombinant DNA techniques also have been used to engineer starter cultures with enhanced bacteriophage resistance (Daly et al., 1996). An alternative approach to the development of phage-resistant strains uses a designed antisense RNA to control the expression of phage genes.

Flavor Production LAB generate a range of flavor products such as diacetyl, which has a buttery aroma and is a highly desirable product in many foods (Cogan and Accolas, 1996). Strategies involving metabolic engineering, whereby metabolic pathways are manipulated to overproduce specific products, have been used to develop cultures that elaborate elevated levels of diacetyl. Proteolytic activity of LAB is of major significance in contributing to the liberation of small peptides and amino acids which either add flavor directly or are likely to be flavor precursors.

Commercial Application

DAIRY PRODUCTS Fermented dairy products are enjoying increased popularity as convenient, nutritious, stable, natural, and healthy foods. LAB are the principal organisms involved in the manufacture of cheese, yogurt, sour cream, and cultured butter. In some fermented dairy products, additional bacteria, referred to as secondary microflora, are added to produce carbon dioxide, which influences the flavor and alters the texture of the final product (Early, 1998). Two LAB, *Leuconostoc* species and strains of *Lactobacillus lactis* subsp *lactis*, which are capable of metabolizing citric acid, are added to produce aroma compounds and carbon dioxide in cultured buttermilk and certain cheeses. *Propionibacterium freudenreichii* subsp. *shermanii* is added to Swiss-type cheeses primarily to metabolize L-lactic acid to propionic acid, acetic acid, and carbon dioxide. Carbon dioxide forms the eyes in Swiss-type cheeses. Other types of secondary microflora include undefined mixtures of yeasts, molds, and bacteria (Ray, 1996). These microorganisms are added directly to the milk or are smeared, sprayed, or rubbed onto the cheese surface. This group of microorganisms has extremely varied and complex metabolic activities, their main function being to produce unique flavors. LAB and the predominant microbes used to make fermented dairy products are listed in Table 4 (Johnson and Steele, 1997).

Cultured Butter and Buttermilk Cultured butter is made from milk fat to which a mesophilic starter culture has been added to enhance its flavor, principally that of diacetyl. Diacetyl, made from citrate by LAB, enhances buttermilk's storage properties. *Lactobacillus lactis* or mixed cultures that contain *Lb. lactis*, *Leuconostoc citrovurum*, and *Leu. dextranicum* are used (Early, 1998). Fat (cream) is separated from skim milk by centrifugation of milk. The cream is pasteurized and inoculated with selected starter cultures. The ripened cream is then churned. The cream separates again into cream butter and its byproduct sour buttermilk, which has limited use because of its high acidity.

An alternative process has been developed to produce cultured butter without the formation of sour buttermilk. In this process lactose-reduced whey inoculated with *Lactobacillus helveticus*, and skim milk inoculated with a starter culture to produce aroma compounds and lactic acid, are added to the pasteurized cream. The cream is further churned and worked. The resulting butter is known as sour aromatic butter and the liquid phase is sweet buttermilk, which is not as acidic as sour buttermilk.

Yogurt Yogurt is produced by fermenting milk, usually cow's milk, with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Chandan and Shahani, 1993). Initial acid production is largely due to *Str. thermophilus*, but the characteristic yogurt flavor is produced by *Lb. bulgaricus*. Both yogurt cultures may produce extracellular polymers, which contribute to the viscosity of yogurt. It is desirable that the starter

Table 4. Microorganisms involved in the manufacture of fermented dairy products.

Product	Principal acid producers	Secondary microflora
Cheese		
Colby, Cheddar, cottage, cream	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	None
Blue	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	Cit$^+$ *Lactococcus lactis* subsp. *lactis* *Penicillium roqueforti*
Mozzarella, provolone, Romano, parmesan	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus helveticus*	None
Swiss	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus helveticus*	*Propionibacterium freudenreichii* subsp. *shermanii*
Fermented milk		
Yogurt	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus*	None
Buttermilk	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	*Leuconostoc* sp. Cit$^+$ *Lactococcus lactis* subsp. *lactis*
Sour cream	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	None

cultures be present in approximately equal numbers to ensure the characteristic flavor, consistency and odor; otherwise *Lb. bulgaricus* becomes dominant.

Yogurt is generally fermented in batches. Standardized milk, with a fat content of 0.5 to 3.0% and milk solids of 14 to 16%, is homogenized and pasteurized. After cooling to the incubation temperature, the batch is inoculated with starter cultures and incubated for 16 h at 30°C or 4 h at 45°C. The product is then cooled to 2 to 4°C and packaged and stored.

Cheese Cheese making is essentially a dehydration process in which milk casein, fat, and minerals are concentrated 6 to 12-fold, depending on the variety. Although the manufacturing protocols for individual varieties differ, the basic steps common to most varieties are acidification, coagulation, dehydration, and salting. Acid production is the major function of the starter bacteria. Lactic acid is responsible for the fresh acidic flavor of unripened cheese and is of importance in coagulation of milk. Coagulation of the casein is accomplished by the concerted action of rennet and by acidification. Starters play other essential roles in the production of volatile flavor compounds (e.g., diacetyl, aldehydes), the synthesis and release of the intracellular proteolytic and lipolytic enzymes involved in the cheese ripening (Steele, 1995), and the suppression of pathogens and other spoilage microorganisms (Lewus et al., 1991). For cheddar cheese production, starters include mixed strains of *Lactobacillus lactis* subsp. *cremoris* or *lactis*. *Leuconostoc* may be added for flavor development. *Streptococcus thermophilus* and *Lb. helveticus* are used in Swiss cheese primarily for acid production, whereas *Propionibacterium* spp. is a secondary culture added for eye formation, taste, and flavor. Similar to Cheddar cheese production, blue cheese production requires *Lb. lactis* subsp. *cremoris* or *lactis* and *Leuconostoc* spp, but the mold *Penicillium roqueforti* is also added as a secondary culture for flavor and blue appearance.

Meat Products Fermented meat products are defined as meats that are deliberately inoculated during processing to ensure sufficient controlled microbial activity to alter the product's sensory characteristics (Luche, 1994). Dry and semidry sausages represent the largest category of fermented meat products, with many present-day processing practices having their origin in the Mediterranean region. Fermented sausages are chopped or ground meat products that, as a result of bacterial action, reach a pH of 5.3 or less and are then dried. Based on final moisture content, fermented sausages are classified as dry (25–45% moisture content) or semidry (40–50% moisture content). To ensure products of consistent flavor, texture and shelf stability, as well as to improve product safety, most processors have developed pure microbial cultures to control the fermentation of their sausage product. It is evident that with a starter culture, the pH drops much more rapidly, hence the entire manufacturing process is accelerated, leading to economic benefits for the processor. Most starter cultures are natural isolates of the desirable microorganisms found normally in sausage (Egan, 1983). A wide variety of species has been used as starter cultures in meat and poultry products (Table 5; Riche and Keeton, 1997).

Table 5. Bacteria used as starter cultures in meat and poultry products.

Products	Bacteria
Semi-dry fermented meat sausages	
Lebanon bologna	Mixture of *Pediococcus cerevisiae/actobacillus plantarum*
Summer sausage	*P. cerevisiae* or mixture/*Lb. plantarum*
Cerevelat	*P. cerevisiae* or mixture/*Lb. plantarum*
Thuringer	*P. cerevisiae*
Teewurst	*Lactobacillus* species
Pork roll	*P. cerevisiae*
Dry fermented meat sausages	
Pepperoni	*P. cerevisiae*/*Lb. plantarum*
Dry sausage	*P. cerevisiae*
European dry sausage	*Micrococcus* spp. or *Micrococcus*/*Lactobacillus* spp.
Salami	*Micrococcus*/*Lactobacillus* spp. or *Lb. plantarum*
Hard salami, Genoa	*Micrococcus* spp.
	Micrococcus spp./*P. cerevisiae; Micrococcus* spp./*Lb. plantarum*
Fermented poultry sausages	
Semi-dry turkey sausage	*P. cerevisiae*
Dry turkey sausage	*P. cerevisiae*/*Lb. plantarum*

Vegetable and Fruit Products The fermentation of vegetables and fruits can be affected by many different microorganisms (Daeschel and Fleming, 1984). LAB and yeasts are preferentially used in the western hemisphere, whereas in the Orient a large number of victuals are fermented by molds. However, the most extensively used procedure for biopreservation of vegetables involves lactic acid fermentation. Almost all vegetables can be fermented through natural processes because they contain fermentable carbohydrates and harbor many types of LAB. Therefore, many fermented vegetable products are produced by natural fermentation, and some such as cucumbers are now being produced by controlled fermentation (Buckenhuskes, 1997). Although many different vegetables are commercially fermented, at present only olives, cabbage for sauerkraut, and cucumbers for pickles are of major economic importance (Buckenhuskes, 1993).

Sauerkraut Sauerkraut is produced from the natural lactic acid fermentation of cabbage that has been shredded and salted. It is a major fermented vegetable food in Europe and the United States. At the beginning of the fermentation, some oxygen remains in the shredded cabbage. Plant cells, aerobic bacteria, yeasts and molds consume this remaining oxygen and then die off as the oxygen supply diminishes. The facultative anaerobes then increase in number. Initially, coliform species (e.g., *Enterobacter cloacae*) and *Flavobacterium* species grow to produce gas, volatile fatty acids and flavors. As the acidity increases, these bacteria are normally replaced by *Leuconostoc mesenteroides*, which become the predominant microbes. They are subsequently succeeded by *Lactobacillus brevis*, *Pediococcus pentosaccus* and *Lb. Plantarum*, which raise the acid level to about 2% and decrease the pH to 3.4–3.6.

Cucumber Pickles The starter culture for cucumber fermentation usually consists of the normal mixed flora of cucumbers, including *Lb. mesenteroides*, *Enterococcus faecalis*, *Pediococcus cerevisiae*, *Lb. brevis*, and *Lb. plantarum*. Of these, the pediococci and *Lb. plantarum* are the most involved, with *Lb. brevis* being undesirable because of its ability to produce gas. *Lb. plantarum* is the most essential species in pickle production, as it is for sauerkraut. The natural fermentation of cucumbers, though in practice for many years, can lead to pickle spoilage and thereby serious economic loss. A controlled fermentation of cucumbers brined in bulk has been developed, and this process not only reduces economic losses but also leads to a more uniform product over a shorter period of time (10–12 days). This method employs a chlorinated brine of 25° salinometer, acidification with acetic acid, the addition of sodium acetate, and inoculation with *P. cerevisiae* and *Lb. plantarum* or with *Lb. plantarum* only.

Olives Like cucumbers, olives are fruits that are categorized as vegetables and are fermented under conditions similar to those of other vegetable products. The microbial population responsible for the fermentation of olives differs from that of sauerkraut and pickles mainly because the higher salt concentration of the brine prevents many salt-sensitive strains from growing and provides an advantage to salt-tolerant strains. LAB become prominent during the intermediate stage of fermentation. *Lb. mesenteroides* and *P. cerevisiae* are the first lactics to become predominant, followed by lactobacilli, with *Lb. plantarum* and *Lb. brevis* being the most important. The fermentation may require as long as 6–10 months to complete, and the final pH of the product is 3.8 to 4.0.

Vinegar Vinegar (acetic acid) is used as an acidulant and flavor compound in processed foods. It is produced by an alcoholic fermentation of sugar-containing plant extracts followed by a microbial oxidation of ethanol to acetic acid (Sievers and Teuber, 1995). The slow process of natural acetification of wines and ciders is the oldest method of making vinegar. The Orleans process, developed in France for the industrial production of vinegar at the end of the 14th century, is also a slow fermentation in which the substrate "wine" is placed in barrels and inoculated with fresh vinegar or with the slimy, thick microbial film formed on the surface of the fermenting mash during acetification. Technological advances have led to a submerged culture process in which a suspension of *Acetobacter* spp. grows in an ethanol-containing substrate with constant agitation and small air bubbles. Commercial strains of *Acetobacter* include low cost, higher yields, less space required and low evaporation loss.

Malolactic Fermentation in Wine Malolactic fermentation (carried out by many LAB) involves the decarboxylation of malic acid to lactic acid and CO_2. In wine making as well as in production of cider and perry, LAB reduce acidity by converting malic to lactic acid and modify flavor and texture, which in part mature a beverage (Henick-Kling, 1995). Red and white wines are commonly produced by a yeast alcoholic fermentation of musts prepared from grapes. After alcoholic fermentation, wines frequently undergo malolactic fermentation. This process occurs naturally at or near the completion of alcoholic

fermentation. Wines produced from grapes cultivated in cool climates generally have higher concentrations of malic acid, which can mask the varietal character of the wine. Decreasing acidity by malolactic fermentation produces a wine with a softer and more mellow taste. LAB resident in wine are responsible for the malolactic fermentation. However, since the early 1980s, commercial starter cultures consisting of *Leuconostoc oenos*, *Lb. plantarum*, and *Lb. hilgardii* as single- or multiple-strain preparations have been available for the induction of malolactic fermentation (Buckenhuskes, 1993). The malolactic fermentation is more commonly used in red wines, although recently it is increasingly used in white wines.

Indigenous Fermented Beverages Production of indigenous fermented beverages (examples listed in Table 6) often involves complex biochemical, sensory and nutritional changes that can result from more or less controlled microbial activity in a range of raw materials (Beuchat, 1997). The preparation of many indigenous or traditional fermented beverages is a household art. Thus, they are often produced regionally or by different ethnic groups and are not commerically available (Steinkraus, 1983). The microbiology of indigenous fermented beverages is less clear. In many cases, LAB and yeasts both contribute to the fermentation process.

Probiotic Bacteria and Competitive Exclusion

A probiotic is a mono- or mixed culture of live microorganisms that, applied to animals or humans, beneficially affects the host by improving the properties of the gastrointestinal normal flora (Abe et al., 1995; Holzapfel et al., 1998; Hove et al., 1999). But it is restricted to products that contain live organisms, improve the health and well-being of animals or humans, and can have their effect on all host mucosal surfaces. Most microbial species used or tested for probiotic efficacy are LAB. Some bifidobacteria and yeasts are also used in the dairy industry. LAB have been used in foods for centuries and most strains are considered commensal microorganisms with no pathogenic potential. Their omnipresence on the intestinal epithelium of the human gastrointestinal tract, their traditional use in fermented foods without significant health-associated problems, and their health-promoting benefits make this group of bacteria ideal candidates for use as a probiotic (Naidu et al., 1999).

Probiotic Bacteria in Food Incorporating probiotic bacteria into foods to counteract harmful bacteria in the intestinal tract has been a renewed interest in health promotion and disease prevention in recent years (Gilliland, 1990; Gorbach, 1990; Holzapfel et al., 1998; Hove et al., 1999). Foods containing probiotic microorganisms range from dairy foods to infant formulas, baby foods, fruit juice-based products, cereal-based products, and pharmaceuticals. New and more specific strains of probiotic bacteria are being sought. Many studies have been conducted during the last 30 years to determine specific health benefits from the consumption of live cells of these organisms (Gorbach, 1990; Hove et al., 1999). There are three major sources of the live cells: 1) fermented milk products, such as yogurt containing live cells of *Lb. delbrueckii* subsp. *bulgaricus* and *Str. thermophilus* and acidophilus milk containing *Lb. acidophilus*; 2) supplementation of foods and drinks with live cells of one or more types of probiotic bacteria, such as *Lb. acidophilus*, *Lb. reuteri*, *Lb. casei*, and *Bifidobacterium* species; and 3) pharmaceutical products in the form of tablets, capsules, and granules. The beneficial effects of probiotic bacteria include protection against enteric pathogens (Jeppesen and Huss, 1993; Lewus et al.,

Table 6. Examples of indigenous fermented beverages.

Product	Geography	Substrate	Microorganism(s)	Product use
Busa	Tartars of Krim, Turkestan, Egypt	Rice or millet, sugar	*Lactobacillus* and *Saccharomyces* spp.	Drink
Mahewu (Magou)	South Africa	Maize	LAB	Sour and nonalcoholic drink
Pito	Nigeria	Guinea corn or maize or both	Yeast, LAB	Drink
Sorghum beer	South Africa	Sorghum, maize	LAB, yeast	Acidic and weakly alcoholic drink
Soybean milk yogurt	China, Japan	Soybeans	LAB	Drink
Pulque	Mexico	Agave	LAB, *Saccharomyces* spp., *Zymomonas mobilis*	Alcoholic drink

LAB, lactic acid bacteria.

1991; Okereke and Montville, 1991; Rodriguez et al., 1997), detoxification and improved digestion by means of enzymes to metabolize some food nutrients (e.g., lactase to hydrolyze lactose; Gilliland, 1990) and remove some harmful food components and metabolites (El-Nezami and Ahokas, 1998), stimulation of the intestinal immune system (Salminen and Deighton, 1992; Salminen and Salminen, 1997), and improvement of intestinal peristaltic activity (Rafter, 1995).

Selection Criteria for Probiotic Bacteria The theoretical base for selection of probiotic microorganisms includes safety, functional aspects (survival, adherence, colonization, antimicrobial production, immune stimulation, antigenotoxic activity, and prevention of pathogens), and technological aspects (growth in milk or other food base, sensory properties, stability, phage resistance, and viability; Salminen et al., 1998).

Competitive Exclusion Concept The competitive exclusion concept was originated by Nurmi and Rantala (1973), based on the study of *Salmonella* in chickens. Newly hatched birds on modern poultry farms are not able to obtain the normal gut flora of adult birds. As a result, the intestines of chicks are easily colonized by pathogens. When the chickens were inoculated immediately after birth with the intestinal content of a *Salmonella*-free adult bird, the frequency of Salmonella infections was radically reduced and the number of *Salmonella* needed to colonize the ceca of chicks increased. Hence, the normal gastrointestinal microflora of adult chickens can competitively exclude *Salmonella* from colonizing the naïve intestinal tract of chicks.

Competitive Exclusion of Foodborne Pathogens After establishment of the basic concept of competitive exclusion, considerable research has been done to identify the mechanisms by which bacteria are competitively excluded from host sites (Nurmi et al., 1992; Zhao et al., 1995). Responsible factors include competition for receptor sites on the intestinal epithelium, production of volatile fatty acids and/or other antibacterial substances, and competition among different bacteria for limited nutrients. The use of LAB as a probiotic for live poultry and livestock has been extensively studied and has potential to reduce carriage of pathogens and increase growth rates (Abe et al., 1995; Hammes and Tichaczek, 1994). Cecum-colonizing bacteria including *Escherichia coli* that produce inhibitory metabolites to *Campylobacter jejuni* have been used successfully to reduce intestinal carriage of *C. jejuni* by poultry (Aho et al., 1992; Schoeni and Doyle, 1992). Similarly, *E. coli* strains that produce antimicrobial metabolites to *E. coli* O157:H7 have been confirmed effective in reducing or eliminating carriage of *E. coli* O157:H7 by cattle (Zhao et al., 1998).

Prospects

For many years bacteria, mainly LAB, have been involved in the fermentation of foods from raw agricultural materials such as milk, meat, vegetables, fruits, and cereals. Fermented foods are a significant part of the food processing industry and are often produced using bacteria that have been selected for their ability to effectively produce desired products or changes. The interaction of LAB and other bacteria in enhancing the physiology, nutrition, and metabolism of humans and animals, and their involvement in promoting health and reducing disease, are additional roles that prokaryotes can serve by their presence in foods. Over the past decade, there have been major developments in furthering our understanding of both the biochemistry and physiology of bacteria involved in food fermentations. Advanced molecular techniques have served as invaluable tools for the development of defined mutants that have enabled basic studies on proteolysis, peptidase action, and peptide transport. Knowledge from such studies is invaluable for the design and modification of commercially useful strains. The application of genetic engineering technology to improve existing strains through enhanced bacteriophage resistance or more efficient metabolic characteristics such as proteolytic activities or to develop novel strains for fermentations has greatly contributed to the success of the fermentation industry through more consistent production of high quality, uniform products and less fermentation failure.

Literature Cited

Abe, F., N. Ishibashi, and S. Shimamura. 1995. Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Dairy Sci. 78:2838–2846.

Aho, M., L. Nuotio, E. Nurmi, and T. Kiiskinen. 1992. Competitive exclusion of campylobacters from poultry with K-bacteria and Broilact. Int. J. Food Microbiol. 15:265–275.

Axelsson, L. 1998. Lactic acid bacteria: classification and physiology. *In:* S. Salminen, and A. von Wright (Eds.) Lactic Acid Bacteria—Microbiology and Functional Aspects. Marcel Dekker, Inc. New York, 1–72.

Beuchat, L. R. 1997. Traditional fermented foods. *In:* M. Doyle, L. Beuchat, and T. Montville (Eds.) Food Microbiology—Fundamentals and Frontiers. ASM Press. Washington DC, 629–648.

Buckenhuskes, H. 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiol. Rev. 12:253–272.

Buckenhuskes, H. 1997. Fermented vegetables. *In:* M. Doyle, L. Beuchat, and T. Montville (Eds.) Food Microbiology—Fundamentals and Frontiers. ASM Press. Washington DC, 595–609.

Chandan, R., and K. Shahani. 1993. Yoghurt. *In:* Y. Hui (Ed.) Dairy Science Technology Handbook. VCH. New York, 2–53.

Chebbi, N. B., H. Chander, and B. Ranganathan. 1977. Casein degradation and amino acid liberation in milk by two highly proteolytic strains of lactic acid bacteria. Acta. Microbiol. Pol. 26:281–284.

Cogan, T. 1996. Cogan, T., and J. AccolasDairy Starter Cultures. VCH. New York.

Collins-Thompson, D. L., P. J. Slade, and M. Goethals. 1991. Use of low molecular mass RNA profiles to identify lactic acid bacteria and related organisms associated with foods. Int. J. Food Microbiol. 14:135–143.

Daeschel, M., and H. Fleming. 1984. Selection of lactic acid bacteria for use in vegetable fermentations. Food Microbiol. 1:303–313.

Daly, C., G. F. Fitzgerald, and R. Davis. 1996. Biotechnology of lactic acid bacteria with special reference to bacteriophage resistance. Ant. v. Leeuwenhoek 70:99–110.

De Ambrosini, V. M., S. Gonzalez, G. Perdigon, A. P. de Ruiz Holgado, and G. Oliver. 1996. Chemical composition of the cell wall of lactic acid bacteria and related species. Chem. Pharm. Bull. (Tokyo) 44:2263–2267.

De Vos, W. M. 1999. Gene expression systems for lactic acid bacteria. Curr. Opin. Microbiol. 2:289–295.

De Vuyst, L. 1994. De Vuyst, L., and E. VandammeBacteriocins of Lactic Acid Bacteria. Blackie Academic and Professional. Glasgow.

Dinsmore, P. K., and T. R. Klaenhammer. 1995. Bacteriophage resistance in Lactococcus. Mol. Biotechnol. 4:297–314.

Early, R. 1998. Early, R. (Ed.) The Technology of Dairy Products, 2nd ed. Blackie Academic & Professional. London.

Egan, A. F. 1983. Lactic acid bacteria of meat and meat products. Ant. v. Leeuwenhoek 49:327–336.

Eijsink, V. G., M. Skeie, P. H. Middelhoven, M. B. Brurberg, and I. F. Nes. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64:3275–3281.

El-Nezami, H., and J. Ahokas. 1998. Lactic acid bacteria: an approach for detoxification of alfatoxins. *In:* S. Salminen, and A. von Wright (Eds.) Lactic Acid Bacteria—Microbiology and Functional Aspects. Marcel Dekker, Inc. New York, 359–368.

Gasson, M. J. 1990. In vivo genetic systems in lactic acid bacteria. FEMS Microbiol. Rev. 7:43–60.

Geisen, R., and W. H. Holzapfel. 1996. Genetically modified starter and protective cultures. Int. J. Food Microbiol. 30:315–324.

Gilarova, R., M. Voldrich, K. Demnerova, M. Cerovsky, and J. Dobias. 1994. Cellular fatty acids analysis in the identification of lactic acid bacteria. Int. J. Food Microbiol. 24:315–319.

Gilliland, S. E. 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. 7:175–188.

Gorbach, S. L. 1990. Lactic acid bacteria and human health. Ann. Med. 22:37–41.

Hammes, W. P., and P. S. Tichaczek. 1994. The potential of lactic acid bacteria for the production of safe and wholesome food. Z. Lebensm. Unters. Forsch. 198:193–201.

Henick-Kling, T. 1995. Control of malo-lactic fermentation in wine: energetics, flavour modification and methods of starter culture preparation. J. Appl. Bacteriol. 79 Suppl:29s37s.

Holzapfel, W. H., P. Haberer, J. Snel, U. Schillinger, and J. H. Huis in't Veld. 1998. Overview of gut flora and probiotics. Int. J. Food Microbiol. 41:85–101.

Hove, H., H. Norgaard, and P. B. Mortensen. 1999. Lactic acid bacteria and the human gastrointestinal tract. Eur. J. Clin. Nutr. 53:339–350.

Jay, J. 1996. Modern Food Microbiology, 5th ed. Chapman & Hall. New York.

Jeppesen, V. F., and H. H. Huss. 1993. Antagonistic activity of two strains of lactic acid bacteria against Listeria monocytogenes and Yersinia enterocolitica in a model fish product at 5 degrees C. Int. J. Food Microbiol. 19:179–186.

Johnson, M., and J. Steele. 1997. Fermented dairy products. *In:* M. Doyle, L. Beuchat, and T. Montville (Eds.) Food Microbiology—Fundamentals and Frontiers. ASM Press. Washington DC, 581–594.

Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria. Ant. v. Leeuwenhoek 49:209–224.

Klaenhammer, T. R. 1991. Development of bacteriophage-resistant strains of lactic acid bacteria. Biochem. Soc. Trans. 19:675–681.

Klein, G., A. Pack, C. Bonaparte, and G. Reuter. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Microbiol. 41:103–125.

Kuipers, O. P., P. G. de Ruyter, M. Kleerebezem, and W. M. de Vos. 1997. Controlled overproduction of proteins by lactic acid bacteria. Trends Biotechnol. 15:135–140.

Lee, B. 1996. Bacteria-based processes and products. *In:* B. Lee (Ed.) Fundamentals of Food Biotechnology. VEH. New York, 219–290.

Lewus, C. B., A. Kaiser, and T. J. Montville. 1991. Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. Appl. Environ. Microbiol. 57:1683–1688.

Luche, F. 1994. Fermented meat products. Food Res. Intern. 27:299–308.

Makela, P., U. Schillinger, H. Korkeala, and W. H. Holzapfel. 1992. Classification of ropy slime-producing lactic acid bacteria based on DNA-DNA homology, and identification of Lactobacillus sake and Leuconostoc amelibiosum as dominant spoilage organisms in meat products. Int. J. Food Microbiol. 16:167–172.

Mayo, B. 1993. The proteolytic system of lactic acid bacteria. Microbiologia 9:90–106.

McKay, L. L., and K. A. Baldwin. 1990. Applications for biotechnology: present and future improvements in lactic acid bacteria. FEMS Microbiol. Rev. 7:3–14.

Montville, T., and K. Winkowski. 1997. Biologically based preservation systems and probiotic bacteria. *In:* M. Doyle, L. Beuchat, and T. Montville (Eds.) Food Microbiology—Fundamentals and Frontiers. ASM Press. Washington DC, 557–577.

Naidu, A. S., W. R. Bidlack, and R. A. Clemens. 1999. Probiotic spectra of lactic acid bacteria (LAB). Crit. Rev. Food Sci. Nutr. 39:13–126.

Nurmi, E., and M. Rantala. 1973. New aspects of Salmonella infection in broiler production. Nature 241:210–211.

Nurmi, E., L. Nuotio, and C. Schneitz. 1992. The competitive exclusion concept: development and future. Int. J. Food Microbiol. 15:237–240.

Okereke, A., and T. J. Montville. 1991. Bacteriocin-mediated inhibition of Clostridium botulinum spores by lactic acid bacteria at refrigeration and abuse temperatures. Appl. Environ. Microbiol. 57:3423–3428.

Ouwehand, A. 1998. Antimicrobial components from lactic acid bacteria. *In:* S. Salminen, and A. von Wright (Eds.) Lactic Acid Bacteria—Microbiology and Functional Aspects. Marcel Dekker, Inc. New York, 139–159.

Poolman, B., E. Kunji, A. Hagting, V. Juillard, and W. Konings. 1995. The proteolytic pathway of Lactococcus lactis. J. Appl. Bacteriol. (Suppl.) 79:65S–75S.

Pritchard, G. G., and T. Coolbear. 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. 12:179–206.

Rafter, J. J. 1995. The role of lactic acid bacteria in colon cancer prevention. Scand. J. Gastroenterol. 30:497–502.

Ray, B. 1996. Fundamental Food Microbiology. CRC Press. Boca Raton, FL.

Riche, S., and J. Keeton. 1997. Fermented meat, poultry, and fish products. *In:* M. Doyle, L. Beuchat, and T. Montville (Eds.) Food Microbiology—Fundamentals and Frontiers. ASM Press. Washington DC, 610–628.

Rodriguez, A., and D. R. Vidal. 1990. Genetics of lactic acid bacteria with special reference to lactococci. Microbiologia 6:51–64.

Rodriguez, E., J. Tomillo, M. Nunez, and M. Medina. 1997. Combined effect of bacteriocin-producing lactic acid bacteria and lactoperoxidase system activation on Listeria monocytogenes in refrigerated raw milk. J. Appl. Microbiol. 83:389–395.

Salminen, S., and M. Deighton. 1992. Lactic acid bacteria in the gut in normal and disordered states. Dig. Dis. 10:227–238.

Salminen, S., and E. Salminen. 1997. Lactulose, lactic acid bacteria, intestinal microecology and mucosal protection. Scand. J. Gastroenterol. (Suppl.) 222:45–48.

Salminen, S., A. von Wright, L. Morelli, P. Marteau, D. Brassart, et al. 1998. Demonstration of safety of probiotics—a review. Int. J. Food Microbiol. 44:93–106.

Sanders, M. E. 1988. Phage resistance in lactic acid bacteria. Biochimie 70:411–422.

Schoeni, J. L., and M. P. Doyle. 1992. Reduction of Campylobacter jejuni colonization of chicks by cecum-colonizing bacteria producing anti-C. jejuni metabolites. Appl. Environ. Microbiol. 58:664–670.

Sievers, M., and M. Teuber. 1995. The microbiology and taxonomy of Acetobacter europaeus in commercial vinegar production. J. Appl. Bacteriol. (Suppl.) 79:85S–95S.

Stackebrandt, E., and M. Teuber. 1988. Molecular taxonomy and phylogenetic position of lactic acid bacteria. Biochimie 70:317–324.

Steele, J. L. 1995. Contribution of lactic acid bacteria to cheese ripening. Adv. Exp. Med. Biol. 367:209–220.

Steinkraus, K. H. 1983. Handbook of Indigenous Fermented Foods. Marcel Dekker, Inc. New York,

Stiles, M. E., and W. H. Holzapfel. 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36:1–29.

Thompson, J. 1988. Lactic acid bacteria: model systems for in vivo studies of sugar transport and metabolism in gram-positive organisms. Biochimie 70:325–336.

Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60:407–438.

Verrips, C. T., and D. J. van den Berg. 1996. Barriers to application of genetically modified lactic acid bacteria. Ant. v. Leeuwenhoek 70:299–316.

Zhao, S., J. Meng, T. Zhao, and M. Doyle. 1995. Use of vaccine and biological control techniques to control pathogens in animals used for food. J. Food Safety 15:193–199.

Zhao, T., M. P. Doyle, B. G. Harmon, C. A., Brown, P. O., Mueller, and A. H. Parks. 1998. Reduction of carriage of enterohemorrhagic Escherichia coli O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol. 36:641–647.

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CHAPTER 3.6

Bacterial Pharmaceutical Products

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Introduction

In contrast to plant metabolites, whose use against diseases has roots in folk and traditional medicine, bacterial pharmaceutical products are the result of large targeted research efforts, carried out by tens of laboratories all around the world. The original approach (later denoted "screening") of Selman Waksman in 1939–1940 (Waksman and Woodruff, 1940) consisted of 1) systematically collecting soil microorganisms, 2) growing them in axenic culture, 3) testing the culture broths for their ability to inhibit the growth of pathogens, and 4) recovering the active substances produced. It was rapidly found that actinomycetes were most frequently positive; in particular, nearly 50% of *Streptomyces* strains isolated were active, mainly against Gram-positive bacteria. This widely applied approach was very fruitful by the early 1960s, and members of all the main families of clinically useful antibiotics were discovered. With the exception of the penicillins, cephalosporins and a few minor products, all were produced by actinomycetes. Their collective spectra of action covered practically all the important bacterial pathogens.

In the 1960s, the need was felt for a substantial revision of the objectives and the methods of the screening. The number of antibiotics isolated was such that repetitions of discovery became more and more frequent. In fact, 1,300 metabolites had been reported in journals or patents by 1960, and over 2,000 by 1965 (data from Biosearch Italia database, courtesy of G. Toppo). General antibacterial activity appeared to be a less attractive target than activity against fungi, viruses or antibiotic-resistant bacterial strains. Antitumor efficacy was also considered as a possible target. Therefore, the search for novel metabolites was slowly changed in the various laboratories. Some devised methods for mass screening of unusual genera of microorganisms (such as rare actinomycetes) or organisms living in different (e.g., marine) environments. Others relied upon the use of new targets, rather than on isolation of novel producers, to select new products. The result was the discovery of novel and important antimicrobial agents, antitumor substances and inhibitors of mammalian enzymes of potential pharmaceutical interest. Mammalian enzymes have been especially targeted in recent years, with the isolation of pharmacologically active metabolites and of antimicrobial agents occurring at present at about the same frequency. However, the number of new products of proven clinical value has been unfortunately declining.

All these natural products are known as secondary metabolites ("idiolites"). They are low molecular weight products of microbial metabolism (such as antibiotics, pheromones, sex hormones, etc.) that differ from primary metabolites (amino acids, vitamins, purines, pyrimidines, etc.) in that they are not involved in growth processes but rather in mechanisms of survival in nature.

The advent of modern biotechnology opened totally new perspectives for the use of microorganisms as producers of pharmaceutical products. Genetic engineering provided methods (such as altering biosynthetic genes or inserting selected genes into the DNA of an antibiotic-producing strain) for obtaining modified secondary metabolites. Moreover, a number of new metabolites could be obtained by randomly combining the genes of two or more gene clusters governing similar biosynthetic pathways. Even more important, the progress of biotechnology made feasible the industrial production of mammalian peptides by bacteria and other hosts. The stages of this development have been: 1) biosynthesis of known clinically useful proteins (e.g., insulin and human growth hormone) and the generation of monoclonal antibodies to them, 2) identification and cloning of genes encoding physiologically important, lesser-known proteins (e.g., interferons, interleukins, colony stimulation factors, cytokines, thrombolytic agents and vaccines), and finally, 3) provision of macromolecules (e.g., receptors, ligands, enzymes, cytoskeleton proteins, adhesion molecules, signalling proteins and regulatory elements) to be used in screening for modulators. We summarize in this chapter the main results obtained during the past sixty years

in the search and development of the various classes of medically useful substances produced by bacteria.

Secondary Metabolites

Bacterial secondary metabolites are relatively small molecules, each produced by a limited number of strains that appear to have no obvious function in growth. In fact, producer strains that by mutation have lost their production ability exhibit perfectly normal growth rates and characteristics. Secondary metabolites include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumor agents and growth promotants of animals and plants. Nature produces an amazing variety and number of such products. At the end of 1999, over 23,400 microbial secondary metabolites were known. Of these, 42.1% were produced by fungi, 42.4% by actinomycetes and 15.5% by other bacteria (Lazzarini et al., 2001). New bioactive products from microbes continue to be discovered at an amazing pace: 200 to 300 per year in the late 1970s increasing to 500 per year by 1997.

Classical Antibiotics

The most important microbial metabolites so far isolated are antibiotics, i.e., substances (produced by microorganisms) that inhibit at low concentrations the growth of different species of microorganisms. They exert a major effect on the health, nutrition and economics of our society. Over 8,100 were reported by the end of 1999, 45.6% of which were produced by streptomycetes, 16.0% by other actinomycetes, 16.9% by other bacteria and 21.5% by fungi (Lazzarini et al., 2001). In addition to their number, another impressive aspect of antibiotics is the variety of their chemical structures. All the different classes of organic chemistry are represented: aliphatic chains, aromatic rings isolated or condensed, heterocyclic rings (all substituted with any imaginable function) and oligopeptides, oligosaccharides and so on.

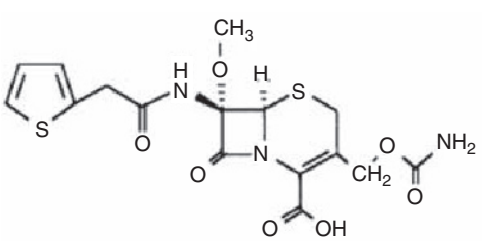
The property that makes antibiotics veritable "wonder drugs" is the selectivity of their mechanisms of antimicrobial action, which distinguishes them from the plethora of synthetic disinfectants and germicides. We have benefited for almost sixty years from their remarkable property of being selectively toxic to some class of organisms. The successes were so impressive that antibiotics were, with a few exceptions, the only drugs utilized for therapy against most pathogenic microorganisms. By 1996, the world market for antimicrobials amounted to $23 billion and involved some 150–300 products (natural, semisynthetic or synthetic), which included cephalosporins (45%), penicillins (15%), quinolones (11%), tetracyclines (6%) and macrolides (5%), thienamycin and others (Strohl, 1997).

Antibiotics are commonly grouped into families. A family is comprised of all the products having a similar chemical structure and sharing the same mechanism of antimicrobial action. We summarize here the characteristics of the classical antibiotic families used in human therapy (Lancini et al., 1995).

β-Lactams: Typical of the chemical structure of these antibiotics is the presence of a four-membered ring closed by an amide bond. They exert a killing action on many bacterial species by inhibiting the assembly of the peptidoglycan component of the cell wall. Fungi produce the classical subfamilies, penicillins and cephalosporins, although bacteria make modified cephalosporins, e.g., cephamycins, etc. Thienamycin, one of the last commercial antibiotics discovered and which possesses a β-lactam ring and an exceptionally broad spectrum of action, is produced by a prokaryote, *Streptomyces cattleya*.

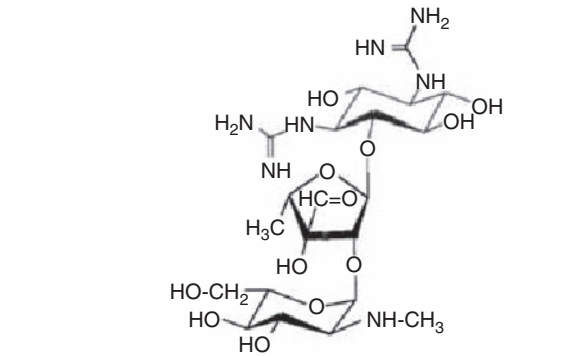
Aminoglycosides: Chemically, these are oligosaccharides comprising aminosugars and an aminocyclitol moiety, i.e., an alicyclic six-membered ring with hydroxyl and amino substituents. They are mainly active on Gram-negative bacteria and act by irreversibly inhibiting protein synthesis. The main products are streptomycin from *Streptomyces griseus*, and gentamicins, produced by *Micromonospora purpurea*.

Tetracyclines: Characteristically composed of four six-membered rings linearly arranged, these broad spectrum antibiotics act by inhibiting protein synthesis at the ribosomal level, but their binding site on the 30S ribosomal subunit differs from that of aminoglycosides. In clinical use are oxytetracycline produced by *Streptomyces rimosus*, tetracycline and chlortetracycline from *Streptomyces aureofaciens*, and a number of their derivatives.

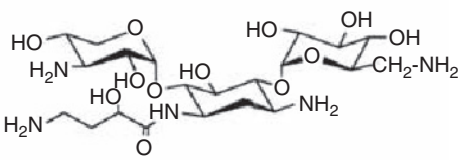


Cefoxitin (a semisynthetic cephamycin)

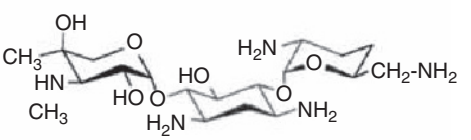
Fig. 1. Cefoxitin (a semisynthetic cephamycin).



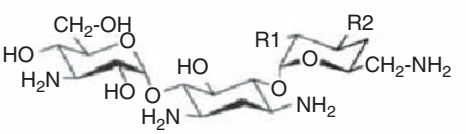
Streptomycin



Amikacin (a semisynthetic aminoglycoside)

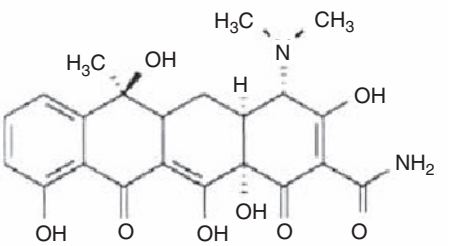


Gentamicin C1a

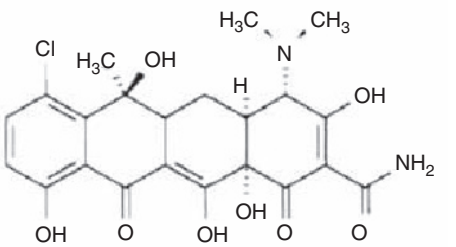


R1	R2	Name
OH	OH	kanamycin A
NH_2	OH	kanamycin B
NH_2	H	Tobramycin

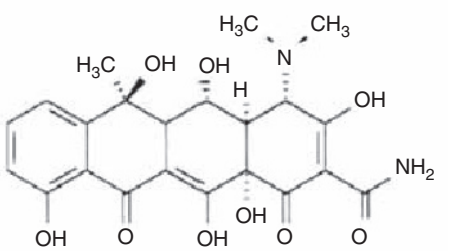
Fig. 2. Aminoglycosides.



Tetracycline



Chlortetracycline



Oxytetracycline

Fig. 3. Tetracyclines.

Antibacterial macrolides: This is a large family of products characterized by a lactone ring of 12 to 16 atoms bearing two or more sugar substituents. The best-known representative is erythromycin, produced by *Saccharopolyspora erythrea*. It inhibits protein synthesis, binding (unlike tetracyclines) to the 50S ribosomal subunit.

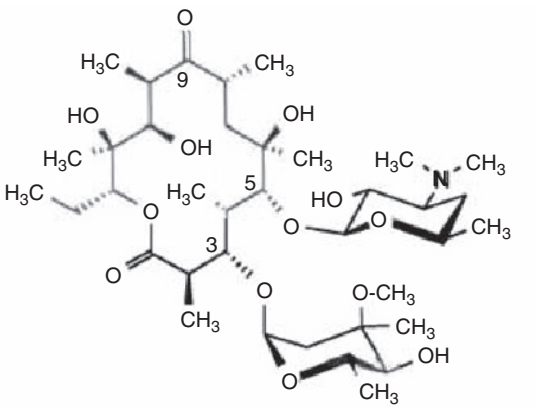
Polyenes: These antifungal macrolides differ from antibacterial macrolides in the size of the lactone ring, ranging from 26 to 38 atoms, and with the presence of a series of conjugated double bonds. The only member systemically used is amphotericin B, produced by *Streptomyces nodosus*. Antifungal activity is due to interference with membrane sterols, resulting in permeability alteration.

Glycopeptides: Among the vast class of peptide antibiotics, the only ones commonly used are members of this family, denoted as "dalbaheptides" in view of their mechanism of action (*D-al*anine-D-alanine *b*inding) and their composition (*he*ptape*ptides*). The most well known are vancomycin and teicoplanin, produced by *Amycolatopsis orientalis* and *Actinoplanes teichomyceticus*, respectively.

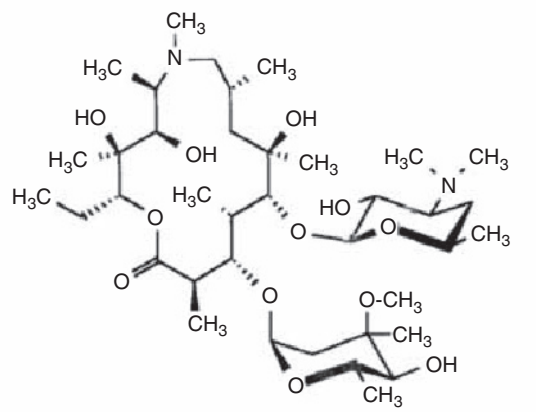
Rifamycins: This family belongs to the large class of ansamycins, molecules characterized by an aromatic nucleus spanned by an aliphatic (the ansa) chain. Their mechanism of action is inhibition of RNA synthesis by binding to RNA poly-

merase. Industrially produced are rifamycin B and rifamycin SV, from *Amycolatopsis mediterranei*. These are the starting materials for the synthesis of rifampicin or rifapentine, clinically used semisynthetic derivatives.

There are a few important antibiotics not belonging to any of the large families. These include chloramphenicol and lincomycin, both having a mechanism of action similar to that of erythromycin. Chloramphenicol was isolated from *Streptomyces venezuelae* but is now produced by chemical synthesis. Lincomycin is the product of *Streptomyces lincolnensis*.



Erythromycin A



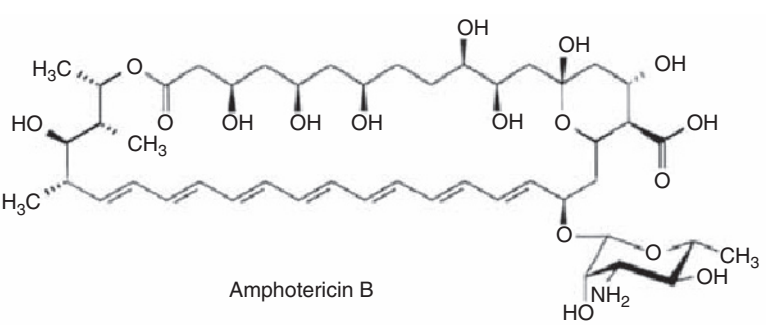
Azithromycin (a semisynthetic erythromycin)

Fig. 4. Macrolides.

Non-classical Antibiotics and Products under Development

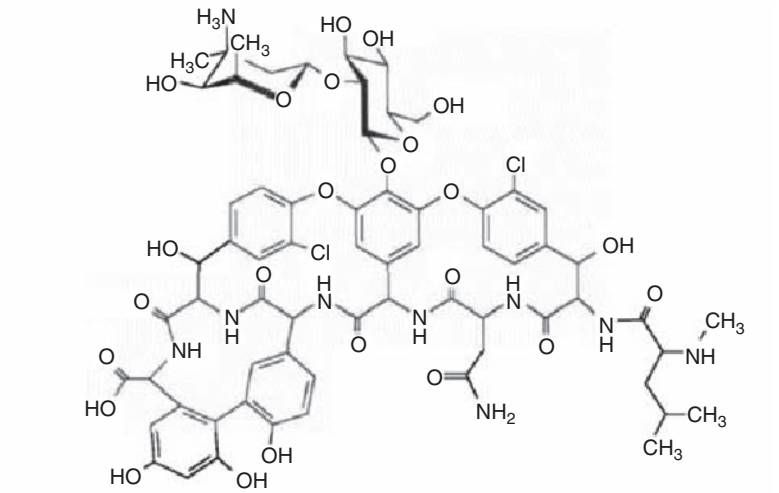
About 30 years ago, the difficulty and high cost of isolating novel structures and agents with new antimicrobial modes of action became apparent and the field looked like it might enter a phase of decline. This is understandable considering the probability of finding useful antibiotics from microbes is one from 10,000 to 100,000 cultures examined (Fleming et al., 1982; Woodruff et al., 1979; Woodruff and McDaniel, 1958). Indeed, the number of anti-infective investigational new drugs (INDs) declined by 50% from the 1960s to the late 1980s (DiMasi et al., 1994).

Prior to the 1980s, bacterially produced antibiotics were the mainstay of control of pathogenic organisms in humans (β-lactams, chloramphenicol, tetracyclines, erythromycin, streptomycin, gentamicin, rifamycin, vancomycin, lincomycin), in foods (nisin), in animals (monensin) and in plants (polyoxins). However, new antibiotics were clearly needed because of 1) the development of resistance in pathogens, 2) the evolution of new diseases (e.g., AIDS, Hanta virus, Ebola virus, *Cryptospiridium*, Legionnaire's disease, Lyme disease and *Escherichia coli* 0157:H7), 3) the existence of naturally resistant bacteria (Stephens and Shapiro, 1997), e.g., *Pseudomonas aeruginosa* (causing fatal wound infections, burn infections and chronic and fatal infections in the lungs of cystic fibrosis patients), *Stenotrophomonas maltophilia*, *Enterococcus faecium*, *Burkholderia cepacia* and *Acinetobacter baumanni* (Tenover and Hughes, 1996), and 4) the toxicity of some of the approved compounds (Strohl, 1997). Other organisms existed which were not normally virulent but did infect immu-

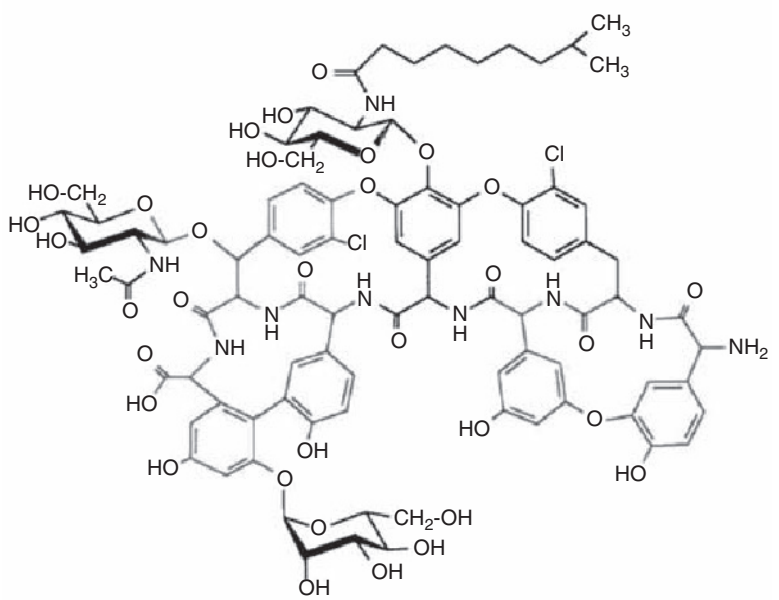


Amphotericin B

Fig. 5. Amphotericin.



Vancomycin



Teicoplanin

Fig. 6. Glycopeptides.

nocompromised patients (Morris et al., 1998). By the technique of semisynthesis, chemists had been improving antibiotics for many years. Despite the success of many semisynthetic antibiotics, new screening techniques were sorely needed in the 1970s and 1980s to isolate new bioactive molecules from nature. Owing to the development of novel target-directed screening procedures, discovery of new effective antibiotics active against cell-wall biosynthesis led to commercial successes. These included fosfomycin (Hendlin et al., 1969) and thienamycin (Birnbaum et al., 1985).

Thienamycin is the most potent and broadest in spectrum of all antibiotics known today. Although a β-lactam, it is not a member of the penicillins or cephalosporins; rather, it is a carbapenem, differing from the conventional β-lactams by the presence of a carbon atom instead of sulfur in the ring condensed to the lactam ring and by the *trans* configuration of the hydrogen atoms of the lactam ring. It is bactericidal against aerobic and anaerobic bacteria, both Gram-positive and Gram-negative, including *Pseudomonas*. This novel structure was isolated in Spain from a new soil species, which was named *S.*

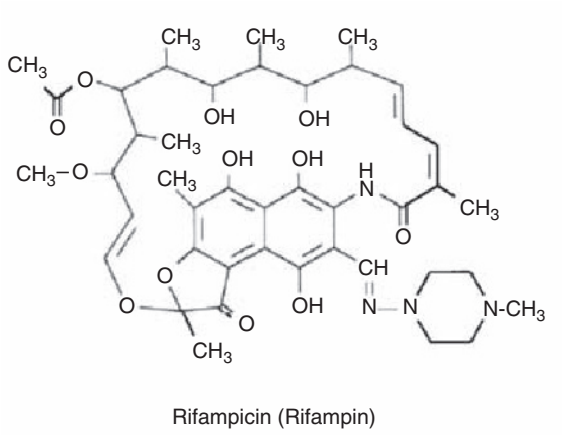


Fig. 7. Rifampicin (Rifampin).

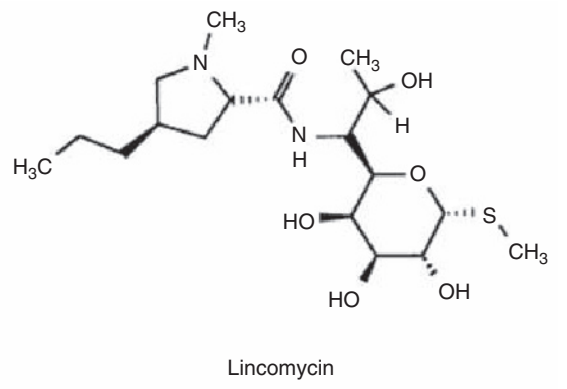


Fig. 8. Lincomycin.

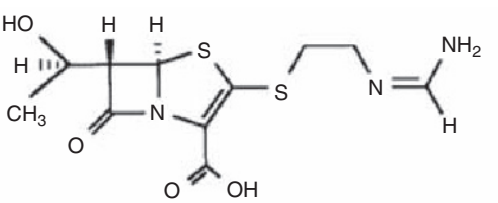


Fig. 9. Imipenem (a semisynthetic thienamycin).

cattleya (Kahan et al., 1979). Interestingly, this culture also produces penicillin N and cephamycin C.

Similar to those of the other bacterial β-lactams, thienamycin's mode of action is inhibition of peptidoglycan synthesis; however, it is not affected by most bacterial β-lactamases and is therefore active against resistant strains. It is chemically rather unstable but chemists were able to eliminate this problem by modifying the molecule. The new derivative, named imipenem, was not only more stable but also twice as active as the original molecule. In humans, imipenem was found to be metabolized by an enzyme in the kidney, renal dehydropeptidase-I, which acts as a β-lactamase.

Because the enzyme appeared to serve no essential role in human metabolism, scientists were able to develop a synthetic competitive inhibitor, cilastatin, which they then used with imipenem to produce the combination drug, primaxin (Tienam). Primaxin was introduced into medical practice in 1985. The unique activity of thienamycin is due to several factors: 1) it permeates the Gram-negative outer cell membrane through porin channels at 10–20 times the rate of classical β-lactams; 2) it is not destroyed by the β-lactamases of the periplasmic space; 3) it binds to and inhibits all penicillin-binding proteins (PBPs) but is principally active against PBPs-2 and -1b; sequential inhibition of PBPs-2 and -1b converts the cells of the pathogen to nongrowing spheroplasts, which rapidly die; and 4) after removal of thienamycin, there is a long delay before regrowth of any unkilled spheroplasts. The only limitation in thienamycin use is that it is not active against methicillin-resistant staphylococcal strains. If thienamycin is so potent, why did it take over 25 years of worldwide screening before it was discovered? Today, we know that carbapenems are not rare. Many members of this family have been isolated in laboratories all over the world although none equals thienamycin in potency and spectrum of activity. The answer probably lies in its extremely low level of production by wild strains and its instability. Conventional screening procedures evidently missed this activity and only after the development of specific and sensitive modern assay procedures was it found. Thienamycin was discovered by a sensitive mode of action screen, the details of which have never been revealed.

Microbiologists knew in the 1970s–1980s that antibiotic technology had not yet defeated infectious microorganisms owing to resistance development in pathogenic microbes. Indeed, antibiotics probably can never win the war against infection permanently and we have to be satisfied to stay one step ahead of the pathogens for a long time to come. Thus, the search for new natural product drugs and their improved semisynthetic derivatives slowed down but, fortunately, was not stopped. As a result, new products are in clinical testing. Clinical isolates of penicillin-resistant *Streptococcus pneumoniae*, the most common cause of bacterial pneumonia, increased in the United States from 1987 to 1992 by 60-fold (Breiman et al., 1994). Methicillin-resistant *Staphylococcus* infections increased to

an alarming extent throughout the world (Goldman et al., 1996). At present, vancomycin is still the molecule of choice to treat these infections; however, resistance is developing to this glycopeptide antibiotic, especially in the case of nosocomial *Enterococcus* infections. Fortunately, some vancomycin-resistant enterococci are treatable by the related glycopeptide, teicoplanin. Teicoplanin is not, at present, available in the United States but is available in Europe only; however, a derivative discovered by Biosearch Italia, BI 397, endowed with similar but improved characteristics, is under clinical development in the United States (Candiani et al., 1999). In addition, ramoplanin, another antibiotic discovered by the same group, is being developed for the prevention of enterococcal infections in patients carrying vancomycin-resistant enterococci in their gastrointestinal tract (Mobarakai et al., 1994).

A number of "old" compounds are now approaching commercialization. These were not previously developed because their antibacterial spectra were restricted to Gram-positive bacteria. At that time (in the 1970s and 1980s), breadth of spectrum was the commercial goal, but today the major aim is to inhibit resistant Gram-positive pathogens. About 90% of natural antibiotics fail to inhibit Gram-negative organisms such as *E. coli* (Vaara, 1993). The reasons include their outer membrane, which contains 1) narrow porin channels that retard the entry into the cell of even small hydrophilic compounds, and 2) a lipopolysaccharide moiety that slows down the transmembrane diffusion of lipophilic antibiotics. Furthermore, Gram-negative bacteria often possess a multiple-drug efflux pump, which eliminates many antibiotics from the cells (Lewis, 1994).

One group of useful narrow-spectrum compounds is the streptogramins, which are synergistic pairs of antibiotics made by single microbial strains. The pairs are constituted by a (Group A) polyunsaturated macrolactone containing an unusual oxazole ring and a dienylamide fragment and a (Group B) cyclic hexadepsipeptide possessing a 3-hydroxypicolinoyl exocyclic fragment. Such streptogramins include virginiamycin and pristinamycin (Barriere et al., 1998). Although the natural streptogramins are poorly water-soluble and cannot be used intravenously, new derivatives have been made by semisynthesis and mutational biosynthesis. Synercid (RP59500) is a mixture of two water-soluble semisynthetic streptogramins, quinupristin (RP57669) and dalfopristin (RP54476), which has recently been approved for resistant bacterial infections (Nichterlein et al., 1996). The two Synercid components synergistically (100-fold) inhibit protein synthesis and are active against vancomycin-resistant enterococci and methicillin-resistant staphylococci (Stinson, 1996). Another product, called RPR106972, is being developed for oral treatment of community-acquired infections and is in phase II clinical trials. It is a co-crystalline association of two minor natural streptogramins (pristinamycin IB and pristinamycin IIB) produced by *Streptomyces pristinaespiralis*.

Another potentially useful compound is a new everninomycin derivative called "ziracin" (Sch27899), which is being developed for drug-resistant Gram-positive infections (Stead, 1997). Everninomycin was discovered in 1979, but this narrow spectrum molecule, containing oligosaccharide and aromatic moieties, was never developed. Semisynthetic tetracyclines, e.g., glycylcyclines, are being developed for use against tetracycline-resistant bacteria (Sum et al., 1998). New screens for further discovery include those for inhibitors of bacterial signal peptidases (Black and Bruton, 1998), non-β-lactam inhibitors of β-lactamase, inhibitors of lipid A biosynthesis and inhibitors of tRNA synthetases (Bush, 1997).

A new and important target for antibiotics is the treatment of ulcers caused by *Helicobacter pylori*. Hundreds of millions of people throughout the world are infected with this Gram-negative bacterium, which causes most gastric and duodenal ulcers. Broths of an actinomycete, *Amycolata* sp., were reported to contain eight novel quinolones that inhibit *H. pylori* at 0.1 ng/ml while not inhibiting other bacteria (Dekker et al., 1997).

Antitumor Agents

Most of the important compounds used for chemotherapy of tumors are antibiotics produced by actinomycetes (Oki and Yoshimoto, 1979; Tomasz, 1995). These include actinomycin D, mitomycin, bleomycins and the anthracyclines, daunorubicin and doxorubicin. A provocative recent finding is that neomycin inhibits human angiogenin-induced angiogenesis in human endothelial cells (Hu, 1998). The mechanism appears to act via neomycin's ability to inhibit phospholipase C. Amazingly, other aminoglycosides (gentamicin, streptomycin, kanamycin, amikacin and paromomycin) are inactive even though paromomycin differs from neomycin by merely having -OH at position 6 of the glucose ring instead of $-NH_2$.

Other Bioactive Metabolites

It was pointed out in the 1970s and 1980s (for review, see Demain, 1983) that compounds with antibiotic activity also exhibit other valuable

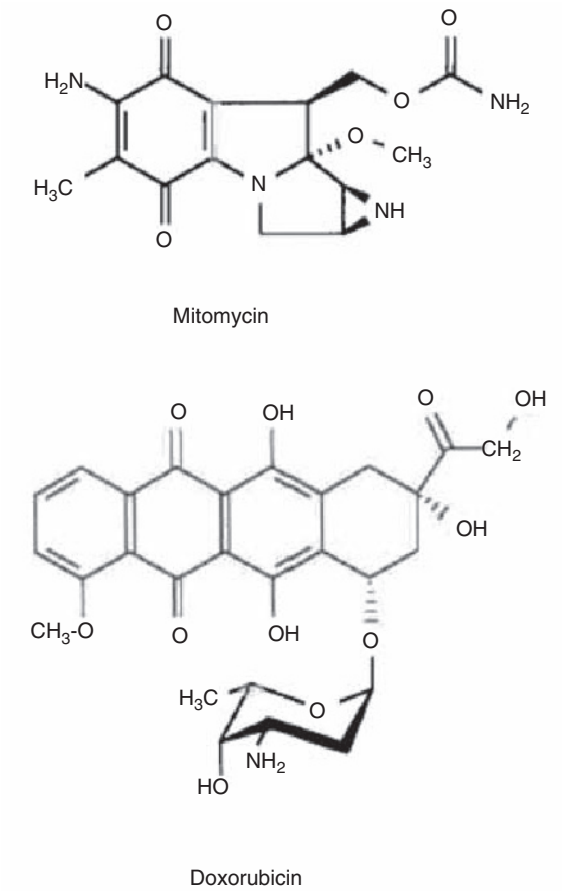


Fig. 10. Antitumor Agents.

activities, some of which had been quietly exploited in the past, and that such broadening of scope should be exploited and expanded in the future. Thus, a broad screening of antibiotically active molecules for activity against organisms other than microorganisms, as well as for activities having other pharmacological applications, was proposed to yield new and useful lives for "failed antibiotics." A large number of simple in vitro laboratory tests were developed to detect, isolate and purify useful compounds. Much of this emphasis was brought about by the writings of Hamao Umezawa (Umezawa, 1972; Umezawa, 1982), who pointed out the potential importance of enzyme inhibitors as drugs. Fortunately, we entered into a new era in which microbial metabolites were applied to diseases (not caused by bacteria and fungi) heretofore only treated with synthetic compounds. Successes were achieved in the following different areas.

Immunosuppressive Agents

Cyclosporin A was originally discovered as a narrow spectrum antifungal peptide produced by a

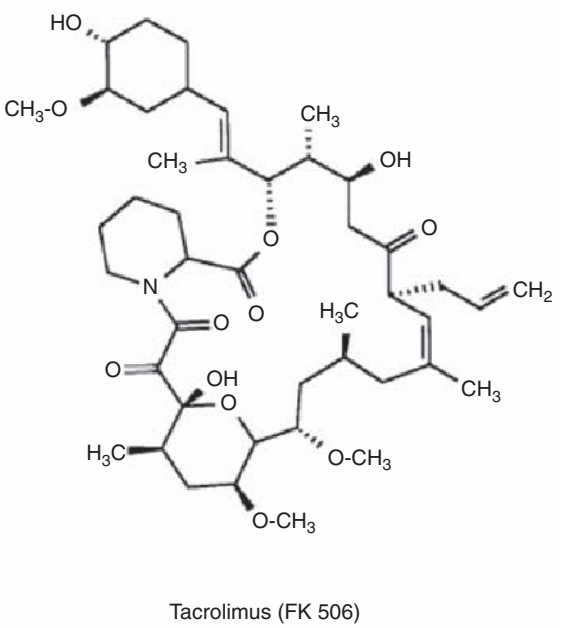


Fig. 11. Tacrolimus (FK-506).

mold (Borel et al., 1976). Discovery of its immunosuppressive activity led to its use in heart, liver and kidney transplants and to the overwhelming success of the organ transplant field. Although cyclosporin A had been the only immunosuppressant on the market for many years, two other products, produced by actinomycetes, are providing new opportunities. These are the polyketides FK-506 (tacrolimus; Kino et al., 1987) and rapamycin (sirolimus; Vezina et al., 1975), both narrow spectrum antifungal agents, which are 100-fold more potent than cyclosporin as immunosuppressants and less toxic. The polyketides FK-506 and rapamycin were approved for use a few years ago and in 1999, respectively.

Studies on the mode of action of these immunosuppressive agents have markedly expanded current knowledge of T-cell activation and proliferation (Liu, 1993). Rapamycin, FK-506 and cyclosporin A all act by a new mechanism of drug action, i.e., interaction with an intracellular protein (an immunophilin) forming a novel complex which selectively disrupts signal transduction events of lymphocyte activation (Dumont and Su, 1996). By binding to its immunophilin (FK506-binding protein; FKBP), rapamycin inhibits a unique growth regulation path utilized by lymphocytes in responding to several cytokines. A previously unknown protein called the "mammalian target of rapamycin" (mTOR; a member of the family of lipid/protein kinases) is part of the rapamycin-sensitive signal transduction pathway.

Enzyme Inhibitors and Chelating Agents

In addition to the enzyme inhibitors produced by fungi used to lower cholesterol (statins), bacterial enzyme inhibitors have also succeeded in the world of medicine. These include clavulanic acid and acarbose. Clavulanic acid is a β-lactam with poor antibiotic activity, produced by *Streptomyces clavuligerus* (Birnbaum et al., 1978). It is an inhibitor of β-lactamases and is thus included with penicillins in combination therapy of penicillin-resistant bacterial infections (Brown, 1986). Acarbose, used as an inhibitor of intestinal α-glucosidase in diabetes and hyperlipoproteinemia, is produced by *Actinoplanes* sp. SE50 (Truscheit et al., 1981). Desferal is a siderophore (produced by *Streptomyces pilosus*; Winkelmann, 1986) whose high level of activity has led to its use in iron-overload diseases (hemochromatoses) and aluminum overload in kidney dialysis patients.

Antiparasitic Agents

One of the major economic diseases of poultry is coccidiosis caused by species of the parasitic protozoan, *Eimeria*. For years, this disease was treated solely by synthetic chemicals and indeed only synthetic compounds were screened for coccidiostatic activity. Although they were generally effective, resistance developed rapidly in the coccidia and new chemical modifications of the existing coccidiostats had to be made. Surprisingly, a parenterally toxic and narrow spectrum antibiotic, monensin, was found to have extreme potency against coccidia (Haney and Hoehn, 1968). Even with this finding, there were grave doubts that the fermentation process for this polyether compound could be improved to the point where monensin would become economical for use on the farm. However, the power of industrial genetics and biochemical engineering is so great that there is almost no limit to the improvements possible in a fermentation process. As a result, the polyether antibiotics, especially monensin (produced by *Streptomyces cinnamonensis*), lasalocid (produced by *Streptomyces lasaliensis*) and salinomycin (produced by *Streptomyces albus*; Westley, 1977) dominate the commercial coccidiostat field today.

Another major veterinary problem has been the infection of farm animals by worms. The predominant type of screening effort over the years was the testing of synthetic compounds against nematodes, and some commercial products did result. Certain antibiotics also had been shown to possess antihelminthic activity against nematodes or cestodes but these failed to compete with the synthetic compounds. Further screening of microbial broths for antihelminthic activity yielded a nontoxic fermentation broth that killed the intestinal nematode, *Nematosporoides dubius*, in mice. The *Streptomyces avermitilis* culture, which was isolated by Omura and coworkers at the Kitasato Institute in Japan (Stapley, 1982; Ikeda and Omura, 1997), produced a family of secondary metabolites having both antihelminthic and insecticidal activities, which were named "avermectins." They are disaccharide derivatives of macrocyclic lactones with exceptional activity against parasites, i.e., at least ten times higher than any synthetic antihelminthic agent known. Despite their macrolide structure, avermectins lack activity against bacteria and fungi, do not inhibit protein synthesis, nor are they ionophores; instead, they interfere with neurotransmission in many invertebrates. They have activity against both nematode and arthropod parasites in sheep, cattle, dogs, horses and swine. A semisynthetic derivative, 22,23-dihydroavermectin B1 (Ivermectin) is one thousand times more active than thiobenzole and is a commercial veterinary product. The avermectins are closely related to the milbemycins, a group of nonglycosidated macrolides produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus* (Mishima et al., 1983). These compounds possess activity against worms and insects. A new avermectin, called "Doramectin" (cyclohexylavermectin B1), was recently developed by the technique of mutational biosynthesis (McArthur, 1998). Indeed, it was the first commercially successful example of mutational biosynthesis. The natural avermectins contain C-25 side chains of 2-methyl butyryl ("a" components) or isobutyryl ("b" components). Branched-chain 2-keto acid dehydrogenase was eliminated from the parent culture by knocking out gene *bkd* (Hafner et al., 1991). This enzyme normally supplies the 2-methylbutyryl-CoA and isobutyryl-CoA starter units of avermectins from isoleucine and valine, respectively (Chen et al., 1989; MacNeil et al., 1994). The mutant produced no avermectin unless fed isobutyric acid or (S)-2-methylbutyric acid. Upon feeding other fatty acids, novel avermectins were made (Dutton et al., 1991). More than 800 fatty acids were tested, yielding over 60 avermectins including cyclohexyl B1 avermectin (Doramectin), which resulted from incorporation of cyclohexane carboxylic acid. Doramectin is claimed to have some commercial advantages over Ivermectin (Goudie et al., 1993).

A fortunate fallout of the avermectin work was the finding that Ivermectin has activity against the black fly vector of human onchocerciasis (river blindness). It interferes with transmission of the filarial nematode, *Onchocerca volvulus*, to the human population. Because this disease affects 40 million people, the decision by

Merck to supply Ivermectin free of charge to the World Health Organization for use in humans in the tropics was met with great enthusiasm and hope for conquering this parasitic disease.

Biochemical and Genetic Aspects of Secondary Metabolism

There is no longer any doubt that antibiotics and other secondary metabolites are produced under natural growth conditions and have a physiological role. Over 40% of actinomycetes produce antibiotics when they are freshly isolated from nature. Soil, straw and agricultural products often contain antibacterial and antifungal substances. Bacterially produced siderophores have been found in soil, and microcins, enterobacterial antibiotics, have been isolated from human fecal extracts. The microcins are thought to be important in bacterial colonization of the human intestinal tract early in life.

Antibiotic production has been shown in unsterilized, unsupplemented soil, in unsterilized soil supplemented with clover and wheat straws, in mustard, pea, and maize seeds, and in unsterilized fruits. A further indication of natural antibiotic production is the possession of antibiotic-resistance plasmids by most soil bacteria. The widespread nature of secondary metabolite production and the preservation of the multigenic biosynthetic pathways in nature indicate that secondary metabolites serve survival functions in organisms that produce them. There are a multiplicity of such functions, some dependent on antibiotic activity and others independent of such activity. Indeed, in the latter case, the molecule may possess antibiotic activity but may be used in nature for an entirely different purpose. These functions include metal transport, bacteria-plant symbiosis, plant growth stimulation, bacteria-nematode symbiosis, and induction of morphological and chemical differentiation (Demain, 1989).

It is now widely accepted that secondary metabolism represents a form of cellular differentiation. In prokaryotes and in other microorganisms, differentiation is comprised of morphological differentiation (morphogenesis, sporulation and germination) and physiological (or chemical) differentiation (production of secondary metabolites). One important difference is that morphological differentiation is characteristic of a taxonomic group of organisms, normally a genus, whereas production of a given secondary metabolite is a characteristic of one or a few strains not necessarily related taxonomically. In the cases thoroughly studied, such as *Bacillus subtilis* and *Streptomyces coelicolor*, it has been observed that the two forms of differentiation share some of the early genes involved in regulation. In *Bacillus*, the transcription of the sporulation genes, as well as that of genes involved in secondary metabolism, is totally dependent on the *spoOA* gene product. The activated forms of gene products SpoOA and SpoOF set off the sporulation regulatory cascade. In this cascade, one of the steps is the repression by SpoOA of the *abrB* gene. The *abrB* gene product represses transcription of genes *tycA* and *tycB* governing the biosynthesis of tyrocidine. Derepression of transcription initiates the synthesis of this antibiotic. In *S. coelicolor*, inactivation of the *bld* genes hinders both the formation of aerial mycelium and the production of antibiotics. Inactivation of the *whi* genes (that follow the *bld* genes in the morphological differentiation cascade) blocks the development of aerial mycelium into spores but allows the production of the antibiotics. Vice versa, mutations in genes *absA* and *absB* result in normal sporulation but absence of antibiotic production (Chater and Bibb, 1997).

Further evidence on the connection of secondary metabolism and morphological differentiation is provided by the function of a number of small molecules, themselves secondary metabolites, called "autoregulators." The best studied is A-factor, originally isolated from *S. griseus*, the producer of streptomycin. Mutants defective in A-factor production are unable to sporulate and to produce the antibiotic. A-factor acts by binding to a protein (A-factor binding protein), which represses the translation of a gene *x*, coding for a regulatory protein X. In the presence of A-factor, the repression is relieved and protein X can, on the one hand, initiate the cascade leading to morphological differentiation, and, on the other hand, activate the transcription of gene *adp*, encoding another positive regulator protein acting on genes *strR* and *aphD* involved in streptomycin biosynthesis (Horinouchi, 1993).

In prokaryotes, the secondary metabolism genes, whether chromosomal or (very rarely) plasmid-borne, are usually clustered, not necessarily as single operons but also in the form of regulons and even modulons. Each cluster includes, besides structural genes governing the synthesis of the enzymes of antibiotic biosynthesis, regulatory genes and genes determining self-resistance to the antibiotic produced. Expression of these genes is under strong individual and global control by nutrients, inducers, products, metals and growth rate. In the few cases studied, it appears that regulation is normally at the level of transcription, as revealed by the absence of mRNA encoding secondary metabolite synthases until growth rate has decreased; translational control is less common.

Secondary metabolism occurs best at submaximal growth rates after growth has slowed down. The distinction between the growth phase ("trophophase") and production phase ("idiophase") is sometimes very clear, but in many cases, idiophase overlaps trophophase. The timing between the two phases can be manipulated, i.e., the two phases are often distinctly separated in a medium favoring rapid growth, but overlap partially or even completely in a medium supporting slower growth. A secondary metabolite is "secondary" because it has no apparent involvement in the vegetative growth of the producing culture, not because it is produced after growth. Thus, elimination of production of a secondary metabolite by mutation will usually not stop or slow down growth; indeed, it may increase the growth rate.

The factors controlling the onset of secondary metabolism are complex and not well understood (Chater and Bibb, 1997). Growth rate is important, but we do not fully know the mechanism(s) involved. Limitations of certain nutritional factors such as carbon, nitrogen or phosphate sources are important, but again we are unclear on the basic mechanisms involved. The temporal nature of secondary metabolism is certainly genetic in nature, but expression can be influenced greatly by environmental manipulations. Recent experiments have shown that the effect of starvation for certain nutrients on the onset of antibiotic production is mediated by the formation of guanine 5′-diphosphate 3′-diphosphate (ppGpp). However, this requirement is not absolute because mutations in a streptomycin-binding ribosomal protein (L11 in *S. coelicolor*, S12 in *B. subtilis*) result in antibiotic production without involvement of ppGpp (Hosoya et al., 1998). Similarly, certain rifamycin-resistant RNA polymerases possess alterations that make antibiotic production independent of ppGpp.

The delay often seen before onset of secondary metabolism was probably established by evolutionary pressures. Many secondary metabolites have antibiotic activity and could kill the producing culture if made too early. Of course, the resistance of antibiotic producers to their own metabolites is well known. Antibiotic-producing species possess suicide-avoiding mechanisms such as 1) enzymatic detoxification of the antibiotic, 2) alteration of the antibiotic's normal target in the cell, and 3) modification in permeability to allow the antibiotic to be pumped out of the cell and to restrict its reentry. Such mechanisms are often inducible, but in some cases are constitutive. In the case of inducible resistance, death could result when the antibiotic is produced too early and induction is slow. Delay in secondary metabolite production until the starvation phase makes sense if the product is used as a competitive weapon in nature or endogenously as an effector of differentiation. In nutritionally rich habitats, such as the intestines of mammals where enteric bacteria thrive, secondary metabolite production is not as important as in soil and water, where nutrients limit microbial growth. Thus secondary metabolites tend not to be produced by enteric bacteria such as *E. coli* but by soil and water inhabitants such as bacilli, actinomycetes and fungi. Nutrient deficiency in nature often induces morphological and chemical differentiation, i.e., sporulation and secondary metabolism, respectively; both are beneficial for survival in the wild. Thus, the regulation of the two types of differentiation is often related.

The study of the biosynthesis of secondary metabolites requires identification of the sequence of reactions by which one or more primary metabolites is converted into the final molecule. The biosynthetic pathways of a vast number of antibiotics have been elucidated, both as the result of academic interest and for the practical outcome that such research can give, e.g., in increasing production yields of antibiotics of commercial interest and in obtaining molecules modified in their biological activity.

In contrast to the huge variety of chemical structures presented by antibiotic molecules, the sequences of biological reactions by which they are made can be grouped into the following three classes (Lancini and Lorenzetti, 1993).

The Three Classes of Biological Reactions

CLASS I REACTIONS. These are the series of reactions by which a primary metabolite is converted into a small antibiotic molecule or into an intermediate of the synthesis of larger antibiotics. Typically, these series of reactions can be conveniently classified according to the pathways of primary metabolism with which they are related. We thus have antibiotics, or antibiotic moieties, whose biosynthesis is tied to amino acid synthesis or catabolism, nucleoside metabolism or coenzyme synthesis (Lancini and Demain, 1999). An example of the synthesis of a small antibiotic molecule through Class I reactions is the biosynthesis of chloramphenicol: a variation of the pattern of tyrosine biosynthesis leads to formation of *p*-aminophenylalanine, which is subsequently modified by hydroxylation, acylation, reduction and oxidation reactions into the final molecule. An example of the synthesis of an intermediate is the biosynthesis of 3-amino-5-hydroxybenzoic acid, the initiator of several ansamycin and other metabolite biosyntheses, occurring by a series of reactions closely paralleling the well-known shikimate pathway of aromatic amino acid biosynthesis.

CLASS II REACTIONS. The key steps in the biosynthesis of a number of antibiotics are polymerization reactions, by which several similar units are linked together to form the backbone of a larger molecule. On the biochemical basis and according to the similarity of the genes involved, the following types of polymerization reaction are recognized in bacteria:

1. Polyketide Synthesis. This is a mechanism of polymerization of small acid units (most often acetate or propionate; the actual molecules involved in the condensation process are malonate or methylmalonate) closely analogous to the mechanism of fatty acid synthesis (Katz and Donadio, 1993). Two types of polyketide synthases (PKSs) are recognized:

a) Polyketide synthases of type II. These PKSs are characterized by an organization of the enzymes similar to that of fatty acid synthases (FAS) of most bacteria, in which each of the reactions necessary for the addition of a unit (condensation, reduction, etc.) is catalyzed by a structurally distinct and functionally different polypeptide. The mechanism is iterative, in the sense that the same addition operations are repeated several times by the same set of enzymes, giving rise to a chain composed of identical elements. In polyketide antibiotic synthesis, the chain extension molecule is always malonate, and reduction and dehydration steps are generally omitted. The resulting chain is composed of alternating methylene and keto groups (the term "PKS" is derived from the presence of the keto groups). Such a structure is highly unstable and tends to eliminate water by aldol condensation between keto and methylene groups. According to the length of the chain and the nature of the enzymes involved, many different structures can be formed; these tend to be aromatic owing to steric and energy factors. A classical example is the biosynthesis of tetracycline by S. aureofaciens. The initiator molecule in this case is the monoamide of malonic acid and the elongation units are eight molecules of malonic acid. The resulting molecule is methylpreteramide, the earliest identified intermediate in tetracycline biosynthesis, whose structure is comprised of four linearly condensed aromatic rings.

b) Polyketide synthases of type I. These, in contrast to PKSs of type II, are multifunctional enzymes (i.e., one protein may catalyze the several different reactions needed for an elongation step). Various classes of secondary metabolites, each comprising important antibiotics, are biosynthesized by a process similar in its general outline to that of the aromatic polyketides but in which profound modifications may be included. Important sources of variation are the frequent use of methylmalonate as the chain extension unit and the partial or total reduction of most of the keto groups that are formed in the chain extension steps. The chain so formed can thus bear methyl, keto or hydroxy groups and may include double bonds and adjacent methylene groups. As a consequence of these variations in its structure, the chain cannot be converted by the aldol reaction into aromatic rings, and either linear molecules or macrocyclic rings are formed. This is the origin, for instance, of the basic structures of the macrolide antibiotics (e.g., erythromycin), the ansamycins (e.g., rifamycins), and the ionophoric polyethers (e.g., monensin). All of these are synthesized by PKSs of type I having a particular modular organization. It is interesting to note that whereas metabolites made by PKSs of type II are produced by both prokaryotes and fungi, those made by PKSs of type I are produced only by actinomycetes and, to a minor extent, by myxobacteria and pseudomonads. Modular PKSs are multifunctional proteins comprised of a few functional modules; each module possesses all the enzymatic activities needed to perform a given elongation step. Each module recognizes one extension unit (malonate or alkyl malonate), adds it to the growing chain, and performs the appropriate reduction or dehydration reaction. The growing chain is transferred from one module to the adjacent one, so that the order in which the modules are arranged determines the order of the chemical groups on the chain. As an example, erythromycin, a product of *S. erythraea*, is composed of a macrocyclic lactone of fourteen atoms, bearing keto, hydroxy, methyl groups and two sugars. The macrocycle is made by the polyketide process, which consists of extending one propionate (the initiator unit) with six methylmalonate units. The synthase complex, as deduced from the gene organization and sequences, is composed of three multifunctional proteins, each comprising three FAS-like modules. Thus, there are a total of six functional modules, each responsible for one of the six elongation cycles needed to construct the chain. Each cycle includes the condensation of one methylmalonate with the growing chain and the appropriate total, partial, or nonreduction of the α-keto group. The linear chain thus produced is closed, forming the macrocyclic lactone of deoxyerythronolide B, the earliest identified intermediate of the biosynthetic pathway. Subsequent reactions transform it into the antimicrobial erythromycin.

2. Polymerization of Amino Acids by Polypeptide Synthases. Typical of secondary metabolism is a mechanism of formation of polypeptides catalyzed by nonribosomal polypeptide synthetases (NRPSs; Kleinkauf and von Döhren, 1997). The mechanism is similar in several aspects to polyketide synthesis by PKS type I. The NRPSs

catalyze the activation and incorporation of monomers (amino- or hydroxy-acids) into oligomers (peptides or depsipeptides). Similar to PKS I, NRPSs present a modular organization such that one module comprising enzymatic domains is utilized for each monomer incorporated. Therefore, the number of modules corresponds to the number of monomers incorporated and, most relevant, the order in which the monomers are incorporated reflects the order in which the modules are present on the NRPS. Each module generally comprises an adenylation domain, responsible for amino acid recognition and activation; a thiolation domain where the amino acid, and then the growing peptide, are covalently bound; and the condensation domain where the amide bond is formed. Additional specialized domains, such as those for conversion of L- to D-amino acids, also may be present. The steps of the synthesis are as follows: 1) the amino acids are activated by adenylation and each linked, through a thioester bond, to the appropriate module; 2) the first amino acid forms an amide bond with the amino group of the second amino acid, the energy being provided by the breaking of the thioester bond; 3) the dipeptide thioester breaks and the carboxyl group forms a peptide bond with the following monomer; and 4) the cycle is repeated until completion of the chain that is then released by a thioesterase. An important example of oligopeptide synthesis is the formation of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), the first free intermediate in the synthesis of β-lactam antibiotics. The enzyme, ACV synthetase (which has been purified from *S. clavuligerus*), activates and binds the three amino acids as thioesters (α-aminoadipic acid is activated and bound at its γ-carboxyl group), performs the inversion of L-valine into D-valine, and carries out the condensation steps. Sequencing of the genes coding for ACV synthetase has revealed the presence of three highly repetitive regions, each containing sequences corresponding to enzyme domains that activate an amino acid.

3. Ribosomal Peptide Synthesis. A small group of large peptide antibiotics has been described that are made by the general transcription and translation system of ribosomal protein synthesis (Jack et al., 1997). These are known as the lantibiotics, owing to the presence in their molecules of several lanthionine moieties derived from sulfide bridge formation between cysteine and serine (or threonine) residues. Nisin, an antibiotic used in foods, is produced by unicellular bacteria; others, such as ancovenin and actagardine, are made by actinomycetes. Studies on their biosyntheses have demonstrated that the final antibiotic molecules are derived from larger ribosomally synthesized peptides called "prelantibiotics." These comprise a leader peptide and an amino acid sequence that give rise to the antibiotic structure through extensive post-translational processing. The last biosynthetic step is the cleavage of the leader peptide.

4. Condensation of Carbohydrate Units (Often Amino Sugars) to Form Oligosaccharides. In a number of important antibiotics, an aminocyclitol moiety is included instead of a sugar, resulting in the formation of a pseudosaccharide. The available evidence indicates that antibiotics having an oligosaccharide structure are formed by the assembly of monomers activated as nucleoside diphosphates at the anomeric carbon (Piepersberg and Distler, 1997). In this respect, their biosynthesis is not different from that of the normal polysaccharides present in bacterial cell walls or surfaces. The components of the oligosaccharide antibiotics are most often unusual carbohydrates. Two patterns may be distinguished with regard to their formation: a) sugars normally found in primary metabolism are assembled and then modified; typical of this pattern is the biosynthesis of the antibiotics of the gentamicin family; and b) common sugars, such as glucose or glucosamine, are converted into unusual carbohydrates and then condensed stepwise to give the final molecule; this is the case of streptomycin biosynthesis. In the latter case, the transformation reactions also occur, as a rule, on sugars activated as nucleoside diphosphates at the anomeric carbon. However, there is not a rigid distinction between the two cases; both patterns may be present in a single biosynthetic pathway.

These mechanisms of polymerization are similar to those of primary metabolism, which are involved in building molecules that are components of cell envelopes. Polyketide synthesis and, to some extent, nonribosomal polypeptide synthesis appear to be derived from the mechanism of membrane fatty acid biosynthesis, whereas the biosynthesis of sugar-derived antibiotics is similar to that of the polysaccharides present in the external layers of bacterial envelopes, such as the O-antigens of Gram-negative organisms. It is of interest that isoprenoid synthesis, very common in fungal secondary metabolism, is almost absent in biosyntheses of bacterial secondary metabolites.

CLASS III REACTIONS. In general, the basic structure produced by a polymerization mechanism, or by condensation of one or more subunits, is further modified by enzymatic reactions to give the final antibiotic molecule (Lancini and Lorenzetti, 1993). These "tailoring" reactions are normally very common ones: hydroxylations, double-bond saturations and other reductions, methylations, transaminations,

glycosylations and acylations. However, they are most relevant because the modifications are usually essential for activity. Tailoring is often responsible for the production, by many strains, of complexes of antibiotic molecules, i.e., structurally related substances quite similar in their biological activity. A complex may arise when an intermediate of the biosynthesis is the substrate of different reactions and therefore yields different products. One example is teicoplanin produced by *A. teichomyceticus*. The antibiotic is a complex of several molecules differing in the structure of the acyl substituents linked to a glucosamine moiety. The relative amounts of the components depend on the amounts of the fatty acids, which are the substrates of the acylation reaction. A typical example of the importance of tailoring is the formation of tetracyclines. Here, the common intermediate methylpretetramide is converted through reductions, amination and methylation into the final tetracycline molecule. In certain strains, carbon 7 of the molecule is chlorinated, giving rise to chlortetracycline; in contrast, other strains hydroxylate carbon 5, yielding oxytetracycline. Another example is the synthesis of erythromycins: the product of PKS action, deoxyerythronolide B, is first hydroxylated and then glycosylated with two sugars. The resulting molecule can be hydroxylated on the ring, giving erythromycin C, or methylated on one of the sugars, giving erythromycin B, or submitted to both these reactions, giving rise to erythromycin A, thus producing a mixture of the three antibiotics.

Products from Modern Technology

Although industrial microbiology is not a new field, the recombinant DNA discoveries made in 1972 in the laboratories of Stanford University and the University of California at San Francisco propelled the field to new heights and led to the establishment of a new biotechnology industry in the United States and around the world. The revolutionary exploitation of basic biological discoveries did not take place in a vacuum but heavily depended upon the solid structure of the fermentation industry. Before the new biotechnology era began, there had already been a rapid application of fundamental biological knowledge, thanks to the earlier developments of industrial microbiology. The period from 1910 to 1950 had featured the first large-scale anaerobic fermentations devoted to manufacture of chemicals (acetone and butanol) and the aerobic production of citric acid and then those of penicillin and streptomycin. The scale-up investigations of the penicillin and streptomycin fermentations, a joint effort between Merck & Co. and Princeton University, gave birth to the new field of biochemical (we prefer microbiological) engineering. On the heels of this major development was the academic development of mutational microbial genetics, which evolved into the new technology of strain improvement. With microbiological engineering and strain improvement at hand, the pharmaceutical industry wasted no time in bringing laboratory discoveries to the commercial scale.

Modern biotechnology encompasses recombinant DNA technology, enzyme and cell immobilization ("enzyme engineering"), cell fusion, monoclonal antibodies and DNA probes as diagnostics, and in vitro mutagenesis ("protein engineering"). It interacts strongly with industrial microbiology and microbial engineering in areas of fermentation, microbial physiology, high throughput screening for novel metabolites, automation and miniaturization in screening and strain improvement, bioreactor design and downstream processing. Biotechnology is having a major effect on health care, diagnostics and agriculture and promises to make inroads in the practices of other industries such as petroleum, mining, foods and chemicals. The progress in biotechnology has been truly remarkable. Within four years of the development of recombinant DNA technology, genetically engineered bacteria were making human insulin and human growth hormone (hGH). This led to an explosion of investment activity in new companies, mainly dedicated to innovation via genetic approaches. Newer companies entered the scene in various niches such as biochemical engineering and downstream processing. Auxiliary industries also made their mark in the commercial world of biotechnology by offering equipment, supplies, information, mechanisms of communication, and patent protection.

The recombinant DNA pharmaceutical market consists of four principal areas: 1) blood products: thrombolytics, dismutases, septic shock drugs, clotting agents and erythropoeitin (EPO); 2) immunotherapy products: α, β and γ interferons, interleukins, and colony stimulating factors (granulocyte-colony stimulating factor [G-CSF] and granulocyte-macrophage-colony stimulating factor [GM-CSF]); 3) infectious disease combatants: hepatitis B vaccine; and 4) growth factors for mammalian cells: epidermal growth factor (EGF), insulin-like growth factors (IGF I and IGF II), fibroblast growth factors (FGFs), transforming growth factors (TGFa and TGFb), platelet-derived growth factor (PDGF), growth hormone releasing factor (GRF), lung surfactants, and tumor necrosis factor (TNF). By 1998, the biotechnology industry in the United States had revenues of $19.6 billion, sales of $13.4 billion and 153,000 employees. In that year, four-

teen new biopharmaceuticals were approved and more than 300 drugs were in phase II/III and phase III trials.

It became clear that recombinant DNA technology yielded purer proteins and was much more economical than conventional techniques. As a result, a large number of mammalian peptide genes were cloned and expressed in *E. coli*, *B. subtilis* and other bacilli, *Saccharomyces cerevisiae* and other yeasts, *Aspergillus niger*, insect cells and mammalian cells. The benefits of *E. coli* as a recombinant host included 1) ease of quickly and precisely modifying the genome, 2) rapid growth, 3) ease of fermentation, 4) ease of reduction of protease activity, 5) ease of avoidance of incorporation of amino acid analogs, 6) ease of promoter control, 7) ease of alteration of plasmid copy number, 8) ease of alteration of metabolic carbon flow, 9) ease of formation of intracellular disulfide bonds, 10) growth to very high cell densities, 11) accumulation of heterologous proteins up to 50% of dry cell weight, 12) survival in a wide variety of environmental conditions, 13) inexpensive medium ingredients, 14) reproducible performance especially with computer control, and 15) high product yields (Swartz, 1996). As a result, *E. coli* became a preferred host for production of recombinant polypeptides. In 1993, *E. coli* recombinant processes were responsible for almost $5 billion worth of products, i.e., human insulin, hGH, interferons and G-CSF (Swartz, 1996). *Escherichia coli* could produce 5.5 grams per liter of α-consensus interferon (Fieshko, 1989). High cell densities are often the key to successful production of recombinant proteins. The main obstacle to high cell density is production of acetate, which is toxic. Success is attained when acetate production is limited by exponential feeding of glucose, which keeps the specific growth rate below that which brings on acetate production. A literature comparison of different host organisms reveals dry cell concentrations of 110–175 g/liter for *E. coli* (Shay et al., 1987; Lee, 1996), 110 g/liter for *Bacillus megaterium*, 84 g/liter for *Pseudomonas putida*, 130 g/liter for methylotrophic yeasts (Gellissen et al., 1991) and 200 g/liter for *S. cerevisiae* (Fieshko et al., 1987).

Not all mammalian polypeptides are produced best in *E. coli*. Heterologous proteins, often produced as inclusion bodies in *E. coli* are inactive, aggregated and insoluble, usually possessing non-native intra- and intermolecular disulfide bonds and unusual free cysteines. To get active protein, these bodies must be removed from the cell by homogenization, washing, and centrifugation and solubilized by denaturants (guanidine·HCl, urea and sodium dodecyl sulfate [SDS]), which unfold the protein and (with reducing agents) break the disulfide bonds. Refolding is carried out by removal of denaturant and reducing agent. The renaturation processes are 1) air oxidation, 2) the glutathione reoxidation system, and 3) the mixed disulfides of protein-S-sulfonate and protein-S-glutathione systems. However, heterologous recombinant proteins can be made in biologically active soluble form at high levels when their genes are fused to the *E. coli* thioredoxin gene (LaVallie et al., 1993). Many murine and human proteins are produced at levels of 5–20% of total protein as fusions in *E. coli* cytoplasm. Some fusions retain the thioredoxin properties of being released by osmotic shock or freeze/thaw methods and high thermal stability. Thioredoxin is small (11 kDa) and is normally produced at 40% of total cell protein in soluble form (Lunn et al., 1984). Another useful method of reducing the formation of inclusion bodies containing heterologous proteins in *E. coli* is to lower the temperature of growth from 37 to 30°C (Schein, 1989).

Another problem with *E. coli* is the formation of analogue polypeptides. For example, norleucine can enter proteins cloned in *E. coli* by competing with methionine (Bogosian et al., 1989). Because norleucine is formed by the leucine biosynthetic enzymes from pyruvate or by α-ketobutyrate substituting for α-ketoisovalerate, norleucine contamination can be eliminated by elimination of the leucine biosynthetic operon. It also can be eliminated by adding methionine or 2-hydroxy-4-methylthiobutanoic acid (a methionine precursor) to the medium. It should be noted, however, that several enzymes are known to retain full activity when norleucine substitutes for methionine. Also production of recombinant human macrophage colony stimulating factor (M-CSF) by *E. coli* resulted in up to 20% substitution of methionine by norleucine with no loss of activity.

Bacillus species are looked upon as good hosts for recombinant DNA work, owing to their ability to excrete proteins rather than accumulate intracellular inclusion bodies. However, *B. subtilis* produces many recombinant protein-destroying proteases. It has seven known proteases (He et al., 1991), five of which are extracellular: 1) subtilisin (encoded by the *aprE* gene): major alkaline serine protease; 2) neutral protease (*nprE*): major metalloprotease, containing Zn; 3) minor serine protease (*epr*), inhibited by phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA); 4) bacillopeptidase F (*bpf*): another minor serine protease/esterase inhibited by PMSF; 5) minor metalloesterase (*mpe*); 6) ISP-I (*isp-I*): major intracellular serine protease, requiring Ca; and 7) ISP-II (*isp-II*): minor intracellular serine protease. The first two enzymes account for 96–98% of the extracellular protease activity. Other

research groups have found six extracellular proteases. Wu et al. (1991) removed all six and only 0.32% activity remained. Growth in the presence of 2 mM PMSF eliminated the rest of the activity. A comparison made of host organisms for production of IL-3 (Van Leen et al., 1991) among *E. coli*, *Bacillus licheniformis*, *S. cerevisiae*, *Kluyveromyces lactis* and C127 mammalian cells showed that *B. licheniformis* is best. Owing to its much lower protease activity and production of a proteinase inhibitor, *Bacillus brevis* is another alternative host to *B. subtilis* (Udaka and Yamagata, 1994). Human epidermal growth factor was produced in *B. brevis* at a level of 3 g/liter (Ebisu et al., 1992).

Many benefits to society have resulted from proteins made in *E. coli* (Swartz, 1996). 1) Diabetics do not have to fear producing antibodies to animal insulin. 2) Children deficient in growth hormone no longer have to suffer from dwarfism or fear the risk of contracting Kreutzfeld-Jacob syndrome. 3) Children who have chronic granulomatous disease can have a normal life by taking interferon gamma therapy. 4) Patients undergoing cancer chemotherapy or radiation therapy can recover more quickly with fewer infections when they use G-CSF.

In some cases, unicellular bacteria cannot be used to make recombinant polypeptides because of an inability to glycosylate the polypeptide. Almost all eukaryotic cell-excreted polypeptides are glycosylated. Glycosylation is species, tissue-, and cell-type specific (Parekh, 1989). In some cases, a normally glycosylated protein is active without the carbohydrate moiety and can be made in bacteria. This is the case with interferon-gamma (Rinderknecht et al., 1984). In cases where glycosylation is necessary for stability or proper folding (e.g., of EPO and human chorionic gonadotropin [hGC]), recombinant yeast, mold, insect or mammalian cells provide the means.

Genetic Engineering of Secondary Metabolism

Genetics has had a long history of contributing to the production of bacterial products. The tremendous increases in fermentation productivity and the resulting decreases in costs came about mainly by mutagenesis and screening for higher producing bacterial strains. In the main, this was "brute force" technology, but in recent years, efforts have been devoted to miniaturization and automation of screening procedures and to the development of methods of enhancing the frequency of improved strains by selection procedures (found earlier to be useful in improving production of primary metabolites, e.g., the isolation of antimetabolite-resistant mutants in cases where the natural metabolite is a precursor, an inhibitor, or a corepressor of a biosynthetic pathway). These selective agents include antimetabolites of precursors, 2-deoxyglucose for enzymes and pathways regulated by carbon source regulation, and methylammonium for those regulated by nitrogen source repression. Mutation has served to 1) shift the proportion of metabolites produced in a fermentation broth to a more favorable distribution, 2) elucidate the pathways of secondary metabolism, and 3) yield new compounds. The medically useful products demethyltetracycline and doxorubicin (adriamycin) were discovered by simple mutation of the cultures producing tetracycline and daunorubicin (daunomycin), respectively. The technique of mutational biosynthesis has been used for the discovery of many new aminoglycoside, macrolide and anthracycline antibiotics as well as a commercial antihelminthic agent, Doramectin.

Although mutation was used for forty years to improve antibiotic production, genetic recombination was virtually ignored, the principal reason being the low frequency of recombination, e.g., 10^{-6}. However, application of protoplast fusion changed the situation markedly. Frequencies of recombination increased to even greater than 10^{-1} in some cases, and strain improvement programs routinely incorporate protoplast fusion between different mutant lines. Successes were obtained, for example, in the cephalosporin and cephamycin strain improvement programs. Another use of protoplast fusion involved the recombination of different strains from the same or different species to yield new antibiotic derivatives such as anthracyclines, aminoglycosides and rifamycins. Protoplast fusion has also been useful in elimination of undesirable components from fermentation broths. Of great interest has been the application of recombinant DNA technology to the bacterial production of antibiotics and other secondary metabolites. Many genes encoding individual enzymes of antibiotic biosynthesis have been cloned and expressed. Genes of fungal β-lactam producers such as *Cephalosporium acremonium* and *Penicillium chrysogenum* (e.g., those encoding isopenicillin N cyclase and deacetoxycephalosporin C synthase and hydroxylase) have been cloned in *E. coli* and expressed at levels of 15–20% of total cell protein.

The potential of recombinant DNA technology in antibiotic improvement and discovery has been enhanced by the finding that bacterial antibiotic biosynthetic pathway genes are usually clustered with resistance genes on the chromosome. This arrangement of genes facilitates transfer of an entire pathway in a single manip-

ulation. Continued progress in the application of recombinant DNA to fermentations has led to overproduction of limiting enzymes of important biosynthetic pathways, thus increasing production of the final products. Epirubicin (4′-epidoxorubicin) is a semisynthetic anthracycline with less cardiotoxicity than doxorubicin. A totally biological method of producing it involved genetic engineering of a blocked *Streptomyces peucetius* strain (Madduri et al., 1998). The gene introduced was *avrE* of the avermectin-producing *S. avermitilis* or the *eryBIV* genes of the erythromycin producer, *S. erythrea*. These genes and the blocked one in the recipient are involved in deoxysugar biosynthesis.

Combinational biosynthesis of bacteria is now being used for discovery of new drugs (Hutchinson, 1998). Recombinant DNA techniques are used to introduce genes coding for antibiotic synthases into producers of other antibiotics or into nonproducing strains to obtain modified or hybrid antibiotics. In 1985, this was described in a breakthrough paper by Hopwood et al. (1985). Gene transfer from a streptomycete strain producing the isochromanequinone antibiotic actinorhodin into strains producing granaticin, dihydrogranaticin and mederomycin (which are also isochromanequinones) led to the discovery of two new antibiotic derivatives, mederrhodin A and dihydrogranatirhodin. Since then, many hybrid antibiotics have been produced in bacteria by recombinant DNA technology (Khosla et al., 1993; Katz and Donadio, 1993; Hopwood, 1993) as shown by the following examples.

1) The hybrid antibiotic, isovalerylspiramycin, was obtained by introducing the *carE* gene from the carbomycin producer, *Streptomyces thermotolerans*, into the spiramycin producer, *Streptomyces ambofaciens* (Epp et al., 1989). These are both 16-membered macrolides but one of the two sugars in carbomycin is isovalerylmycarose and the corresponding sugar in spiramycin is mycarose. When the acylating gene was cloned into the non-macrolide producer, *Streptomyces lividans*, the recombinant was capable of converting spiramycin to isovalerylspiramycin.

2) Hybrid anthraquinones and anthracyclines have been obtained by cloning DNA fragments from one polyketide producer into various blocked mutants of anthracycline-producing streptomycetes (Strohl et al., 1989). Cloning of the *actI*, *actIV* and *actVII* genes from *S. coelicolor* into the 2-hydroxyaklavinone producer, *Streptomyces galilaeus* 31671, yielded the novel hybrid metabolites desoxyerythrolaccin and 1-*O*-methyl-desoxyerythrolaccin (Strohl et al., 1991). Cloning of *actI*, *actIII* and *actVII* genes from *S. coelicolor* into other streptomycetes led to the production of the novel metabolite aloesaponarin II (Bartel et al., 1990).

3) Five novel polyketides were produced by combining polyketide-forming genes from different actinomycetes to construct recombinant PKS systems in an *S. coelicolor* host (McDaniel et al., 1993a; McDaniel et al., 1993b).

4) New antibiotic molecules have been produced by genetic manipulation of the erythromycin PKS and other PKSs (Donadio et al., 1993). The manipulations are of the following types (Staunton, 1998): a) Deletion of one of the domains of a particular module; b) adding a copy of the thioesterase (TE) domain to the end of an earlier module, resulting in a shortened polyketide; c) replacing an acyltransferase (AT) domain of a PKS by an AT domain from another PKS, thus changing an extender unit and resulting in addition of a methyl group at a particular site or removal of a methyl group; d) adding a reductive domain or two or three reductive domains to a particular module, thus changing a keto group to a double bond or to a methylene group; e) use of synthetic diketides delivered as *N*-acetylcysteamine thioesters to load onto the active site of the ketosynthase (KS) domain in module 2 and taking them all the way to a novel final product; f) replacing the loading module of one PKS with the loading module of another PKS, thus changing the starter unit from propionate to acetate, for example; and g) replacing the genes encoding hydroxylase or glycosylase enzymes from one pathway to another, thus modifying the ring structure with respect to OH groups and/or sugars.

Genetic engineering of actinomycetes has been limited by restriction barriers hindering DNA introduction and by inhibition of secondary metabolism contributed by self-replicating plasmid-cloning vectors. Despite these problems, remarkable progress has been made (Baltz and Hosted, 1996). Recently, bacterial artificial chromosomes have been developed to transfer 100 kb DNA segments into *Streptomyces* (Sosio et al., 2000a). A useful review on the use of recombinant microorganisms in the industrial production of antibiotics has been published by Diez et al. (1997).

Closing Remarks

The antibiotics produced by bacteria have been useful in our battles against infectious bacteria and fungi for almost 60 years. In addition, many antibiotics are used commercially or are potentially useful in medicine for activities other than their antibiotic action. There has been a major change in the field of discovery and application of secondary metabolites over the past twenty years. This change is characterized by the broadening of the scope of the search. No longer are

bacterial sources looked upon solely as potential solutions for microbial diseases. This change in screening philosophy has been followed by ingenious applications of molecular biology to detect receptor antagonists and agonists and other agents inhibiting or enhancing cellular activities on a molecular level (Tanaka and Omura, 1997).

Secondary metabolites are employed as antitumor agents, immunosuppressive agents, enzyme inhibitors, antiparasitic agents, etc. Many of these products were first discovered as antibiotics that failed in their commercial development as such, although a few successful secondary metabolites appear to have no antibiotic activity. The recently increased development of resistance to older antibacterial and antifungal drugs is being met with the use or clinical testing of older underutilized or previously nondeveloped narrow-spectrum antibacterial products.

Natural products have been an overwhelming success in our society. The doubling of our life span in the twentieth century is mainly due to the use of plant and microbial secondary metabolites (Verdine, 1996). They have reduced pain and suffering and revolutionized medicine by allowing for the transplantation of organs. Natural products are the most important anticancer and antiinfective agents. Over 60% of approved and pre-NDA (new drug application) candidates are natural products or related to them, not including biologicals such as vaccines and monoclonal antibodies (Cragg et al., 1997). Almost half of the best selling pharmaceuticals are natural or are related to natural products. Often, the natural molecule was not used itself but served as a lead molecule for manipulation by chemical or genetic means.

Secondary metabolism has evolved in nature in response to needs and challenges of the natural environment. Nature is continually carrying out its own version of combinatorial chemistry (Verdine, 1996). Bacteria have inhabited the earth for over 3 billion years (Holland, 1998). During that time, there has been an evolutionary process going on in which producers of secondary metabolites have evolved according to their local environments. When the metabolites were useful to the organism, the biosynthetic genes were retained and genetic modifications further improved the process. Combinatorial chemistry practiced by nature is much more sophisticated than combinatorial chemistry in the laboratory, yielding exotic structures rich in stereochemistry, concatenated rings and reactive functional groups (Verdine, 1996). We hear today that combinatorial chemistry will replace natural product efforts for discovery of new drugs. Some companies are even dropping their natural product programs to support combinatorial chemistry efforts. This makes no sense considering that the role of combinatorial chemistry, like those of structure-function drug design and recombinant DNA technology two and three decades ago, is that of complementing and assisting natural product discovery and development, not replacing them. Natural product research is at its highest level now, owing to unmet needs, remarkable diversity of structures and activities, utility as biochemical probes, novel and sensitive assay methods, improvements in isolation, purification and characterization, and new production methods (Clark, 1996). Enormous diversity exists in secondary metabolism, as illustrated by the following example: 10,000 polyketides are known, many of which are produced by bacteria (Rawls, 1998). These include antibiotics, antitumor agents, immunosuppressants, antiparasitic agents, antifungals and agricultural products. Many new polyketides have been made by genetic methods involving modification or exchange of polyketide genes between organisms to create hybrid polyketides, i.e., by combinatorial biosynthesis (Hutchinson, 1997; McAlpine, 1998). We should also keep in mind the enormous diversity of bacteria and that only a minor proportion have thus far been examined for secondary metabolite production. It has been estimated that 1 gram of soil contains 1,000 to 10,000 species of undiscovered prokaryotes (Torsvik et al., 1996). In addition, surprisingly, it has been found that well-known actinomyetes (either producing or not producing polypeptide antibiotics) harbor an unsuspected number of gene clusters coding for polypeptide synthases (Sosio et al., 2000b). It is conceivable that these could be activated to produce novel metabolites. The main factors important for the discovery of useful compounds in the future are commitment, ingenuity, and the ability to exploit nature's biodiversity and to devise simple in vitro high-throughput screening procedures for desirable activities.

Genetic engineering and its associated disciplines, as first developed in bacteria, have already made major impacts in the world of medicine and finance and are changing the face of agriculture and industry today and will continue to do so. Bacteria have been employed to produce many mammalian polypeptides as pharmaceuticals of major importance to modern medicine. The improvement of secondary metabolite production processes and the discovery of new drugs (by combinatorial biosynthesis) can be attributed to the remarkable developments of recombinant DNA technology.

Molecular biology of bacteria led the way for the development of the biotechnology industry and today molecular biology is the major driving force in pharmaceutical research. In addition to natural polypeptides, analogues can be produced

by recombinant DNA technology. Second generation recombinant polypeptides include modifications of first generation products to alter specificity of targets, distribution in tissues, pharmacokinetics, side effects and stability (Huber, 1989). Third-generation peptides will fuse coding sequences of different genes, e.g., an enzyme's catalytic sequence to a cell-specific receptor-binding sequence, to achieve targeted drug delivery. In the future, we shall see the replacement of polypeptide drugs by small molecules (Moore, 1992) produced by chemical synthesis or by bacteria. Protein drugs are difficult to use because delivery systems are often inappropriate. They cannot be orally administered because they are enzymatically degraded in the stomach. Market size is limited by the need for injection. Polypeptides generally have to be administered by intravenous injection of hospitalized patients. Low molecular mass drugs might be given by intradermal, intraperitoneal or oral route and will probably be more stable and nonimmunogenic. Because bacteria are such excellent producers of small peptides, we can expect full use of these prokaryotes as means of production of these pharmaceuticals of the future.

Literature Cited

Baltz, R. H., and T. J. Hosted. 1996. Molecular genetic methods for improving secondary-metabolite production in actinomycetes. TibTech 14:245–250.

Barriere, J. C., N. Berthaud, D. Beyer, S. Dutka-Malen, J. M. Paris, and J. F. Desnottes. 1998. Recent developments in streptogramin research. Curr. Pharm. Design 4:155–180.

Bartel, P. L., C. B. Zhu, J. S. Lampel, D. C. Dosch, N. C. Connors, W. R. Strohl, J. M. Beale Jr., and H. G. Floss. 1990. Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosyntheis genes in streptomycetes: Clarification of actinorhodin gene functions. J. Bacteriol. 172:4816–4826.

Birnbaum, J., E. O. Stapley, A. K. Miller, H. Wallick, D. Hendlin, and H. B. Woodruff. 1978. Cefoxitin, a semisynthetic cephamycin: A microbiological overview. J. Antimicrob. Chemother. 4, Suppl. B:15–32.

Birnbaum, J., F. M. Kahan, H. Kropp, and J. S. MacDonald. 1985. Carbapenems, a new class of beta-lactam antibiotics: Discovery and development of imipenem/cilastatin. Am. J. Med. 78, Suppl. 6A:3–21.

Black, M. T., and G. Bruton. 1998. Inhibitors of bacterial signal peptidases. Curr. Pharmaceut. Design 4:133–154.

Bogosian, G., B. N. Violand, E. J. Dorward-King, W. E. Workman, P. E. Jung, and J. F. Kane. 1989. Biosynthesis and incorporation into protein of norleucine by Escherichia coli. J. Biol Chem. 264:531–539.

Borel, J. F., C. Feurer, H. U. Gabler, and H. Stahelin. 1976. Biological effects of cyclosporin A: A new antilymphocytic agent. Agents and Actions 6:468–475.

Breiman, R., J. Butler, F. Tenover, J. Elliot, and R. Facklam. 1994. Emergence of drug-resistant pneumococcal infections in the United States. JAMA 271:1831–1835.

Brown, A. G. 1986. Clavulanic acid, a novel β-lactamase inhibitor—a case study in drug discovery and development. Drug Design Deliv. 1:1–21.

Bush, K. 1997. Antimicrobial agents. Curr. Opin. Chem. Biol. 1:169–175.

Candiani, G., M. Abbondi, M. Borgonovi, G. Romanò, and F. Parenti. 1999. In-vitro and in-vivo antibacterial activity of BI 397, a new semi-synthetic glycopeptide antibiotic. J. Antimicrob. Chemother. 44:179–192.

Chater, K. F., and M. J. Bibb. 1997. Regulation of bacterial antibiotic production. *In:* H. Kleinkauf and H. von Döhren (Eds.) Biotechnology. VCH. Weinheim, Germany. 7:57–105.

Chen, T., B. Arison, V. Gullo, and E. Inamine. 1989. Further studies on the biosynthesis of the avermectins. J. Indust. Microbiol. 4:231–238.

Clark, A. M. 1996. Natural products as a resource for new drugs. Pharmaceut. Res. 13:1133–1141.

Cragg, G. M., D. J. Newman, and K. M. Snader. 1997. Natural products in drug discovery and development. J. Nat. Prod. 60:52–60.

Dekker, K. A., T. Inagaki, E. Nomura, T. Gootz,. L. H. Huang, W. E. Kohlbrenner, Y. Matsunaga, P. R. McGuir, T. Sakakibara, S. Sakemi, Y. Suzuki, Y. Yamauchi, Y. Kojima, and N. Kojima. 1997. Novel anti-Helicobacter pylori antibacterials. *In:* C. R. Hutchinson, J. McAlpine (Eds.) Biotechnology of Microbial Products Conference (BMP99) Soc. Indust. Microbiol. Fairfax, VA. 88.

Demain, A. L. 1983. New applications of microbial products. Science 219:709–714.

Demain, A. L. 1989. Function of secondary metabolites. *In:* C. L. Hershberger, S. W. Queener, and G. Hegeman (Eds.) Genetics and Molecular Biology of Industrial Microorganisms. ASM Press. Washington DC, 1–11.

Diez, B., E. Mellado, M. Rodriguez, R. Fouces, and J. L. Barredo. 1997. Recombinant microorganisms for industrial production of antibiotics. Biotech. Bioeng. 55:216–226.

DiMasi, J., M. Seibring, and L. Lasagna. 1994. New drug development in the United States from 1963 to 1992. Clin. Pharmacol. Ther. 55:609–622.

Donadio, S., J. B. McAlpine, P. A. Sheldon, M. A. Jackson, and L. Katz. 1993. An erythromycin analog produced by reprogramming of polyketide synthesis. Proc. Natl. Acad. Sci. USA 90:7119–7123.

Dumont, F. J., and Q. Su. 1996. Mechanism of action of the immunosuppressant rapamycin. Life Sci. 58:373–395.

Dutton, C., S. Gibson, A. Goudie, K. Holdom, M. Pacey, and J. Ruddock. 1991. Novel avermectins produced by mutational biosynthesis. J. Antibiot. 44:357–365.

Ebisu, S., H. Takagi, K. Kadowaki, H. Yamagata, and S. Udaka. 1992. Production of human epidermal growth factor by Bacillus brevis increased with use of a stable plasmid from B. brevis 481. Biosci. Biotech. Biochem. 56:812–813.

Epp, J. K., M. L. B. Huber, J. R. Turner, T. Goodson, and B. E. Schoner. 1989. Production of a hybrid macrolide antibiotic in Streptomyces ambofaciens and Streptomyces lividans by introduction of a cloned carbomycin biosynthetic gene from Streptomyces thermotolerans. Gene 85:293–301.

Fieshko, J. C., K. Egan, T. Ritch, R. A. Koski, M. Jones, and G. A. Bitter. 1987. Controlled expression and purification of human immune interferon from high-cell-density fermentations of Saccharomyces cerevisiae. Biotech. Bioeng. 29:1113–1121.

Fieshko, J. C. 1989. Fermentation technology using recombinant microorganisms. *In:* G. K. Jacobson and S. O. Jolly (Eds.) Biotechnology. VCH. Weinheim, Germany. 7b:117–140.

Fleming, I. D., J. Nisbet, and S. J. Brewer. 1982. Target directed antimicrobial screens. *In:* J. D. Bu'lock, L. J. Nisbet, and D. J. Winstanley (Eds.), Bioactive Microbial Products: Search and Discovery. Academic Press. London, 107–130.

Gellissen, G., Janowicz, Z. A., Merckelbach, A., Piontek, M. Keup, P., Weyemann, U., Hollenberg, C. P., and Strasser, A. W. M. 1991. Heterologous gene expression in Hansenula polymorpha: Efficient secretion of glucoamylase. Bio/Technology 9:291–295.

Goldman, D. A., R. A. Weinstein, R. P. Wenzel, O. C. Tablan, R. J. Duma, R. P. Gaynes, J. Schlosser, and W. J. Martone. 1996. Strategies to prevent and control the emergence and spread of antimicrobial-resistant microorganisms in hospitals. JAMA 275:234–240.

Goudie, A. C., N. A. Evans, K. A. F. Gration, B. F. Bishop, S. P. Gibson, K. S. Holdom, B. Kaye, S. R. Wicks, D. Lewis, A. J. Weatherley, C. I. Bruce, A. Herbert, and D. J. Seymour. 1993. Doramectin—a potent novel endectocide. Vet. Parasitol. 49:5–15.

Hafner, E. W., B. W. Holley, K. S. Holdom, S. E. Lee, R. G. Wax, D. Beck, H. McArthur, and W. C. Wernau. 1991. Branched-chain fatty acid requirement for avermectin production by a mutant of Streptomyces avermitilis lacking branched-chain 2-oxo acid dehydrogenase activity. J. Antibiot. 44:349–356.

Haney Jr., M. E., and M. M. Hoehn. 1968. Monensin, a new biologically active compound. 1: Discovery and isolation. Antimicrob. Agents Chemother. 1967:349–352.

He, X.-S., R. Brückner, and R. H. Doi. 1991. The protease genes of Bacillus subtilis. Res. Microbiol. 142:797–803.

Hendlin, D., E. O. Stapley, M. Jackson, H. Wallick, A. K. Miller, F. J. Wolf, T. Miller, L. Chaiet, F. M. Kahan, E. L. Foltz, H. B. Woodruff, J. M. Mata, S. Hernandez, and S. Mochales. 1969. Phosphonomycin: A new antibiotic produced by strains of Streptomyces. Science 166:122–123.

Holland, H. D. 1998. Evidence for life on earth more than 3850 million years ago. Science 275:38–39.

Hopwood, D. A., F. Malpartida, H. M. Kieser, H. Ikeda, J. Duncan, I. Fujui, B. A. M. Rudd, H. G. Floss, and S. Omura. 1985. Production of "hybrid" antibiotics by genetic engineering. Nature 314:642–644.

Hopwood, D. A. 1993. Genetic engineering of Streptomyces to create hybrid antibiotics. Curr. Opin. Biotechnol. 4:531–537.

Horinouchi, S. 1993. "Eukariotic" signal transduction systems, in the bacterial genus Streptomyces. Actinomycetologica 7:68–87.

Hosoya, Y., S. Okamoto, H. Muramatsu, and K. Ochi. 1998. Acquisition of certain streptomycin-resistant (str) mutations enhances antibiotic production in bacteria. Antimicrob. Agents Chemother. 42:2041–2047.

Hu, G. F. 1998. Neomycin inhibits angiogenin-induced angiogenesis. Proc. Natl. Acad. Sci. USA 95:9791–9795.

Huber, B. E. 1989. Therapeutic opportunities involving cellular oncogenes: Novel approaches fostered by biotechnology. FASEB J. 3:5–13.

Hutchinson, C. R. 1997. Antibiotics from genetically engineered microorganisms. *In:* W. Strohl (Ed.) Biotechnology of Antibiotics, 2nd ed.Marcel Dekker. New York, NY. 683–702.

Hutchinson, C. R. 1998. Combinatorial biosynthesis for new drug discovery. Curr. Opin. Microbiol. 1:319–329.

Ikeda, H., and S. Omura. 1997. Avermectin biosynthesis. Chem. Rev. 97:2591–2609.

Jack, R., F. Gotz, and G. Jung. 1997. Lantibiotics. *In:* H. Kleinkauf and H von Döhren (Eds.) Biotechnology. VCH. Weinheim, Germany. 7:323–370.

Kahan, J. S., Kahan, F. M., Geogelman, R., Currie, S. A., Jackson, M., Stapley, E. O., Miller, T. W., Hendlin, D., Mochales, S. Hernandez, S., Woodruff, H. B., and Birnbaum, J. 1979. Thienamycin, a new β-lactam antibiotic: Discovery, taxonomy, isolation, and physical properties. J. Antibiot. 32:1–12.

Katz, L., and S. Donadio. 1993. Polyketide synthesis: Prospects for hybrid antibitics. Ann. Rev. Microbiol. 47:875–912.

Khosla, C., R. McDaniel, S. Ebert-Khosla, R. Torres, D. H. Sherman, M. J. Bibb, and D. A. Hopwood. 1993. Genetic construction and functional analysis of hybrid polyketide synthases containing heterologous acyl carrier proteins. J. Bacteriol. 175:2197–2204.

Kino, T., H. Hatanaka, Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, and H. Imanaka. 1987. FK-506, a novel immunosuppressant isolated from Streptomyces. 1: Fermentation, isolation and physicochemical and biological characteristics. J. Antibiot. 40:1249–1255.

Kleinkauf, H., and H. von Döhren. 1997. Peptide antibiotics. *In:* H. Kleinkauf and H. von Döhren (Eds.) Biotechnology. VCH. Weinheim, Germany. 7:277–324.

Lancini, G. C., and R. Lorenzetti. 1993. Biotechnology of Antibiotics and Other Bioactive Microbial Metabolites. Plenum Press. New York, NY. 95–132.

Lancini, G. C., F. Parenti, and G. G. Gallo. 1995. Antibiotics: A Multidisciplinary Approach. Plenum Press. New York, NY.

Lancini, G. C., and A. L. Demain. 1999. Secondary metabolism in bacteria: Antibiotic pathways, regulation, and function. *In:* J. W. Lengeler, G. Drews, and H. G. Schlegel (Eds.) Biology of the Prokaryotes. Thieme Verlag. Stuttgart, Germany. 627–651.

LaVallie, E. R., E. A. DiBlasio, S. Kovacic, K. L. Grant, P. F. Schendel, and J. M. McCoy. 1993. A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. Bio/Technology 11:187–193.

Lazzarini, A., L. Cavaletti, G. Toppo, and F. Marinelli. 2001. Potentialities of rare actinomycetes as producers of new antibiotics. Ant. v. Leeuwenhoek in press.

Lee, S. Y. 1996. High cell-density culture of Escherichia coli. TibTech 14:98–105.

Lewis, K. 1994. Multidrug resistance pumps in bacteria: Variations on a theme. Trends Biochem. Sci. 19:119–123.

Liu, J. 1993. FK506 and ciclosporin: Molecular probes for studying intracellular signal transduction. Trends Pharm. Sci. 14:182–188.

Lunn, C. A., S. Kathju, B. J. Wallace, S. R. Kushner, and V. Pigiet. 1984. Amplification and purification of plasmid-encoded thioredoxin from Escherichia coli K12. J. Biol. Chem. 259:10469–10474.

MacNeil, D. J., J. L. Occi, K. M. Gewain, and T. MacNeil. 1994. Correlation of the avermectin polyketide synthase genes to the avermectin structure: Implications for designing novel avermectins. Ann. NY Acad. Sci. 721:123–132.

Madduri, K., J. Kennedy, G. Rivola, A. Inventi-Solari, S. Filippini, G. Zanuso, A. L. Colombo, K. M. Gewain, J. L. Occi, D. J. MacNeil, and C. R. Hutchinson. 1998. Production of the antitumor drug epirubicin (4′ epidoxorubicin) and its precursor by a genetically engineered strain of Streptomyces peucetius. Nature Biotechnol. 16:69–74.

McAlpine, J. 1998. Unnatural natural products by genetic manipulation. *In:* D. M. Sapienza and L. M. Savage (Eds.) Natural Products II: New Technologies to Increase Efficiency and Speed. International Business Community. Southborough, MA. 251–278.

McArthur, H. I. A. 1998. The novel avermectin, Doramectin—a successful application of mutasynthesis. *In:* C. R. Hutchinson and J. McAlpine (Eds.), Proceedings, Biotechnology of Microbial Products Conference. (BMP 97) Soc. Indust. Microbiol. Fairfax, VA. 43–48.

McDaniel, R., S. Ebert-Khosla, D. A. Hopwood, and C. Khosla. 1993a. Engineered biosynthesis of novel polyketides. Science 262:1546–1550.

McDaniel, R., S. Ebert-Khosla, D. A. Hopwood, and C. Khosla. 1993b. Engineered biosynthesis of novel polyketides: Manipulation and analysis of an aromatic polyketide synthase with unproven catalytic specificities. J. Am. Chem. Soc. 115:11671–11675.

Mishima, H., J. Ide, S. Muramatsu, and M. Ono. 1983. Milbemycins, a new family of macrolide antibiotics: Structure determination of milbemycins D, E, F, G, H, J and K. J. Antibiot. 36:980–990.

Mobarakai, N., J. M. Quile, and D. Landman. 1994. Bactericidal activities of peptide antibiotics against multidrug-resistant Enterococcus faecium. Antimicrob. Agents Chemother. 38:385–387.

Moore, M. 1992. Strategic alliances: Technological value in pharmaceutical drug discovery. Biofut. Eur. 9:138–143.

Morris, A., J. D. Kellner, and D. E. Low. 1998. The superbugs: Evolution, dissemination and fitness. Curr. Opin. Microbiol. 1:524–529.

Nichterlein, T., M. Kretschmar, and H. Hof. 1996. RP 59500, a streptogramin derivative, is effective in murine listerosis. J. Chemother. 8:107–112.

Oki, T., and A. Yoshimoto. 1979. Antitumor antibiotics. *In:* D. Perlman (Ed.) Annual Reports on Fermentation Processes. Academic Press. New York, NY. 3:215–251.

Parekh, R. 1989. Polypeptide glycosylation and biotechnology. Biotech. Eur. 6:18–21.

Piepersberg, W., and J. Distler. 1997. Aminoglycosides and sugar components in other secondary metabolites. *In:* H. Kleinkauf and H. von Döhren (Eds.) Biotechnology. VCH. Weinheim, Germany. 7:397–488.

Rawls, R. L. 1998. Polyketides: Research increases on modular synthesis of these biomolecules by enzymes. Chem. Eng. News 76:29–30.

Rinderknecht, E., B. H. O'Connor, and H. Rodriguez. 1984. Natural human interferon-β: Complete amino acid sequencing and determination of site of glycosylation. J. Biol. Chem. 259:6790–6797.

Schein, C. H. 1989. Production of soluble recombinant proteins in bacteria. Bio/Technology 7:1141–1148.

Shay, L. K., H. R. Hunt, and G. H. Wegner. 1987. High-productivity fermentation process for cultivating industrial microorganisms. J. Ind. Microbiol. 2:79–85.

Sosio, M., F. Giusino, C. Cappellano, E. Bossi, A. M. Puglia, and S. Donadio. 2000a. Artificial chromosomes for antibiotic-producing actinomycetes. Nature Botechnol. 18:343–345.

Sosio, M., E. Bossi, A. Bianchi, and S. Donadio. 2000b. Multiple gene synthetase gene clusters in actinomycetes. Mol. Gen. Genet. 264:213–221.

Stapley, E. O. 1982. Avermectins, antiparasitic lactones produced by Streptomyces avermitilis isolated from a soil in Japan. *In:* H. Umezawa, A. L. Demain, R. Hata, and C. R. Hutchinson (Eds.), Trends in Antibiotic Research-Japan Antibiotic Research Association. Tokyo, 154–170.

Staunton, J. 1998. Combinational biosynthesis of erythromycin and complex polyketides. Curr. Opin. Chem. Biol. 2:339–345.

Stead, P. 1997. Natural products drug discovery—new technologies and approaches. Drug Discov. Today 2:256–259.

Stephens, C., and L. Shapiro. 1997. Bacterial protein secretion—a target for new antibiotics? Chem. Biol. 4:637–641.

Stinson, S. C. 1996. Drug firms restock antibacterial arsenal. Chem. Eng. News 74:75–100.

Strohl, W. R., P. L. Bartel, N. C. Connors, C. B. Zhu, D. C. Dosch, J. M. Beale, H. G. Floss, K. Stutzman-Engwall, S. L. Otten, and C. R. Hutchinson. 1989. Biosynthesis of natural and hybrid polyketides by anthracycline-producing streptomycetes. *In:* C. L. Hershberger, S. W. Queener, and G. Hegeman (Eds.) Genetics and Molecular Biology of Industrial Microorganisms. American Society for Microbiology. Washington DC, 68–84.

Strohl, W. R., P. L. Bartel, Y. Li, N. C. Connor, and R. H. Woodman. 1991. Expression of polyketide biosynthesis and regulatory genes in heterologous streptomycetes. J. Ind. Microbiol. 7:163–174.

Strohl, W. R. 1997. Biotechnology of Antibiotics, 2nd ed. Marcel Dekker. New York, NY.

Sum, P. E., F. W. Sum, and S. J. Projan. 1998. Recent developments in tetracycline antibiotics. Curr. Pharm. Design 4:119–132.

Swartz, J. R. 1996. Escherichia coli recombinant DNA technology. *In:* F. C. Neidhardt (Ed.), Escherichia coli and Salmonella: Cellular and Molecular Biology, 2nd ed. American Society of Microbiology Press. Washington DC, 1693–1711.

Tanaka, H., and S. Omura. 1997. Screening of novel receptor-active compounds of microbial origin. *In:* H. J. Rehm, G. Reed, H. Kleinkauf, and H. von Döhren (Eds.) Biotechnology, 2nd ed. VCH. Weinheim, Germany. 7:107–132.

Tenover, F. C., and J. M. Hughes. 1996. The challenges of emerging infectious diseases. JAMA 275:300–304.

Tomasz, M. 1995. Mitomycin C: Small, fast and deadly (but very selective). Curr. Biol. 2:575–579.

Torsvik, V., R. Sorheim, and J. Goksoyr. 1996. Total bacterial diversity in soil and sediment communities—a review. J. Indust. Microbiol. 17:170–178.

Truscheit, E., W. Frommer, B. Junge, L. Müller, D. D. Schmidt, and W. Wingender. 1981. Chemistry and biochemistry of microbial α-glucosidase inhibitors. Angewandte Chemie, International Ed. in English 20:744–761.

Udaka. S., and H. Yamagata. 1994. Extremely efficient protein secretion system in Bacillus brevis. *In:* Y. Murooka and T. Imanaka (Eds.), Recombinant Microbes for Industrial and Agricultural ApplicationsMarcel Dekker. New York, NY. 71–79.

Umezawa, H. 1972. Enzyme Inhibitors of Microbial Origin. University Park Press. Baltimore, MD.

Umezawa, H. 1982. Low-molecular-weight inhibitors of microbial origin. Ann. Rev. Microbiol. 36:75–99.

Vaara, M. 1993. Antibiotic-supersusceptible mutants of Escherichia coli and Salmonella typhimurium. Antimicrob. Agents Chemother. 37:2255–2260.

Van Leen, R. W., J. G. Bakhuis, R. F. van Beckhoven, H. Burger, L. C. Dorssers, R. W. Hommes, P. J. Lemson, B. Noordam, N. L. Persoon, and G. Wagemaker. 1991. Production of human interleukin-3 using industrial microorganisms. Bio/Technology 9:47–52.

Verdine, G. L. 1996. The combinatorial chemistry of nature. Nature 384 (Suppl.):11–13.

Vezina, C., A. Kudelski, and S. N. Sehgal. 1975. Rapamycin (AY 22,989), a new antifungal antibiotic. 1: Taxonomy of the producing streptomycete and isolation of the active principle. J. Antibiot. 28:721–726.

Waksman, S. A., and H. B. Woodruff. 1940. The soil as a source of microorganisms antagonistic to disease-producing bacteria. J. Bacteriol. 40:581–599.

Westley, J. W. 1977. Polyether antibiotics: Versatile carboxylic acid ionophores produced by Streptomyces. Adv. Appl. Microbiol. 22:177–223.

Winkelmann, G. 1986. Iron complex products (siderophores). *In:* H. J. Rehm and G. Reed (Eds.) Biotechnology. VCH. Weinheim, Germany. 4:215–243.

Woodruff, H. B., and L. E. McDaniel. 1958. Antibiotic approach in strategy of chemotherapy. Soc. Gen. Microbiol. Symp. 8:29–48.

Woodruff, H. B., S. Hernandez, and E. D. Stapley. 1979. Evolution of antibiotic screening programme. Hindustan Antibiot. Bull. 21:71–84.

Wu, X.-C., W. Lee, L. Tran, and S.-L. Wong. 1991. Engineering a Bacillus subtilis expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. 173:4952–4958.

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CHAPTER 3.7

Biosurfactants

EUGENE ROSENBERG

Introduction

Microorganisms are specialists. In any particular ecological niche, one microorganism or a limited number of strains dominates. These microorganisms have evolved the ability to survive in this niche for long periods when growth is impossible, and then when nutrients become available, they can outgrow their competitors. The fast growth of microorganisms depends largely on their high surface-to-volume ratio, which allows for the efficient uptake of nutrients and release of waste products. The price that the microorganism pays for the high surface-to-volume ratio is that it is totally exposed. All the components outside of the cell must function under the specific conditions of the ecological niche. Probably for this reason, the diversity of the microbial world is best expressed on the outside of the cell.

In any heterogenous system, boundaries are of fundamental importance to the behavior of the system as a whole. Therefore, it is not surprising that microorganisms, having a large surface-to-volume ratio, produce a variety of surface active agents (surfactants) that adsorb to and alter the conditions prevailing at interfaces. Surfactants concentrate at interfaces because they are amphipathic, i.e., they contain both hydrophobic and polar groups. Biosurfactants can be divided into low molecular weight molecules that efficiently reduce surface and interfacial tensions and high molecular weight polymers that bind tightly to surfaces. Table 1 and Table 2 list some microbially produced surfactants that have been studied.

During the last few years, numerous reviews have examined various aspects of biosurfactants such as their role in bacterial adhesion (Neu, 1996) and in growth of bacteria on hydrocarbons (Rosenberg and Ron, 1996), surface active polymers from the genus *Acinetobacter* (Rosenberg and Ron, 1998), biochemistry of surfactin (Peypoux et al., 1999), microbial surfactants (Rosenberg and Ron, 1999), biosurfactant assay (Lin, 1996), surfactants from *Rhodococci* (Land and Philp, 1998), production (Wang and Wand, 1990), molecular genetics (Sullivan, 1998), and commercial applications (Fiechter, 1992), including enhanced oil recovery (Banat, 1995) and bioemulsans (Rosenberg and Ron, 1997). In this review we will present some of the chemical and surface properties of biosurfactants and the genetics and regulation of their production, and then we will discuss what functions they may play for the producing strains and what their potential is for commercial application.

When considering the natural roles and potential applications of biosurfactants, it is important to emphasize that a wide variety of diverse microorganisms (only some of which are shown in Table 1 and Table 2) make these molecules and that biosurfactants have very different chemical structures and surface properties. It is therefore reasonable to assume that different groups of biosurfactants have different natural roles in the growth of the producing microorganisms. Similarly, since their chemical structures and surface properties are so different, it is likely that each group of biosurfactants will have a specific use. This diversity makes it difficult to generalize about the natural role of biosurfactants and provides both advantages and disadvantages for potential commercial exploitation.

Chemical and Surface Properties of Biosurfactants

Glycolipids

The low molecular weight biosurfactants are generally glycolipids or lipopeptides (Table 1). The best studied glycolipid bioemulsifiers, rhamnolipids, trehalolipids and sophorolipids, are disaccharides that are acylated with long-chain fatty acids or hydroxy fatty acids (Figure 1, Figure 2, and Figure 3). Interest in trehalose lipids as general surfactants can be traced back to the discovery that the emulsion layer of *Arthrobacter paraffineus* culture broths contained trehalose dimycolates when the cells were grown on hydrocarbon substrates (Suzuki et al., 1969). Wagner and co-workers (1983) have studied trehalose dimycolates produced by

Table 1. Low molecular mass microbially produced surfactants.

Surfactant	Producing microorganisms	References
Rhamnolipids	*P. aeruginosa*	Rendell et al., 1990
		Sim et al., 1997
		Lang and Wullbrandt, 1999
		Arino et al., 1996
	Pseudomonas spp.	Parra et al., 1989
Trehalose lipids	*R. erythropolis*	Ristau and Wagner, 1983
		Kim et al., 1990
		Lang and Philip, 1998
	Arthrobacter sp.	Li et al., 1984
	Mycobacterium sp.	Cooper et al., 1989
Sophorolipids	*T. bombicola* (yeast)	Inoue and Itoh, 1982
		Davila et al., 1997
Glucose lipids	*A. borkumensis*	Yakimov et al., 1998
		Abraham et al., 1998
	C. borgoriensis (yeast)	Cutler and Light, 1979
Viscosin	*P. fluorescens*	Neu and Poralla, 1990
Surfactin	*B. subtilis*	Arima et al., 1968
		Wei and Chu, 1998
		Grau et al., 1999
Gramicidin S	*B. brevis*	Katz and Demain, 1977
Polymyxins	*B. polymyxa*	Suzuki et al., 1965
Serrawettin	*S. marcescens*	Matsuyama et al., 1991
		Pruthi and Cameotra, 1997
Lipopeptide	*B. Licheniformis*	Horowitz and Griffen, 1991
		Lin et al., 1994
		Yakimov et al., 1995
Lipopeptide	*S. liquefaciens*	Lindum et al., 1998
Streptofactin	*S. tendae*	Richter et al., 1998
Corynomycolic acids	*N. erythropolis*	MacDonald et al., 1981
	C. lepus	Cooper et al., 1981
Phospholipids	*Acinetobacter* spp.	Kaeppeli and Finnerty, 1980
	T. thiooxidans	Beebe and Umbreit, 1971
Fatty acids	Widespread	MacDonald et al., 1981

Table 2. High molecular mass microbially produced surfactants.

Surfactant	Producing microorganisms	References
RAG-1 emulsan	*A. calcoaceticus* RAG-1	Rosenberg et al., 1979a
BD4 emulsan	*A. calcoaceticus* BD413	Kaplan and Rosenberg, 1982
Alasan	*A. radioresistens* KA53	Navon-Venezia et al., 1995
Biodispersan	*A. calcoaceticus* A2	Rosenberg, 1993
Mannan-lipid-protein	*C. tropicalis*	Kaeppeli et al., 1984
Liposan	*C. lipolytica*	Cirigliano and Carman, 1984
Emulsan 378	*P. fluorescens*	Persson et al., 1988
Protein complex	*M. thermoautotrophium*	De Acevedo and McInerney, 1996
Insecticide emulsifier	*P. tralucida*	Appaiah and Karanth, 1991
Thermophilic emulsifier	*B. stearothermophilus*	Gunjar et al., 1995
Acetylheteropolysaccharide	*S. paucimobilis*	Ashtaputre and Shah, 1995
Food emulsifier	*C. utilis*	Shepherd et al., 1995
Sulfated polysaccharide	*H. eurihalinia*	Calvo et al., 1998
PM-factor	*P. marginalis*	Burd and Ward, 1996
Emulcyan	*Phormidium J-1*	Fattom and Shilo, 1985

Rhodococcus erythropolis extensively with special reference to their interfacial activities and possible application in enhanced oil recovery (Kim et al., 1990). When *R. erythropolis* was grown in batch fermentor cultures on 2% (w/w) C_{12}-C_{18} n-alkanes, the maximum amounts of trehalose lipids and dry cell mass were 2.1 and 19 g/liter, respectively (Rapp et al., 1979). Yields of trehalose lipids were increased to 4 g/liter when the bacteria were grown on 10% (w/v) n-alkanes and the trehalose lipids were continuously extracted. Recently, the yield of rhamnolipids

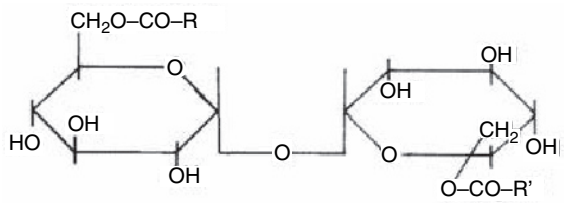

Fig. 1. β, β-Trehalose-6,6′-dicarboxylic ester. Trehalose lipids differ in the structure of the R and R′ groups.

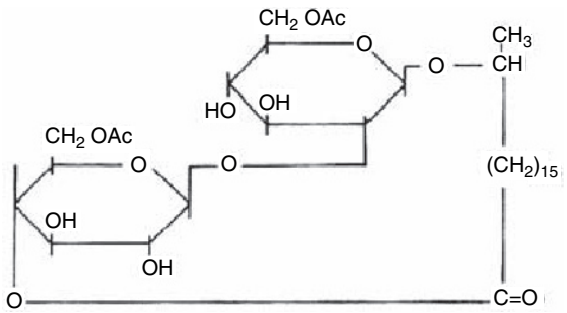

Fig. 2. Structure of the lactonic sophorose lipid of *Torulopsis bombicola*.

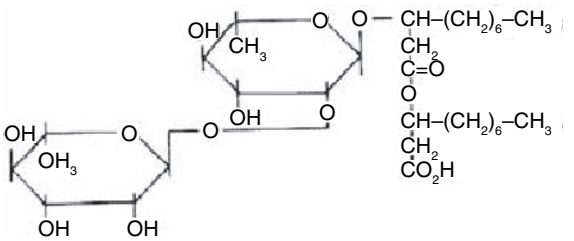

Fig. 3. Structure of the rhamnolipid of *Pseudomonas aeruginosa*.

was increased to 24.3 g/liter in media containing 6% canola oil (Sim et al., 1997). Surfactant production is growth associated (Espuny et al., 1996). Essentially all of the trehalose lipids of *R. erythropolis* are cell bound and extractable with n-hexane. The surfactant properties of the fractionated cell-bound lipids of *R. erythropolis* were measured by Kretschmer et al., 1982. The minimal interfacial tensions (between aqueous salt solutions and n-hexadecane) achieved with corynomycolic acids, trehalose monocorynomycolates, and trehalose dicorynomycolates were 6, 16, and 17 mN/m, respectively. However, the critical micelle concentration (CMC) for the trehalose lipids (ca. 2 mg/liter) was more than 100 times lower than that for the free corynomycolic acids. Trehalose mycolates reduced the surface tension of water from 72 to 26 mN/m (Lang and Philip, 1998).

Different species of the yeast *Torulopsis* produce extracellular sophorolipids, which consist of two glucose units linked β-1,2. The 6 and 6′ hydroxyl groups are generally acetylated. The lipid portion is connected to the reducing end through a glycosidic linkage. The terminal carboxyl group of the fatty acid can be in the lactonic form (as shown in Figure 2) or hydrolyzed to generate an anionic surfactant. High yields of sophorose lipids can be obtained from the extracellular fluid of cultures of *T. bombicola* grown on glucose and oil. For example, Itoh and Inoue, 1982 reported yields of 32 g/liter of a crude sophorolipid mixture when the bacteria were grown in shaker flasks on a glucose-safflower oil medium. Under controlled conditions in a 7-liter fermentor, the maximum yield of sophorose lipids from *T. bombicola* ATCC 22214 was about 70 g/liter when the medium contained both glucose and a vegetable oil as substrates (Cooper and Paddock, 1983). More recently, yields have improved to over 150 g/liter (Davila et al., 1997; Rau et al., 1996; Zhou and Kosaric, 1995), making potential commercial exploitation more feasible. The sophorose lipids lower surface and interfacial tensions, although they are not effective emulsifying agents (Cooper and Paddock, 1983). The pure lactonic sophorose lipid (10 mg/liter) lowered the interfacial tension between n-hexadecane and water from 40 to about 5 mN/m, relatively independently of pH (6 to 9), salt concentration and temperature (20 to 90°C). The minimum interfacial tensions brought about by a mixture of acidic sophorose lipids against hexadecane or several vegetable oils were 1 to 2 mN/m.

Certain species of *Pseudomonads* are known to produce large quantities of a glycolipid (Hauser and Karnovsky, 1954) consisting of 2 mol of rhamnose and 2 mol of β-hydroxydecanoic acid. The hydroxyl group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, whereas the hydroxyl group of the second acid is involved in ester formation. Since one of the carboxylic groups is free, the rhamnolipids are anions above pH 4. Rhamnolipid was reported (Hisatsuka et al., 1971) to lower surface tension, emulsify hydrocarbons and stimulate growth on n-hexadecane of *P. aeruginosa* strains but not other hydrocarbon-degrading bacteria. Formation of rhamnolipids by *P. aeruginosa* has been studied (Wagner et al., 1983; Guerra-Santos et al., 1986; Lang and Wullbrandt, 1999). More than 100 g/liter rhamnolipids were produced from 160 g/liter soybean oil (Lang and Wullbrandt, 1999). The pure rhamnolipid lowered the interfacial tension against n-hexadecane to about 1 mN/m and had a CMC of 10 to 30 mg/liter,

depending on the pH and salt conditions (Lang and Wagner, 1987; Parra et al., 1989).

Zhang and Miller, 1995 reported that the interfacial tension between hexadecane and water was decreased to less than 0.1 mN/m by a methyl ester of rhamnolipid, whereas the free acid decreased the interfacial tension only to 5 mN/m. Mannosylerythritol lipids (extracellular microbial surfactants) have several interesting biological properties. They inhibit growth of human promyelocytic leukemia cell lines and induce monocytic differentiation (Isoda et al., 1997). Furthermore, the surfactant inhibited serine/threonine phosphorylations in intact leukemia cells. The authors suggest that the differentiation-inducing ability of mannosylerythritol lipids is not due to a simple detergent-like effect, but rather is the result of a specific action on the plasma membrane.

Recently, a new class of glycolipid, glucose lipids, has been described (Yakimov et al., 1998; Abraham et al., 1998). These glucose lipids are produced by a new bacterial genus and species, *Alcanivorax borkumensis* (Golyshin et al., 1998). During cultivation on n-alkanes as sole source of carbon and energy, this novel marine bacterium produced extracellular and cell-bound surface-active glucose lipids which reduced the surface tension of water from 72 to 29 mN/m. Ten different glucose lipids were separated and their structures elucidated. They all consist of an anionic glucose lipid with a tetrameric oxyacyl side chain. The glucose lipids extracted from the cell envelope were N-terminally esterified with glycine. The glucose lipids differ in the chain length of one or two of the four β-hydroxy fatty acids.

Lipopeptides

Several lipopeptide antibiotics show potent surface active properties. *Bacillus subtilis* produces a cyclic lipopeptide called surfactin or subtilysin (Peypoux et al., 1999; Bernheimer and Avigad, 1970; Arima et al., 1968), which is reputedly the most active biosurfactant known to date (Cooper and Zajic, 1980). The structure of surfactin is shown in Figure 4. Surfactin has a CMC in water of 25 mg/liter and lowers the surface tension to 27 mN/m. The minimum interfacial tension against hexadecane was 1 mN/m. The yield of surfactin produced by *B. subtilis* can be improved to around 0.8 g/liter by continuously removing the surfactant by foam fractionation and addition of either iron or manganese salts to the growth medium (Cooper et al., 1981). Recently, Wei and Chu, 1998 obtained a yield of 3.5 g/liter by carefully controlling iron concentration and pH. A recombinant *B. subtilis*, carrying a gene

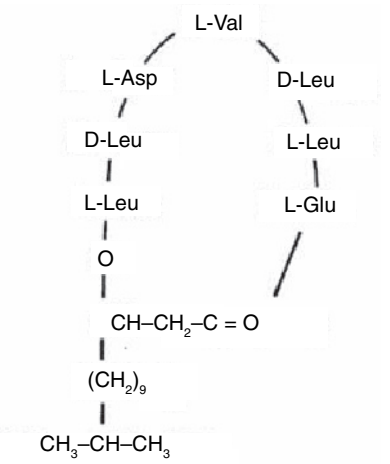

Fig. 4. Surfactin.

for surfactin production, produced new surfactin variants (Nakayama et al., 1997). The amphipathic nature of surfactin may contribute to some of its interesting biological properties, such as the formation of ion-conducting pores in membranes (Grau et al., 1999).

Streptomycestendae produces an extracellular hydrophobic peptide referred to as streptofactin (Richter et al., 1998). Streptofactin is a mixture of structurally related peptides ranging in molecular mass from 1,003 to 1,127 Da. Streptofactin reduced the surface tension of water from 72 mN/m to 39.4 mN/m and had a critical micelle concentration of 36 mg/liter. Interestingly, streptofactin restored the ability of mutants defective in aerial mycelium formation to develop normally. It was suggested that streptofactin plays a role in *Streptomyces* development by allowing for the erection of aerial hyphae by lowering the surface tension of water films enclosing the colonies.

The synthesis of one or more peptide antibiotic during the early stages of sporulation is common to most, if not all, members of the genus *Bacillus* (Katz and Demain, 1977). *B. brevis* produces the cyclosymmetric decapeptide antibiotic gramicidin S. In solution, gramicidin S exists in the form of a rigid ring with the two positively charged ornithine side chains constrained to one side of the ring, an average of 8 nm apart, and the side chains of the remaining hydrophobic residues oriented toward the opposite side of the ring (Krauss and Chan, 1983). As a result, gramicidin S will bind strongly to negative surfaces and polyanions, causing them to become lipophilic. For example, two moles of gramicidin S form a stable coordination complex with one molecule of ATP, which partitions into organic solvents.

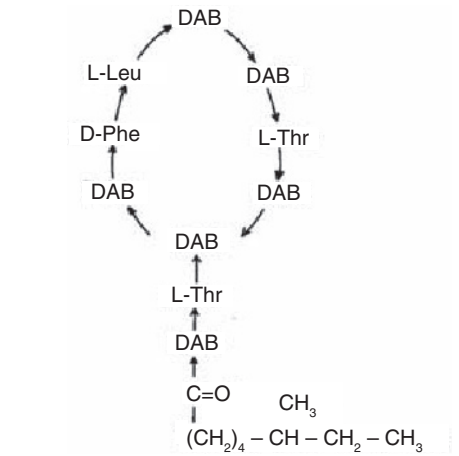

Fig. 5. Polymyxin B. DAB is 2,4-diaminobutyric acid.

The polymyxins are a group of closely related lipopeptide antibiotics produced by *B. polymyxa* and related bacilli. As seen in Figure 5, polymyxin B is a decapeptide in which amino acids 3 through 10 form a cyclic octapeptide. A branched-chain fatty acid is connected to the terminal 2,4-diaminobutyric acid (DAB). The structures of polymyxins differ in substituents at residues 3 (DAB or D-Ser), 6 (D-Leu or L-Ileu), or 7 (D- or L-DAB) (Suzuki et al., 1965). The cationic 7γ-amino groups of the DAB residues, together with the hydrophobic side chain of the fatty acid, give these antibiotics the surface active properties of a cationic detergent. *Pseudomonas* strains produce viscosin, a peptidolipid biosurfactant which lowers surface tension of water to 27 mN/m (Neu et al., 1990).

Fatty Acids and Phospholipids

Fatty acids derived from alkanes have received considerable attention as surfactants. Rehm and Reiff, 1981 have published a detailed list of fatty acids resulting from the microbial oxidation of alkanes. The hydrophilic-lipophilic balance (HLB) of fatty acids is clearly related to the length of the hydrocarbon chain. For lowering surface and interfacial tensions, the most active saturated fatty acids are in the range of C-12 to C-14. In addition to straight-chain fatty acids, microorganisms produce complex fatty acids containing hydroxyl groups and alkyl branches. Some of these complex fatty acids, e.g., the corynomycolic acids, are potent surfactants (MacDonald et al., 1981). Similar to 2-hydroxy fatty acid surface properties, the surface properties of corynomycolic acids are relatively insensitive to pH and ionic strength.

Phospholipids are major components of microbial membranes. When certain hydrocarbon-degrading bacteria or yeast are grown on alkane substrates, the level of phospholipid increases greatly. In the case of hexadecane-grown *Acinetobacter* sp. HO1-N, phospholipid-rich (mainly phosphatidyl ethanolamine) extracellular membrane vesicles accumulate in the medium (Kaeppeli and Finnerty, 1979). The potent surfactant properties of these vesicles are evident from the observation that they are able to generate optically clear microemulsions of alkanes in water (Kaeppeli and Finnerty, 1980). Surfactant properties of cellular phospholipids of *Rhodococcus erythropolis* grown on n-alkanes were examined by Kretschmer et al., 1982. The phosphatidyl ethanolamine fraction was the most potent, lowering the interfacial tension between water and hexadecane to less than 1 mN/m and having a CMC of 30 mg/liter. The surface properties of phospholipids are influenced greatly by changes in pH and ionic strength. Phospholipids produced by *Thiobacillus thiooxidans* have been reported to be responsible for wetting elemental sulfur, which is necessary for growth (Beebe and Umbreit, 1971).

High Molecular Weight Biosurfactants

A large number of bacterial species from different genera produce exocellular polymeric surfactants composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers (Table 2). The best studied are the bioemulsans produced by different species of *Acinetobacter* (Rosenberg and Ron, 1998). The RAG-1 emulsan is a complex of an anionic heteropolysaccharide and protein (Rosenberg et al., 1979b; Rosenberg and Kaplan, 1987). Its surface activity is due to the presence of fatty acids, comprising 15% of the emulsan dry weight, that are attached to the polysaccharide backbone via *O*-ester and *N*-acyl linkages (Belsky et al., 1979). Table 3 summarizes the chemical and physical properties of RAG-1 emulsan. *A. calcoaceticus* RAG-1 excretes maximum amounts of emulsan in shaker flasks when grown in a minimal medium containing 2% ethanol as the sole carbon and energy source. Under these conditions, approximately 80% of the emulsan produced is released when the cells are in stationary phase (Goldman et al., 1982). Emulsan is an effective emulsifier at low concentrations (0.01–0.001%), representing emulsan-to-hydrocarbon ratios of 1:100–1:1000, and exhibits considerable substrate specificity

Fig. 6. The polysaccharide structure of the *A. calcoaceticus* BD4 emulsan (Kaplan et al., 1985)

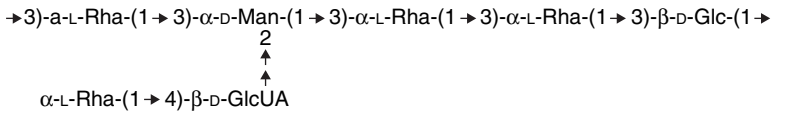

Table 3. Chemical and physical properties of RAG-1 emulsan.[a]

Measurement	Result
Chemical composition	D-galactosamine, 25% L-galactosaminournia acid, 25% Dideoxy-diaminohexose, 25% 3-hydroxydodecanoic acid, 10% 2-hydroxydodecanoic acid, 10% Water and ash, 10%
Intrinsic viscosity	55 cc/g
Diffusion constant	5.3×10^{-8} cm^2/g
Partial molar volume	0.71 cm^3/g
Molecular weight	98×10^5 Da
Dimensions	30 Å × 2000 Å

[a]Data taken from Rosenberg et al., 1979a

(Rosenberg et al., 1979a). The RAG-1 emulsan does not emulsify pure aliphatic, aromatic, or cyclic hydrocarbons; however, all mixtures that contain an appropriate mixture of an aliphatic and an aromatic (or cyclic alkane) are emulsified efficiently. Maximum emulsifying activity is obtained in the presence of divalent cations (2–10 mM Mg^{2+}) at pH values of 5.0–7.5.

Acinetobacter calcoaceticus BD4, initially isolated and characterized by Taylor and Juni, 1961, produces a large polysaccharide capsule. Under certain growth conditions (e.g., 2% ethanol as the carbon and energy source), an enhanced release of the capsules was obtained (Sar and Rosenberg, 1983). However, in contrast to RAG-1, no decrease in capsular polysaccharide was observed; rather, capsular polysaccharide levels remained constant when enhanced levels of extracellular polysaccharide were obtained (Kaplan and Rosenberg, 1982). When the crude capsule was applied to a Sepharose 4B column, it eluted in a single peak containing a polysaccharide-protein complex. The pure polysaccharide component was obtained by deproteinization and its chemical structure elucidated (Fig. 6). The protein component was obtained from the extracellular supernatant fluid of strain BD4-R7, a capsule-negative mutant of BD4 that produces no extracellular polysaccharide. The polysaccharide and protein components had no emulsifying activity by themselves. Mixing the protein and polysaccharide fractions led to a reconstitution of the emulsifying activity (Kaplan et al., 1987). Apparently the protein, which is hydrophobic, binds to the hydrocarbon initially in a reversible fashion. The polysaccharide then attaches to the protein and stabilizes the oil-in-water emulsion.

Alasan, produced by a strain of *Acinetobacter radioresistens*, is a complex of an anionic polysaccharide and protein with a molecular weight of approximately 1×10^6 (Navon-Venezia et al., 1995). However, the polysaccharide component of alasan is unusual in that it contains covalently bound alanine. The protein component of alasan appears to play an important role in both the structure and activity of the complex (Navon-Venezia et al., 1998). Deproteinization of alasan with hot phenol or treatment with specific proteinases caused a loss in most of the emulsifying activity. When a solution of alasan was exposed to increasing temperatures, there were large changes in the viscosity and emulsifying activity of the complex. Between 30 and 50°C, the viscosity increased 2.6 times with no change in activity. Between 50 and 90°C, the viscosity decreased 4.8 times and the activity increased fivefold. None of these changes occurred with the protein-free polysaccharide, indicating that they were due to the interactions of the protein and polysaccharide portions of the complex. Alasan lowers interfacial tension from 69 to 41 mN/m at 20°C and has a CMC of 200 μg/ml (Barkay et al., 1999). Alasan enhanced the aqueous solubility and biodegradation rates of polyaromatic hydrocarbons (Barkay et al., 1999). In the presence of 0.5 mg alasan per ml, the apparent solubilities of phenanthrene, fluoranthene and pyrene were increased 6.6-, 25.7- and 19.8-fold, respectively. Alasan binds polyaromatic hydrocarbons in a reversible manner. The presence of alasan more than doubled the rate of [^{14}C] phenanthrene mineralization by *Sphingomonas paucimobilis*. The solubilization of polyaromatic hydrocarbons and emulsification of hydrocarbons can be accomplished with the purified alasan proteins (Rosenberg et al., 1999).

A. calcoaceticus A2 produces an extracellular anionic polysaccharide surfactant of molecular mass 51.4 kDa that effectively disperses limestone and titanium dioxide (Rosenberg et al., 1988a, b). The biopolymer, referred to as biodispersan, binds to powdered calcium carbonate and changes its surface properties in a way that allows for better dispersion in water. In addition to being a surfactant, biodispersan acts also as a surfractant, aiding in the fracturing of limestone during the grinding process (Rosenberg et al., 1989).

Sar and Rosenberg, 1983 reported that the majority of *Acinetobacter* strains produce extracellular nondialyzable emulsifiers. These strains included both soil and hospital isolates. Marin et al., 1996 have reported the isolation of a strain of *A. calcoaceticus* from contaminated heating oil that emulsifies that substrate. Neufeld and Zajic, 1984 demonstrated that whole cells of *A. calcoaceticus* 2CA2 have the ability to act as emulsifiers, in addition to producing an extracellular emulsifier.

Other High Molecular Mass Biosurfactants

A large number of high molecular weight complex biosurfactants has been reported. In general, little is known about these emulsifiers other than the producing organism and the overall chemical composition of the crude mixture. *Halomonas eurihalina* produces an extracellular sulfated heteropolysaccharide (Calvo et al., 1998). *Pseudomonas tralucida* produced an extracellular acetylated polysaccharide that was effective in emulsifying several insecticides (Appaiah and Karanth, 1991). Several recently reported biosurfactants are effective at high temperature, including the protein complex from *Methanobacterium thermoautotrophium* (De Acevedo et al., 1996) and the protein-polysaccharide-lipid complex of *Bacillus stearothermophilus* ATCC 12980 (Gunjar et al., 1995). An extracellular surfactin with a molecular weight greater than one million was obtained from the culture broth of *Pseudomonas marginalis* PD-14b (Burd and Ward, 1996). The active fraction appeared to be a complex of protein with a very low amount of aromatic amino acids and a lipopolysacccharide.

Yeasts produce a number of emulsifiers, which is particularly interesting because of the food grade status of several yeasts. Liposan is an extracellular emulsifier produced by *Candida lipolytica* (Cirigliano and Carmen, 1984). It is composed of 83% carbohydrate and 17% protein. Mannan-protein emulsifiers are produced by *Saccharomyces cerevisiae* (Cameron et al., 1988). A variety of polymeric bioemulsifiers for potential use in foods was studied by Shepherd et al., 1995.

Genetics and Regulation of Bioemulsifier Production

The genetics of bioemulsifier production has been studied using mutants, naturally occurring or induced by transposition. The screening for such mutants is made difficult by the fact that the loss of ability to produce the emulsifier usually does not result in an easily selectable phenotype. In addition, the genetics of many emulsifier-producing bacteria has not been adequately worked out and important genetic tools (plasmids, transposons, gene libraries) are still to be developed. The regulation of bioemulsifier production has been worked out at the molecular level for the glycolipid rhamnolipid of *P. aeruginosa* and for the lipopeptides of *Bacilli*. In these bacteria, it was shown that emulsifier production is induced by molecular signals involved in quorum sensing. This regulatory feature appears to be general and probably applicable also for the production of high molecular weight emulsifiers, since emulsifier production is concurrent with the onset of the stationary phase of growth.

Lipopeptides of Gram-Positive Bacilli

The two well-studied lipopeptide bioemulsifiers produced by *Bacilli*—surfactin (Peypoux et al., 1999) and lichenysin (Yakimov et al., 1995)—are structurally similar. As already mentioned, both are composed of a cyclic heptapeptide linked to a fatty acid and differ in the last amino acid of the peptide—leucine and isoleucine, respectively. The peptide moiety, like many small peptides in microorganisms, is synthesized non-ribosomally by a multi-enzyme peptide synthetase complex (Marahiel, 1997). The *srfA* operon of *B. subtilis* was defined by a transposon Tn917 insertion and is required for production of surfactin (Nakano et al., 1991). It is an operon of over 25 kb that codes for the peptide synthase and is composed of repeating domains, whose function and order parallel those of the amino acids in the peptide (Cosmina et al., 1993; Fabret et al., 1995; Galli et al., 1994; Marahiel, 1997; Menkhaus et al., 1993). The *sfp* gene is also required for surfactin synthesis and codes for a phosphopantetheinyl transferase that activates the synthetase by post-transcriptional modification (Borchert et al., 1994; Lambalot et al., 1996; Nakano et al., 1992). Still unidentified is the acyl transferase that transfers the fatty acid to the peptide. Lichenysin synthesis is encoded for by the *lchA* operon, which is highly homologous to the *srfA* operon (Konz et al., 1999; Yakimov et al., 1998).

The production of surfactin is regulated by cell density and is involved with the development of competence—a physiological state that enables the uptake of DNA—and with sporulation. Therefore the control of its synthesis is very complex and is affected by cell density. The high cell

density is communicated by several quorum-sensing control elements that either stimulate or inhibit surfactin production. Initiation of transcription from the *srfA* operon is induced by binding of the phosphorylated response regulator, ComA. The ComA molecule and the membrane-bound histidine kinase ComP make up a two-component signal transduction system, which is activated at the membrane by a signal peptide ComX that accumulates at high cell density.The phosphorylation of ComA is also controlled by a signal peptide CSF—competence stimulating factor that is transported across the membrane and inhibits *srfA* expression at high cell densities (Cosby et al., 1998; Grossman, 1995; Lazazzera et al., 1999; Marahiel et al., 1993; Solomon et al., 1995). The involvement of at least two other proteins (a positive regulator ComR and a negative regulator SinR) also has been demonstrated (Liu et al., 1996; Luttinger et al., 1996).

The regulation of lichenysin seems to follow a similar pattern, inasmuch as a homologue of ComA was also identified in *B. licheniformis* and shown to be involved in production of lichenysin (Yakimov and Golyshin, 1997).

Rhamnolipids

The genes required for the synthesis of rhamnolipids in *P. aeruginosa* were identified by Tn5 transposition and screening for transposants that were unable to produce rhamnolipid biosurfactants (Ochsner et al., 1994). Two genes *(rhlAB)* were identified, one coding for the Rh1A protein (32.5 kDa), which has a putative signal sequence, and the other for RhlB protein, which is located in the periplasm. The expression of the *rhlAB* genes in *Escherichia coli* led to the formation of active rhamnosyltransferase. Recently, another gene—*rhlG*—essential for the biosynthesis of rhamnolipids has been identified by insertional mutation (Campos-Garcia et al., 1998). This gene is homologous to the *fabG* gene, which encodes the NADPH-dependent β-ketoacyl-acyl carrier protein (ACP) reductase required for fatty acid synthesis. Insertional mutants in this gene grew normally and the mutation had no apparent effect on the total lipid content of the cells, but the production of rhamnolipids was completely inhibited. These results suggest that the synthetic pathway for the fatty acid moiety of rhamnolipids is separate from the general fatty acid synthetic pathway, starting with a specific ketoacyl reduction step catalyzed by the RhlG protein. The existence of synthetic pathways for emulsifiers that are homologous but distinct from the general fatty acid synthetic pathway may well be a general characteristic of emulsifier biosynthesis, as a *fabG* homologue that is nonessential for growth was also identified in a hydrocarbon-degrading strain of *A. junii* (Sapir, 1998).

Production of rhamnolipids occurs at the stationary phase of growth, and recent experiments demonstrated that it is controlled by quorum sensing (Brint and Ohman, 1995; Ochsner and Reiser, 1995; Pearson et al., 1997). The *rhlAB* gene cluster coding for the biosynthesis of rhamnolipids contains two additional genes that encode regulatory proteins (RhlR and RhlI). These proteins share significant sequence similarity with bacterial autoinducer synthetases of the LuxI type. The RhlR is a putative transcriptional activator and the RhlI protein directs the synthesis of the quorum-sensing inducer N-butyryl homoserine lactone (PAI-2). The RhlR-RhlI regulatory system is essential for the regulation of rhamnolipid production. Moreover, rhamnolipid synthesis also is regulated by another regulatory system, the LasR-LasI system, which controls the biosynthesis of elastase. This is also a quorum-sensing system that consists of a transcriptional activator, LasR, and LasI, which directs the synthesis of the autoinducer *N*-(3-oxododecanoyl) homoserine lactone (PAI-1). There is sequence homology between the LasR-LasI proteins and the RhlR-RhlI proteins and there is cross-communication between the two quorum-sensing systems. It has been shown that LasR together with PAI-1 regulates transcription of the *rhlR* gene (Campos-Garcia et al., 1998; Latifi et al., 1996; Latifi et al., 1995; Ochsner et al., 1994; Pearson et al., 1997; Pesci et al., 1997; Van Delden et al., 1998).

Polymeric Bioemulsifiers

Polymeric bioemulsifiers are more complex than the low molecular weight lipopeptides and rhamnolipids. The synthesis of high molecular weight heteropolysaccharides requires a large number of genes, and the genetics is even more complex for polysaccharide-protein complexes. From the genetics point of view, the best studied polysaccharide bioemulsifier is the one produced by *A. calcoaceticus* BD4. The genes involved in its synthesis were identified in a cosmid library that was used to complement nonproducing mutants (Stark, 1996). These biosynthetic genes are organized in a cluster of about 60 kilobases. The first gene in the biosynthesis of the BD4 emulsifier (Accession no. X89900) was identified as a homologue of genes coding for phosphoglucoisomerase (*pgi*). The product of this gene is a protein of about 60 kDa in molecular weight that carries out the bidirectional conversion of glucose-6-phosphate to fructose-6-phosphate. The gene is highly conserved from bacteria to mam-

mals, with about 40% homology in amino acids. Additional genes in the cluster (*epsX* and *epsM*, Accession no. X81320) show homology to the genes coding for GDP-mannose pyrophosphorylase and phosphomannose isomerase of enteric bacteria (Stark, 1996). It is interesting to note that mutants in the *epsX* and *epsM* genes grow poorly under conditions that favor the formation of the bioemulsifier, and these deleterious mutations can be overcome by an additional (suppressor) mutation in the first gene in the pathway: *pgi*. These results suggest that mutants in the biosynthesis of the polysaccharide accumulate a toxic intermediate, probably fructose-6-phosphate (Fraenkel, 1992). The accumulation of the toxic substance, as well as the inhibition of growth, can be overcome by a mutation in a previous metabolic reaction, since the mutation blocks the synthesis of this toxic intermediate. The finding that some mutants in capsule synthesis grow poorly under conditions that favor capsule synthesis may explain the difficulty often encountered in getting such mutants (as an example, mutants unable to synthesize alasan were not obtained after screening more than 7,000 transposants of *A. radioresistens* KA53; Dahan, 1998). As screening for the mutants is usually performed on media that maximize the contrast between the capsule-producing wild type and mutant organisms (i.e., media that favor capsule formation), it is possible that these conditions are strongly inhibitory, or even lethal, for many of the mutants.

In *Acinetobacter*, the production of polysaccharide bioemulsifiers is concurrent with stationary phase. It has also been suggested that UDP-glucose, one of the precursors in the synthesis of polysaccharide bioemulsifiers, is a signal molecule in the control of σ-S and σ-S-dependent genes (Bohringer et al., 1995). The bioemulsifier of *A. calcoaceticus* BD4 (Kaplan and Rosenberg, 1982), emulsan of *A. calcoaceticus* RAG-1 (Rubinovitz et al., 1982), biodispersan of *A. calcoaceticus* A2 (Rosenberg et al., 1988), alasan of *A. radioresistens* (Dahan, 1998; Navon-Venezia et al., 1995) and an uncharacterized bioemulsifier from *A. junii* (Goldenberg-Dvir, 1998) can be detected in cultures only after more than 10 hours of growth, and maximal production occurs when the cultures have progressed well into the stationary phase. These results suggest the possibility that the high molecular weight bioemulsifiers are also controlled by quorum sensing, although there is, as yet, no direct proof.

The results presented here suggest that production of bioemulsifiers by bacteria is correlated with high bacterial density. This finding may be fortuitous or may reflect an indirect correlation with one or more physiological factors affected by high bacterial density such as availability of energy, nitrogen or oxygen. However, it is possible that the production of bioemulsifiers at high bacterial density has a selective advantage. For emulsifiers produced by pathogens, Sullivan, 1998 suggested that, being virulence factors, they are produced when the cell density is high enough to cause a localized attack on the host. It is easier to explain the need for bioemulsifiers in bacteria growing on hydrocarbons. As these bacteria are growing at the oil-water interphase, production of emulsifiers when the density is high will increase the surface area of the drops, allowing more bacteria to feed. Furthermore, when the utilizable fraction of the hydrocarbon is consumed, as in the case of oil that consists of many types of hydrocarbons, the production of the emulsifiers allows the bacteria to detach from the "used" droplet and find a new one (Rosenberg et al., 1983).

Natural Roles of Biosurfactants

The question "what is the natural role of microbial surfactants" would appear to be of fundamental significance in microbial physiology and of practical value in designing selection methods for improved molecules. However, the question has a basic flaw. There is no reason to suspect that surfactants have *one* natural role. As described in this chapter, microbial surfactants have very different structures, are produced by a wide variety of microorganisms, and have very different surface properties. Thus, it will be necessary to analyze each surfactant, or group of surfactants, separately. Only then may it be possible to draw any generalizations.

There are relatively few data available on the natural roles of biosurfactants, i.e., what function they play for the producing organisms. In only a few cases have nonbiosurfactant-producing mutants been generated and compared to the parent strain (Itoh and Suzuki, 1972; Koch et al., 1991). Thus, most of the concepts have been derived from a consideration of the surface properties of the biosurfactants and experiments in which biosurfactants are added to microorganisms growing on water-insoluble substrates. At least three general hypotheses have emerged.

Increasing the Surface Area of Hydrophobic Water-Insoluble Substrates

Theoretical and experimental studies on the production of single-cell protein from hydrocarbons in fermentors demonstrated that the growth rate can be limited by the interfacial surface area between water and oil (Sekelsky and Shreve,

1999). When the surface area becomes limiting, biomass increases arithmetically rather than exponentially. The evidence that emulsification is a natural process brought about by extracellular agents is indirect, and understanding how emulsification can provide an (evolutionary) advantage for the microoganism producing the emulsifier has certain conceptual difficulties. Stated briefly, emulsification is a cell density-dependent phenomenon; that is, the greater the number of cells, the higher the concentration of extracellular product. The concentration of cells in an open system, such as an oil-polluted body of water, never reaches a high enough value to effectively emulsify oil. Furthermore, any emulsified oil would disperse in the water and not be more available to the emulsifier-producing strain than to competing microorganisms. One way to reconcile the existing data with these theoretical considerations is to suggest that the emulsifying agents do play a natural role in oil degradation, but not in producing macroscopic emulsions in the bulk liquid. If emulsion occurs at or very close to the cell surface, and no mixing occurs at the microscopic level, then each cell creates its own microenvironment and a local cell density dependence would be expected.

Increasing the Bioavailability of Hydrophobic Water-Insoluble Substrates

One of the major reasons for the prolonged persistence of high molecular weight hydrophobic compounds is their low water solubility, which increases their sorption to surfaces and limits their availability to biodegrading microorganisms. When organic molecules are bound irreversibly to surfaces, biodegradation is inhibited (van Loosdrecht et al., 1990). Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility (Deziel et al., 1996). Surfactants that lower interfacial tension dramatically are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation. Low molecular weight biosurfactants that have low CMCs increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Miller and Zhang, 1997). Data have been reported which indicate that biosurfactants can stimulate, inhibit, or have no effect on biodegradation of hydrocarbons (reviewed in Bruheim et al., 1997). In this regard, Arino et al., 1998 have reported that a rhamnolipid-producing strain of *P. aeruginosa* is involved in the degradation of PAHs by a bacterial community. Much less is known on how polymeric biosurfactants increase apparent solubilities of hydrophobic compounds. Recently, it has been demonstrated that alasan increases the apparent solubilities of PAHs 5-20 fold and significantly increases their rate of biodegradation (Rosenberg et al., 1999; Barkay et al., 1999).

Regulating the Attachment-Detachment of Microorganisms to and from Surfaces

One of the most fundamental survival strategies of microorganisms is their ability to locate themselves in an ecological niche where they can multiply. This is true not only for microbes that live in or on animals and plants, but also for those that inhabit soil and aquatic environments. The key elements in this strategy are cell surface structures which are responsible for the attachment of the microbes to the proper surface. Neu, 1996 has reviewed how surfactants can affect the interaction between bacteria and interfaces. If a biosurfactant is excreted, it can form a conditioning film on an interface, thereby stimulating certain microorganisms to attach to the interface while inhibiting the attachment of others. In the case where the substratum is also a water insoluble substrate, e.g., sulfur and hydrocarbons, the biosurfactant stimulates growth (Beebe and Umbreit, 1971; Bunster et al., 1989). If the biosurfactant is cell bound, it can cause the microbial cell surface to become more hydrophobic, depending on its orientation. For example, the cell surface hydrophobicity of *P. aeruginosa* was greatly increased by the presence of cell-bound rhamnolipid (Zhang and Miller, 1994), whereas the cell surface hydrophobicity of *Acinetobacter* strains was reduced by the presence of its cell-bound emulsifier (Rosenberg et al., 1983). These data suggest that microorganisms can use their biosurfactants to regulate their cell surface properties to attach or detach from surfaces according to need. This has been demonstrated for *A. calcoaceticus* RAG-1 growing on crude oil (Rosenberg, 1993). During exponential growth, emulsan is cell-bound in the form of a minicapsule. This bacterium utilizes only relatively long chain n-alkanes for growth. After these compounds are utilized, RAG-1 becomes starved, although it is still attached to the oil droplet enriched in aromatics and cyclic paraffins. Starvation of RAG-1 causes release of the minicapsule of emulsan. It was shown that this released emulsan forms a polymeric film on the n-alkane-depleted oil droplet, thereby desorbing the starved cell (Rosenberg et al., 1983). In effect, the "emulsifier" frees the cell to find fresh substrate. At the same time, the depleted oil droplet has been "marked" as used, because it now has a hydrophilic outer surface to which the bacterium cannot attach.

Potential Commercial Applications

Bioemulsifiers have several important advantages over chemical surfactants, which should allow them to become prominent in several industrial and environmental uses. Bioemulsifiers are produced from renewable resources, are biodegradable, and are active under a variety of conditions. Of special interest are the emulsifiers produced by thermophilic and halophilic bacterial species (Trebbau-de and McInerney, 1996; Makkar and Cameotra, 1997). Although not extensively studied so far, they present unique possibilities for applications involving extreme conditions of pH, salinity and temperature (Desai and Banat, 1997). It is interesting that an arctic strain, *Arthrobacter protophormiae*, produces a heat-stable bioemulsifier (Pruthi and Cameotra, 1997).

One obvious application is in the oil and petroleum industries (Rosenberg, 1993). As surfactants increase the oil-water surface area, they accelerate degradation of various oils by bacteria and improve bioremediation of water and soil (Banat, 1995; Bruheim et al., 1997; Volkering et al., 1997). Surfactants are important for microbially enhanced oil recovery as well as for cleanup of storage tanks and pipes. Moreover, some bioemulsifiers are capable of increasing the bioavailability of poorly soluble organic compounds, such as polycyclic aromatics (PAHs). One such emulsifier—Alasan (Navon-Venezia et al., 1995)—increases the solubility of several PAHs, such as phenanthrene, flourene and pyrene, and significantly accelerates the rate of their mineralization (Barkay et al., 1999). Similar results were obtained for the biodegradation of polychlorinated biphenyls (PCBs) in the presence of a bioemulsifier produced on sunflower (Fiebig et al., 1997; Robinson et al., 1996).

In the detergent and cleaning industries, it is important to remove hydrocarbons or fatty materials. Bioemulsifiers have three major advantages for these applications: the environmental consequences of their use are minimal and their addition can reduce the concentration of chemical detergents that are much more harmful. In addition, the biosurfactants are compatible with the variety of enzymes that are used in the "bio"-detergents that are often inactivated by the chemical detergents. Bioemulsifiers also can be used as substitutes for chlorinated solvents for cleaning of electronic boards, cutting devices and delicate instruments that can be damaged by standard detergents.

Bioemulsifiers are potentially useful in agriculture, especially in various formulations of herbicides and pesticides. The active compounds in these formulations are hydrophobic, and emulsifiers are required for dispersing them in the aqueous solutions. One example is the use of bioemulsifiers—probably glycolipopeptides—produced by strains of *Bacillus* for emulsifying immiscible organophosphorus pesticides (Patel and Gopinathan, 1986).

The class of polymeric (high molecular weight) bioemusifiers offers additional advantages. These emulsifiers coat the droplets of oil, thereby forming very stable emulsions that never coalesce. This property is especially useful for making oil-in-water emulsions for cosmetics and for food (such as salad dressings; Klekner and Kosaric, 1993; Shepherd et al., 1995). In dairy products (soft cheeses and ice creams), the addition of polymeric emulsifiers improves the texture and creaminess. This quality is of special value for low-fat products. Because polymeric emulsifiers adhere to the oil, they concentrate in the oil-water interphase and stay with the oil when the water is removed. Consequently the emulsions are stable even in very dilute solutions. In addition, the emulsifier concentrations in the water are very low, allowing the water to be recycled. These properties are of special importance for various applications in the textile or paper industries (Rosenberg et al., 1989).

At present, the cost of production and insufficient experience in applications limit the use of bioemulsifiers. However, inasmuch as awareness of water quality and environmental conservation is increasing and demand for natural products is expanding, it appears inevitable that the high quality, microbially produced bioemulsifiers will replace the currently used chemical emulsifiers in many of the applications outlined above.

Literature Cited

Abraham, W. R., H. Meyer, and M. Yakimov. 1998. Novel glycine containing glucolipids from the alkane using bacterium Alcanivorax borkumensis. Biochim. Biophys. Acta 1393 (1):57–62.

Appaiah, A. K. A., and N. G. K. Karanth. 1991. Insecticide specific emulsifier production by hexachlorocyclohexane-utilizing Pseudomonas tralucida Ptm+ strain. Biotechnol. Lett. 13:371–374.

Arima, K., A. Kahinuma, and G. Tamura. 1968. Surfactin, a crystalline peptide lipid surfactant produced by Bacillus subtilis: isolation, characterization and its inhibition of fibrin clot formation. Biochem. Biophys. Res. Commun. 31:488–494.

Arino, S., R. Marchal, and J. P. Vandecasteele. 1996. Identification and production of a rhamnolipidic biosurfactant by a Pseudomonas species. Appl. Microbiol. Biotechnol. 45 (1–2):162–168.

Arino, S., R. Marchal, and J. P. Vandecasteele. 1998. Involvement of a rhamnolipid-producing strain of Pseudomonas aeruginosa in the degradation of polycyclic aromatic hydrocarbons by a bacterial community. J. Appl. Microbiol. 84:769–776.

Ashtaputre, A. A., and A. K. Shah. 1995. Emulsifying property of a viscous exopolysaccharide from Sphingomonas paucimobilis. World J. Microbiol. Biotechnol. 11:219–222.

Banat, I. M. 1995. Biosurfactants production and possible use in microbial enhanced oil recovery and oil pollution remediation: a review. Biosource Technol. 51:1–12.

Barkay, T., S. Navon-Venezia, E. Ron, and E. Rosenberg. 1999. Enhancment of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Applied Environ. Microbiol. 65:2697–2702.

Beebe, J. L., and W. W. Umbreit. 1971. Extracellular lipid of Thiobacillus thiooxidans. J. Baceteriol. 108:612–515.

Belsky I., D. L. Gutnick, and E. Rosenberg. 1979. Emulsifier of Arthrobacter RAG-1: determination of emulsifier-bound fatty acids. FEBS Lett. 101:175–178.

Bernheimer, A. W., and L. S. Avigad. 1970. Nature and properties of a cytological agent produced by Bacillus subtilis. J. Gen. Microbiol 61:361–369.

Bohringer, J., D. Fischer, G. Mosler, and R. Hengge-Aronis. 1995. UDP-glucose is a potential intracellular signal molecule in the control of expression of sigma S and sigma S-dependent genes in Escherichia coli. J. Bacteriol. 177:413–422.

Borchert S., T. Stachelhaus, and M. A. Marahiel. 1994. Induction of surfactin production in Bacillus subtilis by gsp, a gene located upstream of the gramicidin S operon in Bacillus brevis. J. Bacteriol. 176:2458–2462.

Brint, J. M., and D. E. Ohman. 1995. Synthesis of multiple exoproducts in Pseudomonas aeruginosa is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. J. Bacteriol. 177:7155–7163.

Bruheim, P., H. Bredholt, and K. Eimhjellen. 1997. Bacterial degradation of emulsified crude oil and the effect of various surfactants. Can. J. Microbiol. 43 (1):17–22.

Bunster, L., N. J. Fokkema, and B. Shippers. 1989. Effect of surface-active Pseudomonas spp. on leaf wettability. App. Environ. Microbiol. 55:1434–1435.

Burd, G., and O. P. Ward. 1996. Physicochemical properties of PM-factor, a surface-active agent produced by Pseudomonas marginalis. Can. J. Microbiol. 42:243–252.

Calvo, C., F. Martinez-Checa, A. Mota, V. Bejar, and E. Quesada. 1998. Effect of cations, pH and sulfate content on the viscosity and emulsifying activity on the Halomonas eurihalina. J. Ind. Microbiol. Biotechnol. 20:205–209.

Cameron, D. R., D. G. Cooper, and R. J. Neufeld. 1988. The mannoprotein of Saccharomyces cerevisiae is an effective bioemulsifier. Appl. Environ. Microbiol. 54:1420–1425.

Campos-Garcia, J., A. D. Caro, R. Najera, R. M. Miller-Maier, R. A. Al-Tahhan, and D. Soberon-Chavez. 1998. The Pseudomonas aeruginosa rhlG gene encodes an NADPH-dependent beta-ketoacyl reductase which is specifically involved in rhamnolipid synthesis. J. Bacteriol. 180:4442–4451.

Cirigliano, M. C., and G. M. Carman. 1984. Purification and characterization of liposan, a bioemulsifier from Candida lipolytica. App. Environ. Microbiol. 50:846–850.

Cooper, D. G., and J. E. Zajic. 1980. Surface active compounds from microorganisms. Adv. Appl. Microbiol. 26:229–253.

Cooper, D. G., C. R. MacDonald, S. J. B. Duff, and N. Kosaric. 1981. Enhanced production of surfactin of B. subtilis by continuous product removal and metal cation additions. Appl. Environ. Microbiol. 42:408–412.

Cooper, D. G., and D. A. Paddock. 1983. Torulopsis petrophilum and surface activity. Appl. Environ. Microbiol. 46:1426–1429.

Cooper, D. G., S. N. Liss, R. Longay, J. E. Zajic. 1989. Surface activities of Mycobacterium and Pseudomonas. J. Ferment Technology 59:97–101.

Cosby, W. M., D. Vollenbroich, O. H. Lee, and P. Zuber. 1998. Altered srf expression in Bacillus subtilis resulting from changes in culture pH is dependent on the Spo0K oligopeptide permease and the ComQX system of extracellular control. J. Bacteriol. 180:1438–1445.

Cosmina, P., F. Rodriguez, F. de Ferra, G. Grandi, M. Perego, G. Venema, and D. van Sinderen. 1993. Sequence and analysis of the genetic locus responsible for surfactin synthesis in Bacillus subtilis. Mol. Microbiol. 8:21–31.

Cutler, A. J., and R. J. Light. 1979. Regulation of hydroxydocosanoic and sophoroside production in Candida bogoriensis by the level of glucose and yeast extract in the growth medium. J. Biol. Chem. 254:1944–1950.

Dahan, O. 1998. Isolation and characterization of alasan mutants in Acinetobacter radioresistens. MSc Thesis, Tel Aviv University.

Davila, A. M., R. Marchal, and J. P. Vandecasteele. 1997. Sophorose lipid fermentation with differentiated substrate supply for growth and production phases. Appl. Microbiol. Biotechnol. 47:496–501.

De Acevedo, G. T., and M. J. McInerney. 1996. Emulsifying activity in thermophilic and extremely thermophilic microorganisms. J. Indust. Microbiol. 16:17–22.

Desai, J., and I. Banat. 1997. Microbial production of surfactants and their commercial potential. R. Microbiol. Mol. Biol. 61:47–48.

Deziel, E., G. Paquette, R. Villemur, F. Lepine, and J. G. Bisaillon. 1996. Biosurfactant production by a soil Pseudomonas strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 62 (6):1908–1912.

Espuny, M. J., S. Egido, I. Rodon, A. Manresa, and M. E. Mercade. 1996. Nutritional requirements of a biosurfactant producing strain Rhodococcus s p. 51T7. Biotechnology Letters 18:521–526.

Fabret, C., Y. Quentin, A. Guiseppi, J. Busuttil, J. Haiech, and F. Denizot. 1995. Analysis of errors in finished DNA sequences: the surfactin operon of Bacillus subtilis as an example. Microbiology 141:345–350.

Fattom, A., and M. Shilo. 1985. Production of emulcyan by Phormidium J-1: its function and activity. FEMS Microbiol. Ecol. 1:1–7.

Fiebig, R., D. Schulze, J. C. Chung, and S. T. Lee. 1997. Biodegradation of polychlorinated biphenyls (PCBs) in the presence of a bioemulsifier produced on sunflower oil. Biodegradation 8:67–75.

Fiechter, A. 1992. Biosurfactants: moving towards industrial application. Trends Biotechnol. 10:208–217.

Fraenkel, D. G. 1992. Genetics and intermediary metabolism. Ann. Rev. Genet. 26:159–177.

Galli, G., F. Rodriguez, P. Cosmina, C. Pratesi, R. Nogarotto, F. de Ferra, and G. Grandi. 1994. Characterization of the surfactin synthetase multi-enzyme complex. Biochim. Biophys. Acta. 1205:19–28.

Goldenberg-Dvir, V. 1998. A new bioemulsifier produced by the oil-degrading Acinetobacter junii V-26. MSc thesis, Tel-Avi University.

Goldman, S., Y. Shabtai, C. Rubinovitz, E. Rosenberg, and D. L. Gutnick. 1982. Emulsan in Acinetobacter calcoaceticus RAG-1: Distribution of cell-free and cell-

associated cross-reacting materials. Appl. Environ. Microbiol. 44:165–170.

Golyshin, P. N., S. Lang, E. R. Moore, W. R. Abraham, H. Lunsdorf, and K. J. Timmis. 1998. Alcanivorax borkumensis gen. nov., sp. nov., a new hydrocarbon-degrading and surfactant-producing marine bacterium. Int. J. Syst. Bacteriol. 48 (2):339–348.

Grau, A., J. C. Gomez Fernandez, F. Peypoux, and A. Ortiz. 1999. A study on the interactions of surfactin with phospholipid vesicles. Biochim. Biophys. Acta. 1418:307–319.

Grossman, A. D. 1995. Genetic networks controlling the initiation of sporulation and the development of genetic competence in Bacillus subtilis. Annu. Rev. Genet. 29:477–508.

Guerra-Santos, L. H., O. Kappeli, and A. Fiechter. 1986. Dependence of Pseudomonas aeruginosa continuous culture biosurfactant production on nutritional and environmental factors. Appl. Microbiol. Biotechnol. 24:443–448.

Gunjar, M., J. M. Khire, and M. I. Khan. 1995. Bioemusifier production by Bacillus stearothermophilus VR8 isolate. Lett. Appl. Microbiol. 21:83–86.

Hauser, G., and M. L. Karnovsky. 1954. Studies on the production of glycolipid by Pseudomonas aeruginosa. J. Bacteriol. 68:645–654.

Hisatsuka, K., T. Nakahara, N. Sano, and K. Yamada. 1971. Formation of rhamnolipid by Pseudomonas aeruginosa and its function in hydrocarbon fermentation. Agr. Biol. Chem. 35:686–692.

Horowitz, S., and W. M. Griffin. 1991. Structural analysis of Bacillus licheniformis 86 surfactant. J. Ind. Microbiol. 7:45–52.

Inoue, S., and S. Itoh. 1982. Sophorolipids from Torulopsis bombicola as microbial surfactants in alkane fermentation. Biotechnol. Lett. 4:308–312.

Isoda, H., H. Shinmoto, D. Kitamoto, M. Matsumura, and T. Nakahara. 1997. Differentiation of human promyelocytic leukemia cell line HL60 by microbial extracellular glycolipids. Lipids 32:263–271.

Itoh, S., and T. Suzuki. 1972. Effect of rhamnolipids on growth of Pseudomonas aeruginosa mutant deficient in n-paraffin-utilizing ability. Agric. Biol. Chem. 36:1233–1235.

Itoh, S., and S. Inoue. 1982. Sophorolipids from Torulopsis bombicola as microbial surfactants in alkane fermentations. Appl. Environ. Microbiol. 43:1278–1283.

Kaeppeli, O., and W. R. Finnerty. 1979. Partition of alkane by an extracellular vesicle derived from hexadecane-grown Acinetobacter. J. Bacteriol. 140:707–712.

Kaeppeli, O., and W. R. Finnerty. 1980. Characteristics of hexadecane partition by the growth medium of Acinetobacter sp. Biotechnol. Bioeng. 22:495–501.

Kaeppeli, O., P. Walther, M. Mueller, and A. Fiechter. 1984. Structure of cell surface of the yeast Candida tropicalis and its relation to hydrocarbon transport. Arch. Microbiol. 138:279–282.

Kaplan, N., and E. Rosenberg. 1982. Exopolysaccharide distribution and bioemulsifier production in Acinetobacter calcoaceticus BD4 and BD413. Appl. Environ. Microbiol. 44:1335–1341.

Kaplan, N., B. Jann, and K. Jann. 1985. Structural studies on the capsular polysaccharide of Acinetobacter calcoaceticus BD4. Eur. J. Biochem. 152:453–458.

Kaplan, N., Z. Zosim, and E. Rosenberg. 1987. Acinetobacter calcoaceticus BD4 emulsan: reconstitution of emulsifying activity with pure polysaccharide and protein. Appl. Environ. Microbiol. 53:440–446.

Katz, E., and A. L. Demain. 1977. The peptide antibiotics of Bacillus: chemistry, biogenesis and possible functions. Bacteriol. Rev. 41:449–458.

Kim, J. S., M. Powalla, S. Lang, F. Wagner, H. Lunsdorf, and V. Wray. 1990. Microbial glycolipid production under nitrogen limitation and resting cell conditions. J. Biotechnol. 13:257–266.

Koch, A. K., O. Kaeppeli, A. Fiechter, and J. Reiser. 1991. Hydrocarbon assimilation and biosurfactant production in Pseudomonas aeruginosa mutants. J. Bacteriol. 173:4212–4219.

Klekner, V., and N. Kosaric. 1993. Biosurfactants for cosmetics. *In*: N. Kosaric (Ed.) Surfactant Science Series. Biosurfactants: production, properties, applications. Marcel Dekker Inc. New York, NY. 48:329–372.

Konz, D., A. Doekel, and M. A. Marahiel. 1999. Molecular and biochemical characterization of the protein template controlling biosynthesis of the lipopeptide lichenysin. J. Bacteriol. 181:133–140.

Krauss, E. M., and S. I. Chan. 1983. Complexation and phase transfer of nucleotides by gramicidin S. Biochemistry 22:4280–4285.

Kretschmer, A., H. Bock, and F. Wagner. 1982. Chemical and physical characterization of interfacial-active lipids from Rhodococcus erythropolis grown on n-alkane. Appl. Environ. Microbiol. 44:864–870.

Lambalot, R. H., A. M. Gehring, R. S. Flugel, P. Zuber, M. LaCelle, M. A. Marahiel, R. Reid, C. Khosla, and C. T. Walsh. 1996. A new enzyme superfamily–the phosphopantetheinyl transferases. Chem. Biol. 3:923–936.

Lang, S., and F. Wagner. 1987. Structure and properties of biosurfactants. *In*: Kosaric, N., W. L. Cairns, and N. C. C. Gray (Eds.) Surfactant Science Series, Biosurfactants and Biotechnology. Marcel Dekker Inc.. New York, 25:21–47.

Lang, S., and J. C. Philip. 1998. Surface active lipids in rhodococci. Anton. Leeuw. Int. 74:59–70.

Lang, S., and D. Wullbrandt. 1999. Rhamnose lipids-biosynthesis, microbial production and application potential. Appl. Microbiol. Biotechnol. 51:22–32.

Latifi, A., M. K. Winson, M. Foglino, B. W. Bycroft, G. S. Stewart, A. Lazdunski, and P. Williams. 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in Pseudomonas aeruginosa PAO1. Mol. Microbiol. 17:333–343.

Latifi, A., M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski. 1996. A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. 21:1137–1146.

Lazazzera, B. A., I. G. Kurtser, R. S. McQuade, and A. D. Grossman. 1999. An autoregulatory circuit affecting peptide signaling in Bacillus subtilis. J. Bacteriol. 181:5193–5200.

Li, Z. Y., S. Lang, F. Wagner, L. Witte, and V. Wray. 1984. Formation and identification of interfacial-active glycolipids from resting microbial cells of Arthrobacter sp. and potential use in tertiary oil recovery. Appl. Environ. Microbiol. 48:610–617.

Lin, S. C., M. A. Minton, M. M. Sharma, and G. Georgiou. 1994. Structural and Immunological characterization of

a biosurfactant produced by Bacillus licheniformis JF-2. Appl. Environ. Microbiol. 60:31–38.

Lin, S. C. 1996. Biosurfactants: recent advances. J. Chem. Technol. Biotechnol. 66 (2):109–120.

Lindum, P. W., U. Anthoni, C. Christophersen, L. Eberl, S. Molin, and M. Givskov. 1998. N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of Serratia liquefaciens MG1. J. Bacteriol. 180:6384–6388.

Liu, L., M. M. Nakano, O. H. Lee, and P. Zuber. 1996. Plasmid-amplified comS enhances genetic competence and suppresses sinR in Bacillus subtilis. J. Bacteriol. 178:5144–5152.

Luttinger, A., J. Hahn, and D. Dubnau. 1996. Polynucleotide phosphorylase is necessary for competence development in Bacillus subtilis. Mol. Microbiol. 19:343–356.

MacDonald, C. R., D. G. Cooper, and J. E. Zajic. 1981. Surface-active lipids from Nocardia erythropolis grown on hydrocarbons. Appl. Environ. Microbiol. 41:117–123.

Makkar, R. S., and S. S. Cameotra. 1997. Biosurfactant production by a thermophilic Bacillus subtilis strain. J. Ind. Microbiol. Biotechnol. 18(1):37–42.

Marahiel, M. A., M. M. Nakano, and P. Zuber. 1993. Regulation of peptide antibiotic production in Bacillus. Mol. Microbiol. 7:631–636.

Marahiel, M. A. 1997. Protein templates for the biosynthesis of peptide antibiotics. Chem. Biol. 4:4561–4567.

Marin, M., A. Pedregosa, and F. Laborda. 1996. Emulsifier production and microscopical study of emulsions and biofilms formed by the hydrocarbon-utilizing bacteria Acinetobacter calcoaceticus MM5. Appl. Microbiol. Biotechnol. 44:660–667.

Matsuyama, T., M. Sogawa, and I. Yano. 1991. Direct colony thin-layer chromatography and rapid characterization of Serratia marcescens mutants defective in production of wetting agents. Appl. Environ. Microbiol. 53:1186–1188.

Menkhaus, M., C. Ullrich, B. Kluge, J. Vater, D. Vollenbroich, and R. M. Kamp. 1993. Structural and functional organization of the surfactin synthetase multienzyme system. J. Biol. Chem. 268:7678–7684.

Miller, R. M., and Y. Zhang. 1997. Measurement of biosurfactant-enhanced solubilization and biodegradation of hydrocarbons. Meth. Biotechnol. 2:59–66.

Nakano, M. M., R. Magnuson, A. Myers, J. Curry, A. D. Grossman, and P. Zuber. 1991. srfA is an operon required for surfactin production, competence development, and efficient sporulation in Bacillus subtilis. J. Bacteriol. 173:1770–1778.

Nakano, M. M., N. Corbel, J. Besson, and P. Zuber. 1992. Isolation and characterization of sfp: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in Bacillus subtilis. Mol. Gen. Genet. 232:313–321.

Nakayama, S., S. Takahashi, M. Hirai, and M. Shoda. 1997. Isolation of new variants of surfactin by a recombinant Bacillus subtilis. Appl. Microbiol. Biotechnol. 48:80–82.

Navon-Venezia, S., Z. Zosim, A. Gottlieb, R. Legmann, S. Carmeli, E. Z. Ron, and E. Rosenberg. 1995. Alasan, a new bioemulsifier from Acinetobacter radioresistens. Appl. Environ. Microbiol. 61:3240–3244.

Navon-Venezia, S., E. Banin, E. Z. Ron, and E. Rosenberg. 1998. The bioemulsifier alasan: role of protein in maintaining structure and activity. Appl. Microbiol. Biotechnol. 49:382–384.

Neu, T. R., and K. Poralla. 1990. Emulsifying agent from bacteria isolated during screening for cells with hydrophobic surfaces. Appl. Microbiol. Biotechnol. 32:521–525.

Neu, T. R. 1996. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. Microbiol. Rev. 60:151–166.

Neufeld, R. J., and J. E. Zajic. 1984. The surface activity of Acinetobacter calcoaceticus sp. 2CA2. Biotechnol. Bioeng. 26:1108–1114.

Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser. 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa. J. Bacteriol. 176:2044–2054.

Ochsner, U. A., and J. Reiser. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA 92:6424–6428.

Parra, J. L., J. Guinea, M. R. Manresa, M. E. Mercade, F. Comelles, and M. P. Bosch. 1989. Chemical characterization and physico-chemical behaviour of biosurfactants. J. Am. Oil. Chem. Soc. 66:141–145.

Patel, M. N., and K. P. Gopinathan. 1986. Lysozyme-sensitive bioemulsifier for immiscible organophosphorus pesticides. Appl. Environ. Microbiol. 52:1224–1226.

Pearson, J. P., E. C. Pesci, and B. H. Iglewski. 1997. Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. 179:5756–5767.

Persson, A., E. Oesterberg, and Dostalek, M. 1988. Biosurfactant production by Pseudomonas fluorescens 378: Growth and product characteristics. Appl. Microbiol. Biotechnol. 29:1–4.

Pesci, E. C., J. P. Pearson, P. C. Seed, and B. H. Iglewski. 1997. Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. J. Bacteriol. 179:3127–3132.

Peypoux, F., J. M. Bonmatin, and J. Wallach. 1999. Recent trends in the biochemistry of surfactin. Appl. Microbiol. Biotechnol. 51:553–563.

Pruthi, V., and S. S. Cameotra. 1997. Production of a biosurfactant exhibiting excellent emusification and surface active properties by Serratia marcescens. World J. Microbiol. Biotechnol. 13 (1):133–135.

Rapp, P., H. Bock, V. Wray, and F. Wagner. 1979. Formation, isolation and characterization of trehalose dimycolates from Rhodococcus erythropolis grown on n-alkanes. J. Gen. Microbiol 115:491–503.

Rau, U., C. Manzke, and F. Wagner. 1996. Influence of substrate supply on the production of sophorose lipids by Candida bombicola ATCC 22214. Biotechnology Letters 18:149–154.

Rehm, H. J., and I. Reiff. 1981. Mechanisms and occurrence of microbial oxidation of long-alkanes. Adv. Biochem. Eng. 19:173–181.

Rendell, N. B., G. W. Taylor, M. Somerville, H. Todd, R. Wilson, and J. Cole. 1990. Characterization of Pseudomonas rhamnolipids. Biochim. Biophys. Acta. 1045:189–193.

Richter, M., M. Willey, R. Suessmuth, G. Jung, and H. P. Fiedler. 1998. Streptofactin, a novel biosurfactant with aerial mycelium inducing activity from Streptomyces tendae Tue 901/8c. FEMS Microbiol. Lett. 163 (2):165–171.

Ristau, E., and F. Wagner. 1983. Formation of novel anionic trehalose-tetraesters from Rhodococcus erythropolis

under growth limiting conditions. Biotechnol. Lett. 5:95–100.

Robinson, K., M. Ghosh, and Z. Shi. 1996. Mineralization enhancement of non-aqueous phase and soil-bound PCB using biosurfactant. Water. Sci. Technol. 34:303–309.

Rosenberg, E., A. Perry, D. T. Gibson, D. Gutnick. 1979a. Emulsifier of Arthrobacter RAG-1: Specificity of hydrocarbon substrate. Appl. Environ. Microbiol. 37:409–413.

Rosenberg, E., A. Zuckerberg, C. Rubinovitz, D. L. Gutnick. 1979b. Emulsifier of Arthrobacter RAG-1: Isolation and emulsifying properties. Appl. Environ. Microbiol. 37:402–408.

Rosenberg, E., A. Gottlieb, M. Rosenberg. 1983. Inhibition of bacterial adherence to hydrocarbons and epithelial cells by emulsan. Infect. Immun. 39:1024–1028.

Rosenberg, E., and N. Kaplan. 1987. Surface-active properties of Acinetobacter expolysaccharides. Inouye, M. Bacteria Outer Membranes as Model Systems. John Wiley & Sons, Inc.. New York, 311–342.

Rosenberg, E., C. Rubinovitz, A. Gottlieb, S. Rosenhak, E. Z. Ron. 1988a. Production of biodispersan by Acinetobacter calcoaceticus A2. Microbiol. Appl. Environ. 54:317–322.

Rosenberg, E., C. Rubinovitz, R. Legmann, E. Z. Ron. 1988b. Purification and chemical properties of Acinetobacter calcoaceticus A2 biodispersan. Appl. Environ. Microbiol. 54:323–326.

Rosenberg, E., Z. Schwartz, A. Tenenbaum, C. Rubinovitz, R. Legmann, E. Z. Ron. 1989. A microbial polymer that changes the surface properties of limestone: Effect of biodispersan in grinding limestone and making paper. J. Dispersion. Sci. Technol. 10:241–250.

Rosenberg, E. 1993. Exploiting microbial growth on hydrocarbon: new markets. Trends Biotechnol. 11:419–424.

Rosenberg, E., and E. Z. Ron. 1996. Bioremediation of petroleum contamination. Crawford, R. L., and D. L. CrawfordBioremediation: Principles and Applications. Cambridge University Press. MA. 100–124.

Rosenberg, E., and Eliora Z. Ron. 1997. Bioemulsans: microbial polymeric emulsifiers. Cur. Opin. in Biotechnol. 8:313–316.

Rosenberg, E., E. Z. Ron. 1998. Surface active polymers from the genus Acinetobacter. Kaplan DLBiopolymers from Renewable Resources. Springer-Verlag. Berlin, 281–291.

Rosenberg, E., and E. Z. Ron. 1999. High and low molecular mass microbial surfactants. Appl. Microbiol. Biotechnol. 52:154–162.

Rubinovitz, C., D. L. Gutnick, and E. Rosenberg. 1982. Emulsan production by Acinetobacter calcoaceticus in the presence of chloramphenicol. J. Bacteriol. 152:126–132.

Rosenberg, E., T. Barkay, S. Navon-Venezia, E. Z. Ron. 1999. Role of Acinetobacter bioemulsans in petroleum degradation. Fass, et. al. Novel Approaches for Bioremediation of Organic Pollution. Kluwer Academic/Plenum Publishers. New York, 171–180.

Sapir, S. 1998. Genes involved in growth of Acinetobacter junii strain V26 on hexadecane. MSc Thesis.

Sar, N., E. Rosenberg. 1983. Emulsifier production by Acinetobacter calcoaceticus strains. Curr. Microbiol. 9:309–314.

Sekelsky, A. M., and G. S. Shreve. 1999. Kinetic model of biosurfactant-enhanced hexadecane biodegradation by Pseudomonas aeruginosa. Biotech. Bioeng. 63:401–409.

Shepherd, R., J. Rockey, I. W. Sutherland, S. Roller. 1995. Novel bioemulsifiers from microorganisms for use in foods. J. Biotechnol. 40:207–217.

Sim, L., O. P., Ward, and Z. Y. Li. 1997. Production and characterization of a biosurfactant isolated from Pseudomonas aeruginosa UW-1. J. Ind. Microbiol. Biol. 19:232–238.

Solomon, J. M., R. Magnuson, A. Srivastava, and A. D. Grossman. 1995. Convergent sensing pathways mediate response to two extracellular competence factors in Bacillus subtilis. Genes Dev 9:547–558.

Stark, M. 1996. Analysis of the exopolysaccharide gene cluster from Acinetobacter calcoaceticus BD4. PhD thesis, Tel-Aviv University.

Sullivan, E. R. 1998. Molecular genetics of biosurfactant production. Curr. Opinion in Biotechnol. 9:263–269.

Suzuki, T., K. Hayashi, K. Fujikawa, K. Tsukamoto. 1965. The chemical structure of polymyxin E. The identities of polymyxin E1 with colistin A and polymyxin E2 with colistin B. J. Biol. Chem. 57:226–227.

Suzuki, T., K. Tanaka, J. Matsubara, S. Kimoshita. 1969. Trehalose lipid and branched-hydroxy fatty acids formed by bacteria grown on n-alkanes. Agric. Biol. Chem. 33:1619–1625.

Taylor, W. H., E. Juni. 1961. Pathways for biosynthesis of a bacterial capsular polysaccharide. I. Characterization of the organism and polysaccharide. J. Bacteriol. 81:688–693.

Trebbau-de, A. G., M. J. McInerney. 1996. Emulsifying activity in thermophilic and extremely thermophilic microorganisms. J. Ind. Microbiol. 16:1–7.

Van Delden, C., E. C. Pesci, J. P. Pearson, and B. H. Iglewski. 1998. Starvation selection restores elastase and rhamnolipid production in a Pseudomonas aeruginosa quorum-sensing mutant. Infect. Immun. 66:4499–4502.

van Loosdrecht, M. C. M., J. Lyklema, W. Norde, A. J. B. Zehnder. 1990. Influence of interfaces on microbial activity. Microbiol. Rev. 54:75–87.

Volkering, F., A. Breure, W. Rulkens. 1997. Microbiological aspects of surfactant use for biological soil remediation. Biodegradation 8:401–417.

Wagner, F., V. Behrendt, H. Bock, A. Kretschmer, S. Lang, and C. Syldatk. 1983. Production and chemical characterization of surfactants from Rhodococcus erythropolis and Pseudomonas sp. MUB grown on hydrocarbons. Zajic, J. E., et al. Microbial Enhanced Oil Recovery. Penwell. Tulsa, OK. 55–60.

Wang, S. D., and D. I. C. Wand. 1990. Mechanisms for biopolymer accumulation in immobilized Acinetobacter calcoaceticus system. Biotech. Bioeng. 36:402–410.

Wei, Y. H., and I. M. Chu. 1998. Enhancement of surfactin production in iron-enriched media by Bacillus subtilis ATCC 21332. Enzyme-Microb. Technol. 22:724–728.

Yakimov, M. M., K. N. Timmis, V. Wray, H. L. Fredrickson. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface Bacillus licheniformis BAS50. Appl. Environ. Microbiol. 61:1706–1713.

Yakimov, M. M., and P. N. Golyshin. 1997. ComA-dependent transcriptional activation of lichenysin A synthetase pro-

moter in Bacillus subtilis cells. Biotechnol. Prog. 13:757–761.

Yakimov, M. M., A. Kroger, T. N. Slepak, L. Giuliano, K. N. Timmis, and P. N. Golyshin. 1998. A putative lichenysin A synthetase operon in Bacillus licheniformis: initial characterization. Biochim. Biophys. Acta. 1399:141–153.

Zhang, Y., R. M. Miller. 1994. Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. App. Environ. Microbiol. 60:2101–2106.

Zhang, Y., and R. M. Miller. 1995. Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of n-alkanes. Appl. Environ. Microbiol. 61 (6):2247–2251.

Zhou, Q. H., and N. Kosaric. 1995. Utilization of canola oil and lactose to produce biosurfactant with Candida bombicola. J. Am. Oil. Chem. Soc. 72:67–71.

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CHAPTER 3.8

Bioremediation

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Introduction

As a result of human ignorance in the safe use of chemicals, carelessness in manufacture of synthetic compounds, occasional accidents, and improper disposal of chemical wastes, toxic anthropogenic chemicals have become ubiquitous contaminants of soils and waters worldwide. These compounds include thousands of individual molecules. They are found in the environment sometimes individually but more often as complex mixtures. Many are overtly toxic (e.g., the metal mercury or the herbicide dinoseb). Many more are chronic toxins, some of them carcinogens (e.g., organic polynuclear aromatic hydrocarbons or metals such as arsenic). They are derived from sources such as petroleum, synthetic organic chemicals, mining activities, and natural or man-made radionuclides. Governments now regulate the use and disposal of toxic chemicals more rigorously than in the past. However, the legacy of environmental damage from previous decades of improper practice now requires cleanup to mitigate, insofar as possible, hazards to human health and the environment.

Traditional environmental cleanup methods include approaches such as excavation and incineration of contaminated soil or pumping and aboveground treatment of groundwater. These techniques are both expensive and politically unpopular. Affordable, innovative technologies are needed by the environmental restoration industry. Bioremediation is such a technology.

Bioremediation

Bioremediation employs living organisms, most often microorganisms, plants, or both, or products produced from living organisms to degrade, detoxify, or sequester toxic chemicals present in natural waters and soils. Bioremediation can be adapted for use to treat soil, sediment, sludge, water, or even air. Treatments can be either ex situ or in situ. Ex situ procedures involve removal of contaminated materials from a polluted site prior to treatment in another location. In situ approaches treat contaminants in place without moving them to a treatment facility. Bioremediation sometimes can be speeded or, in very difficult circumstances, even made possible by augmenting natural systems with exogenous biological materials. This process is called *bioaugmentation*. Bioaugmentation usually involves the use of natural microorganisms or plants grown to large numbers in fermenters or greenhouses and can include the use of genetically engineered microorganisms (GEMs) or plants developed specifically for the purpose. More often bioremediation can be accomplished most efficiently by simply stimulating natural, indigenous, contaminant-transforming microbial or plant populations by providing them the necessary nutrients, environmental conditions, or both needed for growth and metabolism. This process is called *biostimulation*. Yet another bioremedial option is called *intrinsic bioremediation*. This encompasses natural microbial processes that occur without human intervention (self-restoration). Finally, abiotic processes sometimes can be used in combination with biotic processes to degrade particularly recalcitrant molecules. Examples of abiotic catalysts that may enhance biodegradative processes include ultraviolet light, inorganic reductants, and Fenton reagent (iron and hydrogen peroxide). The bioremediation industry has developed many novel approaches for biostimulation, bioaugmentation, combined abiotic/biotic processes, and for monitoring and quantifying intrinsic bioremediation.

Areas of Research and Development

Bioremediation

BIOREACTORS Bioreactors are vessels of various configurations and arrangements (Admassu and Korus, 1996) that contain degradative microbes and possibly other catalytic agents that function cooperatively with microbial systems. They are a common means of ex situ treatment of contami-

nated soil that has been excavated from a polluted site or groundwater that has been pumped from a polluted aquifer.

A common bioreactor design seen in the field is a vessel containing a slurry of soil and water to which nutrients have been added to stimulate indigenous bacteria. Common nutrient additions include sources of nitrogen, phosphorus, and carbon that support the growth and metabolism of desirable indigenous degradative microorganisms. For example, nitrogen can be added to stimulate the growth of bacteria that grow on hydrocarbon contaminants such as polynuclear aromatic compounds and aromatic compounds derived from creosote (Barbeau et al., 1997; Shuttleworth and Cerniglia, 1997). Additions also may include inducer substrates to stimulate particular types of microorganism or microbial activities. For example, biphenyl has been used to stimulate the growth of bacteria able to degrade chlorinated compounds and polychlorinated biphenyls (PCBs; Focht, 1997). If appropriate microorganisms are not present to be stimulated, they can be added (Brunsbach and Reineke, 1994). *Slurry reactors* can be run aerobically by stirring and mixing in either air or pure oxygen (LaGrega et al., 1994). Alternatively, they can be run anaerobically (anoxic conditions) with the economic advantage that little or no mixing is required (Funk et al., 1995a, b). Slurry-phase reactors can be designed to hold a few thousand or millions of gallons of material (Korus, 1997). Aeration systems for aerobic slurry reactors are usually designed to maintain dissolved oxygen concentrations of at least 2.0 mg/liter. Since aeration requires considerable power consumption (pumps, mixers, spargers, etc.) and may require special mechanical designs, it is a major expense for any treatment system. For example, to increase oxygen transfer rates, a system may be sparged with air or pure oxygen introduced through a series of special eductors. At the same time, such systems must minimize air emissions, which often are regulated by state or federal agencies or both. Anaerobic slurry systems usually also require some mixing devices to evenly distribute soil or sediment materials throughout the vessel. However, anoxic processes need not move large amounts of oxygen into the aqueous phase. The choice of anaerobic versus aerobic processes involves, however, more than economic considerations. Some contaminants are degraded poorly or not at all under normal aerobic conditions (e.g., the chlorinated solvent tetrachloroethylene; Wackett, 1997). Other contaminants are not degraded under anaerobic conditions, e.g., many aromatic compounds (polynuclear aromatic hydrocarbons [PAHs]; Shuttleworth and Cerniglia, 1997). Thus, aerobic slurry reactors have been used most commonly to treat soil or water contaminated with compounds such as PAHs and chlorinated compounds (chlorinated phenols; Barbeau et al., 1997). Anaerobic slurry reactors have been used to treat soil- or water-containing compounds such as highly chlorinated solvents and nitrated munitions like 2,4,6-trinitrotoluene (TNT, Figure 1; Funk et al., 1995b) or aerobically recalcitrant herbicides like dinoseb (Kaake et al., 1992).

Water often is treated in simple vessels known as batch reactors, which hold from a few hundred to many thousands of liters. Nutrients, microorganism, or both may be added to promote degradation of particular contaminants. Another variation on reactors designed for treatment of water is the fluidized bed. Fluidized beds usually contain biomass immobilized on or within carriers (e.g., polysaccharide-based beads, plastic saddles, sand particles, etc.). These carrier matrixes are circulated continuously within the vessel to ensure that environmental conditions are maintained uniformly throughout the system. Fluidized beds are particularly useful and frequently used for water treatment (Nyer, 1992). They are run either aerobically or anaerobically (Voice et al., 1995; Sayles and Suidan, 1993). Even very toxic and recalcitrant compounds such as nitrobenzene or aniline have been treated successfully in microbiologically acclimated fluidized beds.

Biomass also can be cultivated on a supporting, but stationary, matrix. The matrix is packed into a vessel such as a column. Such an arrangement is known as a packed bed reactor. Contaminated water passes through the packed matrix where biomass (usually bacteria) eliminates the

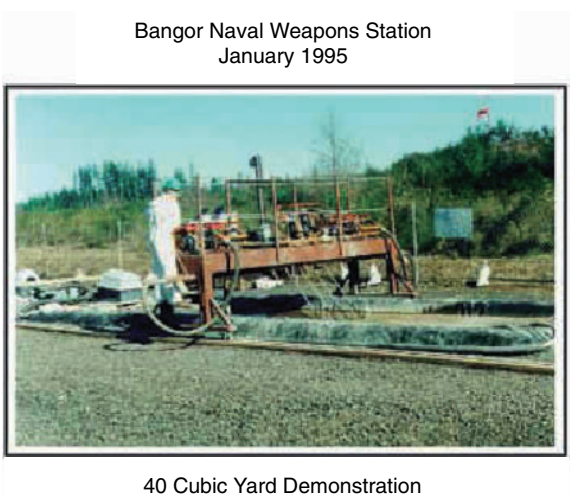

Fig. 1. Anaerobic slurry reactor for treating TNT-contaminated soil (photograph courtesy of Tom Yergovich, J. R. Simplot Company).

contaminants by degradation, absorption, or both (King, 1992). Packed beds work well for water, but also have been successful for the removal of contaminants from the vapor phase. For example, some chlorinated solvents in their vapor phase can be sparged through a packed-bed reactor. Microorganisms on or within the packed matrix material take in and degrade the gaseous molecules as they pass through the bed (Marsman et al., 1994). A particularly useful variation of the packed-bed reactor is the *biotrickling filter*, which also can be employed for treatment of vapor-phase pollutants (Unterman et al., 1996). Within a biotrickling filter the gaseous phase containing contaminants flows through the packed bed in one direction, and an aqueous nutrient solution flows in the opposite direction.

The *up-flow sludge-blanket reactor* is a water treatment approach that uses unique *biogranules* containing anaerobic bacteria that have been acclimated for the degradation of waste components within a particular waste stream (Wu et al., 1993). The granules contain a mixed microflora consortium that acts as a community with biodegradative capacities far beyond those of individual members.

As mentioned previously, bioreactors can be used in unique combinations as treatment trains that vary conditions to obtain specific results not possible with single reactors. The most common approach is the simple system of alternating anaerobic and aerobic conditions for treating mixtures of oxidized and reduced contaminants. Such systems also may work for some highly chlorinated compounds that are first reductively dehalogenated (in an anaerobic reactor), producing products that are then mineralized by aerobic microorganisms in a second reactor (Evans et al., 1996). Aerobic and anaerobic series reactors have been proposed for contaminants like PCBs and solvents like the tetrachloroethylene (perchloroethylene, also known as PCE). Many other variations of serial reactors are possible. For example, an abiotic reactor (UV light or Fenton reagent) can precede a biotic reactor (Büyüksönmez et al., 1998a; Büyüksönmez et al., 1998b).

BIOFILTRATION Another established method for treatment of gas-phase contaminants is biofiltration. An ex situ process (Deshusses, 1997; Leson and Winer, 1991), as shown in Figure 2, biofiltration requires the passing of contaminated air through a bed of soil, peat, or compost. These materials first must be pre-acclimated to the contaminants being treated to select a microbial consortium that can detoxify or completely mineralize the targeted pollutants (Yudelson and Tinari, 1995). Biofiltration works particularly

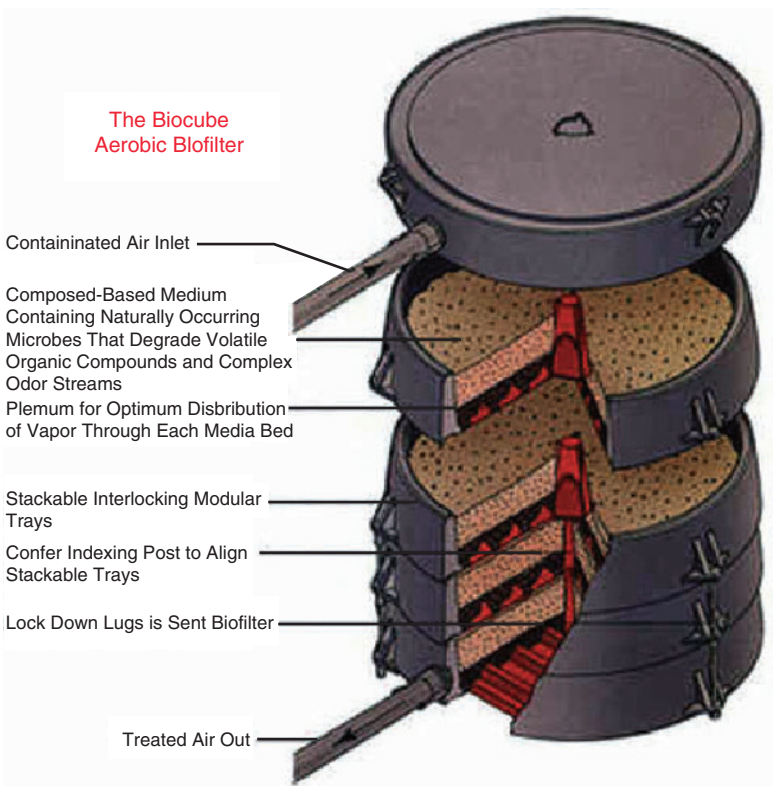

Fig. 2. Example of design of a biofiltration unit for removal of vapor-phase contaminants; illustration from ProAct Services Corporation (http://www.proact-usa.com/biocube.html).

well for volatile components of petroleum. It is commonly used in Europe for odor control (Ottengraf, 1986), and use of the method in the United States is increasing due to its simplicity and effectiveness. The following lists some Internet sites that discuss biofiltration technology:

(http://www.kumc.edu)
(http://www-rcf.usc.edu)
(http://www-scf.usc.edu)
(http://www.cee.uc.edu)
(http://www.inel.gov)
[194.178.172.97/class/ixg02.htm]
[194.178.172.97/aboutgrn.htm]
[online.awma.org]
(http://www.proact-usa.com)

Biofiltration works to degrade a diversity of airborne contaminants, including industrial chemicals like styrene (Arnold et al., 1997), pentane and isobutane mixtures (Barton et al., 1997), toluene (Matteau and Ramsay, 1997), chlorinated benzenes (Oh and Bartha, 1994), dimethylsulfide (Pol et al., 1994), ethylene (Elsgaard, 1998), and other volatile organic compounds (VOCs; Leson and Winer, 1991). Maintenance of good degradative activity of biofilter microbial communities sometimes requires the addition of nutrients to the biofiltration matrix, since materials like peat or wood chips are generally nutrient poor. Adjustments and careful control of environmental variables such as temperature, pH, and availability of moisture (humidity) also are often required (Arnold et al., 1997; Matteau and Ramsay, 1997). Removal rates for contaminants by biofilters can be impressive. For example, removal of vapors of chlorinated compounds (chlorinated benzenes, in one instance) was measured at 300 g of solvent vapor $\cdot$ h^{-1} $\cdot$ m^3 of filter volume (Oh and Bartha, 1994).

Air Sparging Biodegradation of many contaminants requires the availability of an electron acceptor to be reduced at the expense of oxidation of the contaminant(s). In groundwater, electron acceptor concentrations often are limited, especially for oxidative processes. Though numerous compounds can serve as electron acceptors for respiratory processes (oxygen, nitrate, Fe^{3+}, Mn^{3+}, and others), the most effective electron acceptor for the bioremediation of many contaminants is oxygen. Oxygen is present in pristine ground water at only 8–10 mg/liter, and in most ground water the levels are even lower. Oxygen can be introduced into ground water by a process called *air sparging*, an in situ technology (Raymond et al., 1975). Air or even pure oxygen is injected into an aquifer through specially designed sparging wells or infiltration galleries (Bowlen and Kosson, 1995). Oxygen is such a good electron acceptor (effective energy production for cellular metabolism) that air sparging runs the risk of overstimulating microbial growth. A resulting problem can be *biofouling*, where injection wells become plugged with biomass, which obstructs well screens and seals off the aquifer formation from further aeration. Additional problems include the plugging of subsurface geologic formations by iron-containing precipitates formed by the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) and the subsequent formation of ferric oxyhydroxides or other highly insoluble iron minerals. Hydrogen peroxide can be used as a convenient source of oxygen, since it decomposes in the presence of iron or biomass to produce oxygen (Frankenberger et al., 1989). Another potential advantage of hydrogen peroxide is that some iron minerals may catalyze its decomposition to hydroxyl radicals by the well-known Fenton process (Haber and Weiss, 1934).

$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + {}^{-}OH + Fe^{3+}$$

Hydroxyl radicals produced by Fenton reactions are exceptionally reactive and can attack contaminants in groundwater (Tyre et al., 1991), degrading them to products that may be more susceptible to breakdown by microbial populations than the original contaminants. In fact, hydroxyl radicals generated by modified Fenton reactions react with most environmental contaminants at near diffusion-controlled rates ($>10^9$ M^{-1} $\cdot$ s^{-1}). The degradation of xenobiotic chemicals by hydroxyl radicals then proceeds via either hydroxylation or hydrogen atom abstraction:

$$\cdot OH + R \rightarrow \cdot ROH \text{ (reaction 1)}$$

$$\cdot OH + RH_2 \rightarrow \cdot RH + H_2O \text{ (reaction 2)}$$

More work is required to determine how effective this "abiotic/biotic" approach to in situ bioremediation might be (Büyüksönmez, 1998a; Büyüksönmez, 1998b).

Even when oxygen is supplied by sparging, it can be used so rapidly that its supply can still be rate-limiting for bioremediation processes. Thus, the bioremediation industry has developed oxygen-releasing compounds (ORCs) composed of materials such as magnesium peroxide that allow controlled, long-term release of oxygen when introduced into boreholes as a slurry (Kao and Borden, 1994). Most successes with air sparging, as with many other bioremediation systems, have been reported in petroleum-contaminated aquifers. The reintroduction of indigenous microorganisms isolated from a contaminated site after culturing is one approach to in situ hydrocarbon bioremediation. It is effective especially when microorganism growth is supple-

mented by addition of oxygen and nitrogen (Korda et al., 1997). At the Moffett Field (Sunnyvale, Calif.) groundwater test site, in situ removal of *c*-DCE (*cis*-dichloroethylene) and TCE (trichloroethylene) coincided with biostimulation through phenol and oxygen injection and utilization, with *c*-DCE removed more rapidly than TCE. Greater TCE and *c*-DCE removal was observed when the phenol concentration was increased. Over 90% removal of *c*-DCE and TCE was observed in a 2-meter biostimulated zone (Hopkins et al., 1993). Thus, introduction of oxygen into contaminated aquifers can be of use even for recalcitrant compounds such as VOCs.

BIOVENTING Bioventing is an in situ technique and a cousin of air sparging (Kidd, 1996). The objectives of air sparging and bioventing are similar: to provide oxygen to the microbial populations in the subsurface. Bioventing systems use pumps, blowers, and piping systems similar to those used for *soil vapor extraction* (SVE). Where the primary goal of SVE is simply to remove contaminants from the subsurface for physical destruction (e.g., combustion) or adsorption (e.g., to activated carbon), the purpose of bioventing is to promote biological degradation of the vapors as they move through the soil (Kramer and Cullen, 1997). Injection- or extraction wells or specially designed trenches are connected to vacuum pumps or blowers, which either pull or push air through the unsaturated soil horizons (the vadose zone). The movement of air (oxygen) stimulates the microorganisms naturally present that are capable of degrading the targeted contaminants, using them as energy and carbon sources. Oxygen is used as the terminal electron acceptor for oxidation of the pollutants. Enough oxygen must be supplied to the subsurface to promote in situ contaminant degradation. However, the use of too much flow may force undegraded contaminant into the atmosphere. Flows must be carefully regulated to avoid this loss. Thus, bioventing is most effective when applied to sites containing contaminants that are moderately volatile. Examples of such contaminants include diesel or jet fuels and residues of aged petroleum (Bowlen and Kosson, 1995). Bioventing does not work for all sites, even if the site contains an appropriate contaminant. As a general guide, the vadose zone must be permeable enough to allow air to be exchanged at least every 24 hours (Hinchee and Ong, 1992). For some impermeable soils, such as those containing lots of clay or silt, methods have been developed to make soils more porous for better air flow. For example, this has been accomplished by fracturing soils with pressurized hydraulic fluids or by the use of pressurized air injection (Kaplan, 1990). After fractures are

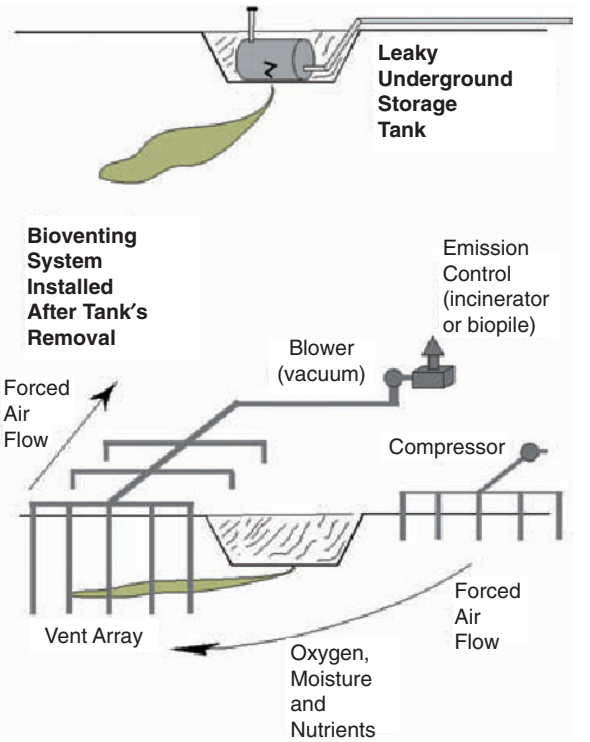

Fig. 3. Typical bioventing operation.

formed, bioventing becomes possible. Bioventing has been employed frequently with great success worldwide in the past decade (Bowlen and Kosson, 1995; Dupont, 1993; Hinchee et al., 1991; Bossert and Compeau, 1995). A typical bioventing system is shown in Figure 3.

During bioventing operations, it is important to ensure that the soil does not become too dry to support microbial growth. Thus, neutron probes sometimes are used for soil moisture monitoring. Soil moisture must be maintained within a range of 6 to 12% for biological processes to proceed effectively. An impermeable liner, usually made of plastic, is commonly placed over the surface of a contaminated area. This prevents infiltration of air that might bypass the desired path of air to the vent wells. Extra phosphorus or nitrogen or both may be needed if the soils being treated are poor in these essential nutrients. Nitrogen, in the form of ammonia, can be provided in the gas phase. It is desirable, if possible, to maintain a carbon/nitrogen/phosphorus ratio of about 100 : 10 : 1. It is not always possible to ensure that all of the contaminant vapors at a particular site are degraded as they pass through a soil column. Also, most engineered systems are subject to occasional failures in operation or design. Thus, a catalytic incinerator to destroy extracted vapor that was not degraded by microbes in the vadose zone is often

installed as a safety device. As mentioned above, not all sites are amenable to bioventing. The Battelle Corporation (Battelle Memorial Institute, Columbus, Ohio) has developed an evaluation protocol that is useful for determining whether a site is suited for remediation by bioventing (Hinchee and Ong, 1992). The evaluation involves four steps, performed sequentially:

A soil-gas survey is conducted to determine if the subsurface is oxygen-limited. Soil gas is analyzed for concentrations of oxygen and carbon dioxide.

1. A soil-gas permeability test is conducted. This determines if air can be injected at sufficient rates to aerate the vadose zone. The actual test involves injection and withdrawal of air, measuring changes in subsurface pressures at specific distances from the injection point.
2. If a soil is found to be oxygen-limited but permeable, it is next subjected to an in situ respiration test. This test measures biological contaminant degradation rates as compared to respiration rates in similar but uncontaminated control soils. For this test, soils are aerated while monitoring the oxygen and carbon dioxide concentrations in the soil-gas phase. Oxygen utilization rates are calculated and used to estimate contaminant degradation rate. These rates can be used later to estimate the time that will be required to treat a site by bioventing.
3. If a site remains a candidate for bioventing after the initial three tests, a pilot-scale bioventing demonstration may be conducted at the site. However, it may be possible to move directly to a full-scale process. This is determined on a site-by-site basis.

Among its advantages, bioventing: 1) uses readily available equipment, easy to install; 2) creates minimal disturbance to site operations; 3) can be used in inaccessible areas (e.g., under buildings); 4) requires short treatment times, usually 6 months to 2 years under optimal conditions; 5) is competitively priced ($45–140 per ton of contaminated soil); 6) is easily combinable with other technologies (e.g., air sparging, groundwater extraction); and 7) may not require costly offgas treatment. Its disadvantages are: 1) high constituent concentrations initially may be toxic to microorganisms; 2) certain site conditions (low soil permeability, high clay content, and others) preclude its use; 3) very low cleanup standards cannot always be achieved; 4) permits are generally required for nutrient injection wells (if used); and 5) only unsaturated (vadose) zone soils can be treated.

LANDFARMING Landfarming is an ex situ process where contaminated soil is excavated, supplemented with nutrients, and spread in layers on a prepared site to promote biodegradation. The nutrients added usually are nitrogen or phosphorus sources or both, which support the microbial catabolism of the added contaminant carbon. The soils are kept moist by irrigation and aerated by periodic tilling (Bowlen and Kosson, 1995). Depending on the contaminants present, soils may or may not be spread directly on the ground at a landfarm site. If there is a danger that the contaminants will leach through the vadose zone to groundwater or vaporize to dangerous levels in the atmosphere, then additional precautions must be considered. For example, soils may be spread on pre-installed plastic liners or clay barriers. Aqueous leachate collection systems may be constructed to prevent the movement of contaminants off-site or to groundwater. Vapor containment structures may be required, especially if the landfarm is located near populated areas. Petroleum, which is readily biodegraded by soil microbial communities, has been commonly treated by landfarming (Bossert and Compeau, 1995). However, highly toxic and/or biologically recalcitrant compounds generally cannot be successfully landfarmed. The presence of heavy metals, such as lead found in some older petroleum products, usually is a negative indication for landfarming. Using this method to treat petroleum-contaminated soil has some advantages: 1) design and implementation are relatively simple; 2) treatment times (usually 6 months to 2 years under optimal conditions) are short; 3) cost ($30–60 per ton of contaminated soil) is competitive; and 4) treatment is effective for organic constituents with slow biodegradation rates. The disadvantages of landfarming of petroleum-contaminated soil are: 1) concentration reductions >95% and constituent concentrations > 0.1 ppm are very difficult to achieve; 2) high constituent concentrations (>50,000 ppm total petroleum hydrocarbons) may reduce or preclude effectiveness; 3) presence of significant heavy metal concentrations (>2,500 ppm) may inhibit microbial growth; 4) volatile constituents tend to evaporate rather than biodegrade during treatment; 5) treatment requires a large land area; 6) dust and vapor generation during aeration may pose air quality concerns; and 7) bottom liners may be required if leaching from the landfarm is a concern. (The data are from US EPA http://www.epa.gov/swerust1/cat/landfarm.htm).

The presence of plants sometimes can enhance the degradation of contaminants during landfarming operations. For example, the degradation of aromatic compounds (PAHs) can be greater in the presence of vegetation than without it. Enhanced microbial activity in the plant root zone seems to contribute to the increased

removal of PAHs. Though PAHs can be detected in the tissues of plants grown on landfarms, overall uptake is generally thought to be insignificant. Planting of vegetation not only may increase contaminant degradation rates but also helps control erosion and can be an esthetic addition to a treatment site. Use of plants therefore can be an inexpensive means of speeding landfarm-based remediation of sites contaminated with petroleum (Schwab and Banks, 1994).

Biopiles Biopiles are variants of landfarms that permit treatment conditions to be more directly controlled. Also known as *soil-heaping* operations, biopiles use less land area and produce fewer air emissions than landfarms. Volatile compounds lost from biopiles are readily captured and treated, a major advantage of this technology (Bossert and Compeau, 1995). To produce a biopile, soil is excavated and then mounded and covered by a plastic sheet within a lined treatment cell. An internal system of perforated pipes is placed within the pile and used to draw air in by means of vacuum pumps. Air also may be blown into a biopile through the internal pumping system. Alternatively, oxygen can be supplied from oxygen-releasing compounds (ORCs, see Air Sparging) and mixed into the piles as they are formed. As for all biological processes, careful control of moisture content and nutrient status (particularly the nitrogen to phosphorus to carbon ratio) is required. Water and nutrients are added as needed. Special microbial cultures can be added (Walter, 1997), though this is rarely done in practice. Most contaminants treated in biopiles are degradable by natural microbial populations already present in the soil, if conditions are made favorable for their growth. Leachates and gaseous emissions are collected for separate treatment. Biopiles can be used for most biodegradable contaminants, but most of the limitations of landfarming still apply. To date, most biopile operations have treated petroleum-contaminated soils (Bossert and Compeau, 1995).

Biomounds are lower cost variations of biopiles and also have been used to treat petroleum-contaminated soil. Biomounding is the addition of bulking materials like animal manure and wood chips to help retain moisture, provide good aeration, and supply readily available sources of nitrogen, phosphorus, and trace nutrients for growing microbial populations capable of hydrocarbon degradation. This simple process usually can reach stringent soil cleanup standards in the range of 10 ppm of residual petroleum in the soil (known as *total petroleum hydrocarbons*, or TPH). As with biopiles, indigenous bacteria present in petroleum-contaminated soil proliferate under biomounding conditions. Addition of exogenous microbes is not normally required. Biomounds thus are relatively inexpensive to manage and fairly fast at the breakdown of petroleum residues in soil. Though the construction seems much like that of a standard compost heap, biomounds are designed not to require continual turning of the soil as is done during traditional composting. Simple passive aeration systems are used that usually include a grid, sometimes with multiple layers of horizontal piping constructed of flexible, perforated drain tile, along with vertical piping (risers) of PVC. Biomounds generate heat from their biological activity, and it is usually desirable to retain much of this heat, along with moisture, by covering mounds with plastic sheeting.

Composting Composting is a familiar and effective process for preparing high quality soil additives from organic materials. It also is of value in treatment of hazardous wastes. In composting of contaminated soil, excavated soil is first mixed with bulking agents like straw, horse manure, or other agricultural residues. The mixture then is formed into piles that are aerated by periodic turning with specially designed machines. Microbial activity in composts can be very vigorous, generating considerable heat. Composts thus often are termed *thermophilic*, since they may operate at temperatures of 50–60°C. Composting has been examined as a treatment for a variety of contaminants. Examples include petroleum sludges (McMillin et al., 1993), chlorophenols (Valo and Salkinoja-Salonen, 1986; Mueller et al., 1991), and residues of explosives (Williams, et al., 1992). Some questions remain as to the overall efficacy and effectiveness of composting for treating certain types of contaminants. For example, during composting some compounds such as the munition compound 2,4,6-trinitrotoluene, or TNT, are polymerized and/or incorporated into compost humus fractions. Thus there is little or no conversion of these contaminants to carbon dioxide or products that might themselves be degradable to carbon dioxide (Williams et al., 1992). The long-term stability and potential for release of toxic compounds from such uncharacterized materials in soil are not known. Some work has implied that mutagenic metabolites of explosives are formed and incompletely degraded during the composting process (Jarvis et al., 1998). In another study, [^{14}C]-TNT was subjected to composting. Much of the ^{14}C became incorporated into the "humic" fraction of the compost. When the composted TNT was fed to rats, about 2.3% of the total ^{14}C dose appeared in the rats' urine during the first 3 days after feeding, and excretion of ^{14}C in the urine

continued for more than 6 months (Palmer et al., 1997). Thus, it is clear that composted TNT is not irreversibly immobilized in the humic fraction of the compost. TNT, or one or more of its biotransformation products in compost, is still bioavailable. Clearly, composting is not the treatment of choice for some wastes, especially when little mineralization of the target pollutant occurs. Thus, composting is still an experimental approach for most hazardous wastes and should be evaluated individually for each new pollutant prior to extensive use. The fates and mechanisms of removal of chemicals like pesticides and other exotic compounds in composting processes are largely a mystery (Fogarty and Tuovinen, 1991; Michel et al., 1995; Kastner et al., 1995). Continued research on composting for treatment of hazardous wastes is slowly building on our knowledge base and overcoming some of the prior limitations. Recent work, for example, indicates that TNT can be degraded more effectively if the compost is allowed to undergo a fairly long anaerobic phase before traditional aerobic composting (Breitung et al., 1996).

Microbiological agents also can pose a hazard in composted materials (Marsh et al., 1979; Millner et al., 1977). The presence of pathogenic bacteria and fungi is of particular concern. Their presence depends largely on composting methods, and additional research is needed on the risks to human health and the environment of exposure to potentially hazardous chemical residues, heavy metals (Keller and Brunner, 1983), and microorganisms in composted waste (Deportes et al., 1995). The danger from pathogenic microorganisms centers on fungi such as *Aspergillus fumigatus*. Spores of this causal agent of the lung disease aspergillosis are abundant in the air near some composting sites (Marsh et al., 1979; Millner et al., 1977). Thus, site location is of considerable importance during design of composting operations. Overall, though the simplicity and affordability of composting for hazardous waste treatment are attractive, the efficacy and safety of this method for treatment of many wastes are still an open question.

Intrinsic Bioremediation Intrinsic remediation is result of the combined effects of all natural processes in contaminated environments that reduce the mobility, mass, and risks of pollutants (Hinchee et al., 1995). The mechanisms of intrinsic remediation include 1) biodegradation or biotransformation of contaminants by indigenous microbial populations, 2) sorption to or trapping within matrixes or on mineral phases that make toxic compounds non-bioavailable, and 3) loss of toxicity by dilution or volatilization (Frankenberger and Karlson, 1991). Intrinsic remediation of petroleum hydrocarbons has been well documented (Rifai et al., 1995; Wiedemeier et al., 1995). Intrinsic remediation also has been confirmed for chlorinated compounds (chlorinated solvents) in anoxic environments that promote reductive dehalogenation of chlorinated hydrocarbons (Hinchee et al., 1995; Rifai et al., 1995). Evidence indicates that natural evolution by genetic exchange within the environment can lead to novel microbial populations that degrade xenobiotic contaminants (van der Meer et al., 1998).

Risk-based assessments become the basis for determining cleanup end-points when intrinsic processes are considered as methods for site remediation. In such cases, the distribution of contaminants must be determined. These analyses include obtaining data that allow an understanding of the extent of contaminant plume migration in relation to sensitive receptors, such as groundwater. These analyses require extensive sampling at most sites of the vadose zone and aquifers. Analyses also must confirm that pollutant remediation is proceeding at a rate sufficient to reduce risks to human health and the environment in an acceptable period. Measurements that can provide the required information include: 1) the contaminant mass and its environmental distribution; 2) the abundance of contaminant-degrading or -transforming microorganisms, as indicated by actual microbial counts or other evidence of their presence; 3) the temperature and pH of the environment to show that they are appropriate for microbial activity; 4) types of electron donors and acceptors present, and changes in their concentrations (e.g., concentrations of nitrate, nitrite, redox-active metal species like iron, methane, ammonia, carbon dioxide, and sulfate); 5) redox potential; and 6) other factors, including abiotic ones, that must be determined on a site-by-site basis and will depend on what contaminant(s) is present. Ideally, the efficacy of intrinsic remediation processes should be predictable at individual sites using computer models, though modelers are a long way from this ideal. It is obvious, however, that intrinsic remediation has its place in the repertoire of environmental restoration tools, but it requires specific, extensive, and long-term activities at a site. It is never a "do-nothing" option.

Phytoremediation Phytoremediation involves the use of plants to remove contaminants from soil or water. Though proposed primarily as a means to remove toxic metals from environmental systems, phytoremediation also has been suggested for bioremediation of some organic pollutants (Bolton and Gorby, 1995). Through a process called *phytoextraction*, contaminants are taken into a plant and concentrated within the

plant tissues (Kelly and Guerin, 1995; Cunningham et al., 1995). When the plants are harvested, the sequestered contaminants are thereby removed from the environment. Some investigators have suggested that bacteria associated with root zones (the rhizosphere) of plants may degrade organic contaminants, also indicating a potential to use phytoremediation for removal of organic pollutants from soil or water. However, additional research is needed to confirm which plants degrade or sequester which contaminants. Plants, particularly those with deep roots, may remove volatile contaminants from soil as they transpire water. The contaminants are carried through the vascular tissue of the plant and released to the atmosphere with transpired water vapor. Recently, Rugh et al. (1998) developed a genetically engineered yellow poplar tree that expresses the bacterial mercury reductase gene (*merA*) in the plant's tissue. Mercury reductase converts nonvolatile Hg^{2+} to volatile Hg^0, which the tree pumps into the atmosphere. Thus, this deep-rooted tree has the potential for use in mercury phytoremediation, if it proves to be effective at getting to the mercury bound in soil, and the release of mercury vapor to the air is judged to be safe. Besides their direct effects on contaminants, plants also serve a useful function in the stabilization of polluted sites. Plant cover reduces soil erosion and the infiltration of water into soil. The latter process may decrease or eliminate the leaching of mobile contaminants into groundwater (Bolton and Gorby, 1995; Cunningham et al., 1995). Plants appear to have special potential for the challenging task of removal of radionuclides from soil or water (Cornish et al., 1995).

In practice, plants are grown on a contaminated site and allowed to concentrate contaminants like metals or radioactive compounds into their tissues. After an appropriate time, they are harvested and burned to reduce the volume of collected material, typically by about 95%. This volume reduction, and the accompanying concentration of hazardous material in the ash, greatly decreases the ultimate cost of waste disposal. Though usually not economically feasible, useful metals might be extracted from the ash as industrial products. For example, after harvest a plant may contain as much as 1% or more by weight of a particular metal. The ash of this plant would contain about 20% by weight of that metal, a higher metal content than is found in many ores. Unfortunately, the amounts of metals that might be obtained in this way are small compared to those obtained from traditional mining processes.

Phytoremediation is not a panacea. It can be a very slow process. Thus, it is not an acceptable option for sites that represent an immediate public health or environmental threat and require immediate cleanup. Plant roots also may not grow deep enough to access contaminants at many sites. Most plants used for phytoremediation send their roots to depths of 0.3–10 m. Some trees used in phytoremediation may send their roots somewhat deeper. However, it is improbable that plant roots, even under the best of circumstances, can contact all the soil within a contaminated zone. Thus, several cropping seasons may be required to phytoremediate any particular site. Furthermore, plants require a narrow range of growth temperatures. They also grow well only in the presence of favorable soil qualities (texture, water-holding capacity, pH, presence of oxygen, etc.). They must obtain enough water to support growth and metabolism, so irrigation may be required at dry sites. Plants also may be inefficient at taking up some pollutants, allowing contaminants to reach groundwater. All of these potentially negative factors must be considered before phytoremediation is chosen as a remedial option for any particular site.

Some plants proposed for use in phytoremediation (http://www.ecological-engineering.com/phytorem.html) are shown in Table 1.

White-Rot Fungi Fungi have been used for bioremediation (Bennett and Faison, 1997). White-rot fungi, as shown in Figure 4, are wood-degrading microorganisms that produce special oxidases, enzymes that help degrade the plant polymer ligninasa as well as a great variety of chemicals, including many environmental pollutants (Paszczynski and Crawford, 1995). Thus, these fungi have been examined extensively as potential bioremediation agents. The peroxidases (known as ligninases or manganese peroxidases) of the basidiomycete *Phanerochaete chrysosporium* are the most thoroughly investigated of the xenobiotic-compound-degrading fungal enzymes. These and other oxidative fungal enzymes like laccase appear to initiate degradation of many xenobiotic molecules, such as the chlorinated compounds (including PCBs; Novotny et al., 1997).

For application in the field, white-rot fungi are grown on a cellulosic substrate such as wood chips. Some commercial companies have developed specially formulated proprietary lignocellulosic (lignocellulose) materials for growing fungal mycelia. These colonized materials provide both a carrier for the organism and a carbon and energy source (cellulose) to support pollutant cometabolism. The carriers are mixed directly into a polluted soil, which then is tilled, watered, and managed to encourage the growth and lignin-degrading activity of the introduced

Table 1. Plants proposed for use in phytoremediation.

Plant	Comment
Alfalfa	Symbiotic with hydrocarbon-degrading bacteria
Arabidopsis	Genetically engineered to carry a bacterial gene that transforms mercury into a gaseous state
Bladder campion	Accumulates zinc and copper
Indian mustard greens (*Brassica juncea*)	Accumulates selenium, sulfur, lead, chromium, cadmium, nickel, zinc, and copper
Boxwood (Buxaceae)	Accumulates nickel
Compositae	Symbiotic with bacteria, accumulates cesium and strontium
Euphorbiaceae	Succulent plants that accumulate nickel
Tomato	Accumulates lead, zinc, and copper
Poplar	Used in the absorption of the pesticide atrazine and solvents like carbon tetrachloride
Alpine pennycress (*Thlaspi caerulescens*)	Accumulates zinc and cadmium

Fig. 4. The white-rot fungus *Fomitopis pinicola* (common name, red-banded conk). Photographed by Dr. Andrzej Paszczynski in the University of Idaho Experimental Forest, Moscow Mountain, Moscow, Idaho.

fungus. As the fungus consumes its lignocellulosic carrier, it simultaneously degrades the contaminants through the fortuitous activities of its oxidases. This technology is still experimental. The fungi used thus far can be inhibited by the contaminant concentrations found in some soils. With some contaminants degradation may be incomplete. Thus, more development work is required before white-rot fungi see significant use in the treatment of contaminated soil.

The focus of many recent studies has been the fungus *Phanerochaete chrysosporium*. For example, this fungus was shown to enhance the indigenous rate of mineralization of aromatic compounds (such as PAHs) in one soil system (Brodkorb and Legge, 1992). Among the P. chrysosporium genes implicated in pollutant degradation are two lignin peroxidase genes. The mRNA transcripts of these genes were detected directly in a soil being treated by fungal inoculation (Lamar et al., 1995). Thus, the use of white-rot fungi appears most promising for specific classes of contaminants including the PAHs (Brodkorb and Legge, 1992) and some chlorinated phenols (Paszczynski and Crawford, 1995). Additional research, however, is required before use of white-rot fungi becomes common practice. There remains the possibility of identifying many more strains of white-rot fungi that might be suitable for use as bioremediation agents. Hundreds of species of white-rot fungi have never been examined in this regard.

Limitations of Bioremediation

Bioremediation has the limitations inherent in any biological system. Thus, bioremediation is not always an appropriate approach for restoration of a contaminated site. The first requirement for use of bioremediation is that environmental conditions must support biological activity. For example, the extreme ranges of pH, temperature, or radioactivity seen at some sites generally are not tolerated by degradative organisms. Microorganisms require nitrogen, phosphorus, sulfur, and a number of trace elements. These must be present and in forms that are available to microbial cells. The presence of overly toxic concentrations of heavy metals like mercury, lead, and zinc or of other antimicrobial substances may inhibit or even prevent bioremediation processes. In other instances the concentrations of contaminants may be so low that they do not provide sufficient energy for microbial growth. If alternative energy sources are unavailable and cannot be added, then even cometabolism may not be an option. Contaminants may not be accessible to microorganisms. This might occur, for example, if they are tightly adsorbed to clays or soil organic matter or have found their way into pores that are smaller than bacteria. Even such non-bioavailable contaminants still may be subject to very stringent remediation standards set

and enforced by regulatory agencies. Contaminants more often than not occur in mixtures. Sometimes these are so complex that the most likely bioremediation processes may remove only some of the waste components. Those that remain still may be regulated. In other instances the site to be treated (e.g., a large, deep aquifer) may not be accessible to manipulations needed to promote bioremediation. Despite the amazing versatility of microorganisms for the degradation of xenobiotic molecules, some man-made compounds simply are not biodegradable. In summary, bioremediation is just one tool in the environmental engineer's repertoire. It will be used frequently, but not for all sites or situations.

Literature Cited

Admassu, W., and Korus, R. A. 1996. Engineering of bioremediation processes: Needs and limitations. R. L. Crawford and D. L. Crawford. Bioremediation: Principles and Applications. Cambridge University Press. Cambridge, UK. 13–34.

Arnold, M., Reittu, A., von Wright, A., Martikainen, P. J., and Suihko, M. L. 1997. Bacterial degradation of styrene in waste gases using a peat filter. Appl. Microbiol. Biotechnol. 48:738–744.

Barbeau, C., Deschenes, L., Karamanev, D., Comeau, Y., and Samson, R. 1997. Bioremediation of pentachlorophenol-contaminated soil by bioaugmentation using contaminated soil. Appl. Microbiol. Biotechnol. 48:745–752.

Barton, J. W., Klasson, K. T., Koran, L. T. Jr., and Davison, B. H. 1997. Microbial removal of alkanes from dilute gaseous waste streams: kinetics and mass transfer considerations. Biotechnol. Prog. 13:814–821.

Bennett, J. W., and Faison, B. D. 1997. Use of fungi in biodegradation. Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenback, L. D., and Walter, M. V. Manual of Environmental Microbiology. ASM Press. Washington, DC, 758–765.

Büyüksönmez, F., Hess, T. F., Crawford, R. L., Paszczynski, A., and Watts, R. A. 1998a. Effects of modified fenton reactions on Xanthobacter flavus FB71. Appl. Environ. Microbiol. 64(10):3759–3764..

Büyüksönmez, F., Hess, T. F., Crawford, R. L., Paszczynski, A., and Watts, R. A. 1998b. Simultaneous abiotic-biotic mineralization of perchlorethylene (PCE). Proceedings, First International Conference on the Remediation of Chlorinated and Recalcitrant Compounds, May 1998. Monterey, CA. Battelle Press. Columbus, OH. 277–282.

Bolton, H. Jr., and Gorby, Y. A. 1995. An overview of the bioremediation of inorganic contaminants. Hinchee, R. E., Means, J. L., and Burris, D. R. Bioremediation of Inorganics. Battelle Press. Columbus, OH. 1–16.

Bossert, I. D., and Compeau, G. C. 1995. Cleanup of petroleum hydrocarbon contamination in soil. Young, L. Y., and Cerniglia, C. E. Microbial Transformation and Degradation of Toxic Organic Chemicals. Wiley-Liss, Inc. New York, NY. 77–125.

Bowlen, G. F., and Kosson, D. S. 1995. In situ processes for bioremediation of BTEX and petroleum fuel products. Microbial Transformation and Degradation of Toxic Organic Chemicals. Wiley-Liss, Inc. New York, NY. 514–542.

Breitung, J., Bruns-Nagel, D., Steinbach, K., Kaminski, L., Gemsa, D., and von Low, E. 1996. Bioremediation of 2,4,6-trinitrotoluene-contaminated soils by two different aerated compost systems. Appl. Microbiol. Biotechnol. 44:795–800.

Brodkorb, T. S., and Legge, R. L. 1992. Enhanced biodegradation of phenanthrene in oil-tar-contaminated soils supplied with Phanerochaete chrysosporium. Appl. Environ. Microbiol. 58:3117–3121.

Brunsbach, F. R., and Reineke, W. 1994. Degradation of chlorobenzenes in soil slurry by a specialized organism. Appl. Microbiol. Biotechnol. 42:415–420.

Cornish, J. E., Goldberg, W. C., Levine, R. S., and Benemann, J. R. 1995. Phytoremediation of soils contaminated with toxic elements and radionuclides. Hinchee, R. E., Means, J. L., and Burris, D. R. Bioremediation of Inorganics. Battelle Press. Columbus, OH. 55–63.

Cunningham, S. D., Berti, W. R., and Huang, J. W. 1995. Remediation of contaminated soils and sludges by green plants. Hinchee, R. E., Means, J. L., and Burris, D. R. Bioremediation of Inorganics. Battelle Press. Columbus, OH. 33–54.

Deportes, I., Benoit-Guyod, J. L., and Zmirou, D. 1995. Hazard to man and the environment posed by the use of urban waste compost: a review. Sci. Total. Environ. 172:197–222.

Deshusses, M. A. 1997. Biological waste air treatment in biofilters. Curr. Opin. Biotechnol. 8:335–339.

Dupont, R. R. 1993. Fundamentals of bioventing applied to fuel-contaminated sites. Environ. Prog. 12:45–53.

Elsgaard, L. 1998. Ethylene removal by a biofilter with immobilized bacteria. Appl. Environ. Microbiol. 64:4168–4173.

Evans, B. S., Dudley, C. A., and Klasson, K. T. 1996. Sequential anaerobic-aerobic biodegradation of PCBs in soil slurry microcosms. Appl. Biochem. Biotechnol. 57/58:885–894.

Focht, D. D. 1997. Aerobic biotransformation of polychlorinated biphenyls. Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenback, L. D., and Walter, M. V. Manual of Environmental Microbiology. ASM Press. Washington, DC, 811–814.

Fogarty, A. M., and Tuovinen, O. H. 1991. Microbiological degradation of pesticides in yard waste composting. Microbiol. Rev. 55:225–233.

Frankenberger, W. T. Jr., Emerson, K. D., and Turner, D. W. 1989. In situ bioremediation of an underground diesel fuel spill: a case history. Environ. Manage. 13:325–332.

Frankenberger, W. T. Jr., and Karlson, U. 1991. Bioremediation of seleniferous soils. Hinchee, R. E., and Olfenbuttel, R. F. On-Site Bioreclamation: Processes for Xenobiotic and Hydrocarbon Treatment. Butterworth-Heinemann. Stoneham, MA. 239–254.

Funk, S. B., Crawford, D. L., Roberts, D. J., and R. L. Crawford. 1995a. Two-stage bioremediation of TNT-contaminated soils. Schepart, B. S. Bioremediation of Pollutants in Soil and Water, ASTM STP 125. American Society for Testing and Materials. Philadelphia, PA. 177–189.

Funk, S. B., Crawford, D. L., Crawford, R. L., Mead, G., and Davis-Hoover, W. 1995b. Full-scale anaerobic bioremediation of trinitrotoluene (TNT)-contaminated soil: a

U.S. EPA SITE program demonstration. Appl. Biochem. Biotechnol. 51/52:625–633.

Haber, F., and Weiss, J. 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. 147:332–351.

Hinchee, R. E., Downey, D. C., Dupont, R. R., Aggarwal, P., and Miller, P. 1991. Enhancing biodegradation of petroleum hydrocarbons through bioventing. J. Haz. Mat. 27:315–325.

Hinchee, R. E., and Ong, S. K. 1992. A rapid in situ respiration test for measuring aerobic biodegradation rates of hydrocarbons in soils. J. Air Waste Manage. Assoc. 42:1305–1312.

Hinchee, R. E., Wilson, J. T., and Downey, D. C. (editors). 1995. Intrinsic Bioremediation. Battelle Press. Columbus, OH.

Hopkins, G. D., Semprini, L., and McCarty, P. L. 1993. Microcosm and in situ field studies of enhanced biotransformation of TCE by phenol-utilizing microorganisms. Appl. Environ. Microbiol. 59:2277–2285.

Jarvis, S. A., McFarland, V. A., and Honeycutt, M. E. 1998. Assessment of the effectiveness of composting for the reduction of toxicity and mutagenicity of explosive-contaminated soil. Ecotoxicol. Environ. Safety 39:131–135.

Kaake, R. H., Roberts, D. J., Stevens, T. O., Crawford, R. L., and Crawford, D. L. 1992. Bioremediation of soils contaminated with 2-sec-butyl-4,6-dinitrophenol (dinoseb). Appl. Environ. Microbiol. 58:1683–1689.

Kao, C.-M., and Borden, R. C. 1994. Enhanced aerobic bioremediation of a gasoline-contaminated aquifer by oxygen-releasing barriers. Hinchee, R. E., Alleman, B. C., Hopple, R. E., and Miller, R. N. Hydrocarbon Bioremediation. Lewis Publishers. Boca Raton, FL. 262–266.

Kaplan, D. L. 1990. Biotransformation pathways of hazardous energetic organo-nitro compounds. Kamely, D., Chakrabarty, A., and Omenn, G. S. Biotechnology and Biodegradation. Portfolio Publishing Co.. Houston, TX. 152–182.

Keller, L., and Brunner, P. H. 1983. Waste-related cadmium cycle in Switzerland. Ecotoxicol. Environ. Safety 7:141–150.

Kelly, R. T., and Guerin, T. F. 1995. Feasibility of using hyperaccumulating plants to bioremediate metal-contaminated soil. Hinchee, R. E., Means, J. L., and Burris, D. R. Bioremediation of Inorganics. Battelle Press. Columbus, OH. 25–32.

Kidd, D. F. 1996. Fracturing. Nyer, E. In Situ Treatment Technology. Lewis Publishers. Boca Raton, FL. 245–269.

King, R. B. 1992. Practical Environmental Bioremediation. CRC Press. Boca Raton, FL.

Kner, M., Lotter, S., Heerenklage, J., Breuer-Jammali, M., Stegmann, R., and Mahro, B. 1995. Fate of 14C-labeled anthracene and hexadecane in compost-manured soil. Appl. Microbiol. Botechnol. 43:1128–1135.

Korda, A., Santas, P., Tenente, A., and Santas, R. 1997. Petroleum hydrocarbon remediation: sampling and analytical techniques, in situ treatments, and commercial microorganisms currently used. Appl. Microbiol. Biotechnol. 48:677–686.

Korus, R. A. 1997. Scale-up of processes for bioremediation. Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenback, L. D., and Walter, M. V. Manual of Environmental Microbiology. ASM Press. Washington, DC, 856–864.

Kramer, J. H., and Cullen. 1997. Bioventilation and modeling of airflow in soil. Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenback, L. D., and Walter, M. V. Manual of Environmental Microbiology. ASM Press. Washington, DC, 746–752.

LaGrega, M. D., Bunkingham, P. L., and Evans, J. C. 1994. Hazardous Waste Management. McGraw Hill. New York, NY.

Lamar, R. T., Schoenike, B., Vanden Wymelenberg, A., Stewart, P., Dietrich, D. M., and Cullen, D. 1995. Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to Phanerochaete chrysosporium-colonized soil. Appl. Environ. Microbiol. 61:2122–2126.

Leson, G., and Winer, A. M. 1991. Biofiltration: an innovative air pollution control technology for VOC emissions. J. Air Waste Manage. Assoc. 41:1045–1054.

Marsh, P. B., Millner, P. D., and Kla, J. M. 1979. A guide to the recent literature on aspergillosis as caused by Aspergillus fumigatus, a fungus frequently found in self-heating organic matter. Mycopathologica 69:67–81.

Marsman, E. H., Appleman, J. M. M., Urlings, L. G., and Bult, B. A. 1994. Biopur, an innovative bioreactor for the treatment of groundwater and soil vapor contaminated with xenobiotics. Hinchee, R. E., Anderson, D. B., and Metting, F. B., Jr. Applied Biotechnology for Site Remediation. Lewis Publishers. Boca Raton, FL. 391–399.

Matteau, Y., and Ramsay, B. 1997. Active compost biofiltration of toluene. Biodegradation 8:135–140.

McMillin, S. J., Kerr, J. M., and Gray, N. R. 1993. Microcosm studies of factors that influence bioremediation of crude oil in soil. Proceedings, SPE/EPA Exploration and Production Environmental Conference, San Antonio, TX, March 7-10, 1993. Society of Petroleum Engineers. Dallas, TX. 389–401.

Michel, F. C., Jr., Reddy, C. A., and Forney, L. J. 1995. Microbial degradation and humification of the lawn care pesticide 2,4- dichlorophenoxyacetic acid during the composting of yard trimmings. Appl. Environ. Microbiol. 61:2566–2571.

Millner, P. D., Marsh, P. B., Snowden, R. B., and Parr, J. F. 1977. Occurrence of Aspergillus fumigatus during composting of sewage sludge. Appl. Environ. Microbiol. 34:765–772.

Mueller, J. G., Lanz, S. E., Baltmann, B. O., and Chapman, P. J. 1991. Bench-scale evaluation of alternative biological treatment processes for the remediation of pentachlorophenol- and creosote-contaminated materials: slurry-phase bioremediation. Environ. Sci. Technol. 25:1055–1061.

Novotny, C., Vyas, B. R., Erbanova, P., Kubatova, A., and Sasek, V. 1997. Removal of PCBs by various white-rot fungi in liquid cultures. Folia Microbiol. (Praha) 42:136–140.

Nyer, K. E. 1992. Groundwater Treatment Technology. Van Nostrand Reinhold. New York, NY.

Oh, Y. S., and Bartha, R. 1994. Design and performance of a trickling air biofilter for chlorobenzene and o-dichlorobenzene vapors. Appl. Environ. Microbiol. 60:2717–2722.

Ottengraf, S. P. P. 1986. Exhaust gas purification. Rehm, H. J., and Reed, G. Biotechnology 8:301–322.

Palmer, W. G., Beaman, J. R., Walters, D. M., and Creasia, D. A. 1997. Bioavailability of TNT residues in composts

of TNT-contaminated soil. J. Toxicol. Environ. Health 51:97–108.

Paszczynski, A., and Crawford, R. L. 1995. Potential for bioremediation of xenobiotic compounds by the white-rot fungus Phanerochaete chrysosporium. Biotechnol. Prog. 11:368–379.

Pol, A., Op den Camp, H. J., Mees, S. G., Kersten, M. A., and van der Drift, C. 1994. Isolation of a demethylsulfide-utilizing Hyphomicrobium species and its application in biofiltration of polluted air. Biodegradation 5:1–5–112.

Raymond, R. L., Jamison, V. W., and Hudson, J. O. 1975. Beneficial Stimulation of Bacterial Activity in Groundwater Containing Petroleum Products. API Publication 4427. American Petroleum Institute. Washington, DC,

Rifai, H. S., Borden, R. C., Wilson, J. T., and Ward, C. W. 1995. Intrinsic bioattenuation for subsurface restoration. Hinchee, R. E., Wilson, J. T., and Downey, D. C. Intrinsic Bioremediation. Battelle Press. Columbus, OH. 1–29.

Sayles, G. D., and Suidan, M. T. 1993. Biological treatment of industrial and hazardous wastewater. Levin, M. A., and Gealt, M. A. Biotreatment of Industrial and Hazardous Wastes. McGraw Hill. New York , NY. 245–268.

Schwab, A. P., and Banks, M. K. 1994. Biologically mediated dissipation of polyaromatic hydrocarbon in the root zone. Anderson, T. A., and Coats, J. R. Bioremediation through Rhizosphere Technology. ACS Symposium Series 563. American Chemical Society. Washington, DC, 132–141.

Shuttleworth, K. L., and Cerniglia, C. E. 1997. Practical methods for the isolation of polycyclic aromatic hydrocarbon (PAH)-degrading microorganisms and the determination of PAH mineralization and biodegradation intermediates. Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenback, L. D., and Walter, M. V. Manual of Environmental Microbiology. ASM Press. Washington, DC, 766–775.

Tyre, B. W., Watts, R. J., and G. C. Miller. 1991. Treatment of four biorefractory contaminants in soils using catalyzed hydrogen peroxide. J. Environ. Qual. 20:832–838.

Unterman, R., Folsom, B. R., and Togna, A. P. 1996. Mechanically mixed packed bed reactor. U.S. patent 5,494,574. U.S. Patent Office. Washington, DC.

Valo, R., and Salkinoja-Salonen, M. 1986. Bioreclamation of chlorophenol-contaminated soil by composting. Appl. Microbiol. Biotechnol. 25:68–75.

van der Meer, J. R., Werlen, C., Nishino, S., and Spain, J. C. 1998. Evollution of a pathway for chlorobenzene metabolism lead to natural attenuation in contaminated groundwater. Appl. Environ. Microbiol. 64:4185–4193.

Voice, T. C., Zhao, X., Shi, J., and Hickey, R. F. 1995. Biological activated carbon fluidized-bed system to treat gasoline-contaminated groundwater. Hinchee, R. E., Sayles, G. D., and Skeen, R. S. Biological Unit Processes for Hazardous Waste Treatment. Battelle Press. Columbus, OH . 29–36.

Wackett, L. P. 1997. Biodegradation of halogenated solvents. Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenback, L. D., and Walter, M. V. Manual of Environmental Microbiology. ASM Press. Washington, DC, 784–789.

Walter, M. V. 1997. Bioaugmentation. Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenback, L. D., and Walter, M. V. Manual of Environmental Microbiology. ASM Press. Washington, DC, 753–757.

Wiedermeier, T. H., Swanson, M. A., Wilson, J. T., Kampbell, D. H., Miller, R. N., and Hansen, J. E. 1995. Patterns of intrinsic bioremediation at two U.S. Air Force bases. Hinchee, R. E., Wilson, J. T., and Downey, D. C. Intrinsic Bioremediation. Battelle Press. Columbus, OH . 31–51.

Williams, R. T., Ziegenfuss, P. S., and Sick, W. E. 1992. Composting of explosives and propellant-contaminated soils under thermophilic and mesophilic conditions. J. Indust. Microbiol. 9:137–144.

Wu, W. M., Bhatnagar, L., and Zeikus, J. G. 1993. Performance of anaerobic granules for degradation of pentachlorophenol. Appl. Environ. Microbiol. 59:389–397.

Yudelson, J. M., and Tinari, P. D. 1995. Economics of biofiltration for remediation projects. Hinchee, R. E., Sayles, G. D., and Skeen, R. S. Biological Unit Processes for Hazardous Waste Treatment. Battelle Press. Columbus, OH. 205–209.

Additional References for Specific Topics

Biofiltration

Alonso, C., P. J. Smith, M. T. Suidan, G. A. Sorial, P. Biswas, F. L. Smith, and R. C. Brenner. 1995. Gas treatment in trickle-bed biofilters: a modeling approach and experimental study. Proceedings of the Water Environmental Federation 68th Annual Conference and Exposition, Miami Beach, FL, October 21-25.

Alonso, C., M. T. Suidan, B. R. Kim, and B. J. Kim. 1996. Dynamical mathematical model for the biodegradation of voc's in a biofilter: biomass accumulation study. Proceedings of the Water Environmental Federation 69th Annual Conference and Exposition, Dallas, TX, October 5-9.

Alonso, C., M. T. Suidan, G. A. Sorial, F. L. Smith, P. Biswas, P. J. Smith, and R. C. Brenner. 1997. Gas treatment in trickle-bed biofilters: biomass, how much is enough? Biotechnol. Bioeng. 54: 583-549.

Alonso, C., X. Zhu, M. T. Suidan, B. R. Kim, and B. J. Kim. 1997a. Effect of nitrate on VOC removal in a gas phase biofilter. Proceedings of the Water Environmental Federation 70th Annual Conference and Exposition, Chicago, IL.

Alonso, C., X. Zhu, M. T. Suidan, B. R. Kim, and B. J. Kim. 1997b. Nutrients and electron acceptors effect in a vapor phase bioreactor. Proceedings of the In Situ and On-Site Bioremediation, 4th International Symposium, New Orleans, LA.

Apel, W. A., J. M. Barnes, and K. B. Barrett. Biofiltration of nitrogen oxides from fuel combustion gas streams. Proceedings of the 88th Annual Meeting of the Air and Waste Management Association, Paper 95-TP9C.04, San Antonio, TX, June 1995.

Apel, W. A., W. D. Kant, F. S. Colwell, B. Singleton, B. D. Lee, G. E Andrews, A. M. Espinosa, and E. G. Johnson. 1994. Removal of gasoline vapors from air streams by biofiltration. In, Emerging Technologies in Hazardous Waste Management, IV, D. W Tedder and F. G. Pohland (eds.), Am. Chem. Soc., 9, pp. 142-159.

Apel, W. A., B. D. Lee, M. R. Walton, L. L. Cook, and K. B. Dinerstein. 1995. Removal of ?-pinene from off-gases by biofiltration. Proceedings of the 88th Annual Meeting of

the Air and Waste Management Association. Paper No. 95-TP9C.07, San Antonio, TX, June 1995.

Apel, W. A. and C. E. Turick. 1993. The use of denitrifying bacteria for the removal of nitrogen oxides from combustion gases. Fuel 72(12): 1715-1718.

Apel, W. A., M. R. Walton, and P. R. Dugan. 1994. An evaluation of autotrophic microbes for the removal of carbon dioxide from combustion gas streams. Fuel Processing Technol. 40: 139-149.

Barnes, J. M., W. A. Apel, and K. B. Barrett. 1995. Removal of nitrogen oxides from gas streams using biofiltration. J. Haz. Mat. 41:315-326.

Bohn, H. 1992. Consider biofiltration for decontaminating gases. Chem. Eng. Prog. 88(4): 34–40.

Devinny, J. S., M. A. Deshusses and T. S. Webster. 1998. Biofiltration for Air Pollution Control. CRC Lewis Publishers, Boca Raton, FL. 320 pp.

Lee, B. D., W. A. Apel, J. M. Barnes, M. R. Walton, K. B. Barrett, and L. L. Cook. 1995. Biofiltration using different modes of biodegradation. In, Proceedings of the 1995 Conference on Biofiltration, University of Southern California, Los Angeles, CA, October 1995, pp. 309-316.

Lee, B. D., W. A. Apel, L. L. Cook, and K. M. Nichols. 1996. Effect of bed medium moisture on alpha-pinene removal by biofilters. Proceedings of the 1996 Conference on Biofiltration. University of Southern California, Los Angeles, CA. October 1996.

Lee, B. D., W. A. Apel, and M. R. Walton. 1997. Utilization of toxic gases and vapors as alternate electron acceptors in biofilters. Proceedings of the 90th Annual Meeting of the Air and Waste Management Association. Paper 97-WA71A.05, Toronto, Canada, June 1997.

Lee, B. D., W. A. Apel, M. R. Walton, and L. L. Cook. 1996. Treatment of methanol contaminated air streams using biofiltration. Proceedings of the 89th Annual Meeting of the Air and Waste Management Association. Paper 96-RP87C.03. Nashville, TN. June 1996.

Smith, F. L., Sorial, G. A., M. T. Suidan, A. W. Breen, P. Biswas, and R. C. Brenner. 1996. Development of two biomass control strategies for extended, stable operation of highly efficient biofilters with high toluene loadings. Environ. Sci. Technol. 30: 1744-1751.

Sorial, G. A., F. L. Smith, M. T. Suidan, and P. Biswas. 1995. Evaluation of trickle bed biofilter media for toluene removal. J. Air Waste Manage. Assoc. 45: 801-810.

Zhu, X, M. J. Rihn, M. T. Suidan, B. J. Kim, and B. R. Kim. 1996. The effect of nitrate on voc removal in trickle bed biofilters. Wat. Sci. Technol. 34, 3-4: 573-581.

Zhu, X, M. J. Rihn, M. T. Suidan, B. J. Kim, B. R. Kim, and Paik, B. C. 1996. Role of nitrate in trickle bed biofilters: nutrient or electron acceptor. Proceedings of the Water Environmental Federation 69th Annual Conference and Exposition, Dallas, TX, October 5-9.

Bioventing

Hinchee, R. E., S. K. Ong, and R. Hoeppel, 1991. A treatability test for bioventing. In, Proceedings of the 84th Annual Meeting and Exhibition, Air and Waste Management Association, Vancouver, BC, 91-19.4.

Hinchee, R. E., S. K. Ong, R. N. Miller, and D.C. Downey, 1992. Report to AFCEE, Brooks AFB, TX.

Hinchee, R. E., 1993. Bioventing of petroleum hydrocarbons. In, Handbook of Bioremediation. Lewis Publication, Boca Raton, FL, pp. 39-59.

Hoeppel, R. E., R. E. Hinchee, and M. F. Arthur, 1991. Bioventing soils contaminated with petroleum hydrocarbons. J. Indust. Microbiol. 8:141-146.

Leeson, A., and Hinchee, R. E. 1996. Principles and Practices of Bioventing, vol. 2: Bioventing Principles. Prepared by Battelle Memorial Institute for U.S. Air Force and U.S. EPA.

Leeson, A., and Hinchee, R. E. 1996. Principles and Practices of Bioventing, vol. 2: Bioventing Design. Prepared by Battelle Memorial Institute for U.S. Air Force and U.S. EPA.

Norris, R. D., Hinchee, R. E., Brown, R. A., McCarty, P. L., Semprini, L., Wilson, J. T., Kampbell, D. H., Reinhard, M., Bower, E. J., Borden, R. C., Vogel, T. M., Thomas, J. M., and C. H. Ward. 1994. Handbook of Bioremediation. Boca Raton, FL: CRC Press.

USAEC, 1997. Bioventing of POL contaminated soils. In, Innovative Technology Demonstration, Evaluation and Transfer Activities, FY 96 Annual Report, Report No. SFIM-AEC-ET-CR-97013, pp. 75-76.

U.S. Environmental Protection Agency (EPA). 1991a. Guide for Conducting Treatability Studies Under CERCLA: Aerobic Biodegradation Remedy Screening. Washington, DC: Office of Emergency and Remedial Response. EPA/540/2-91/013A.

U.S. Environmental Protection Agency (EPA). 1991b. Soil Vapor Extraction Technology: Reference Handbook. Cincinnati, OH: Office of Research and Development. EPA/540/2-91/003.

U.S. Environmental Protection Agency (EPA). 1991c. Guide for Conducting Treatability Studies Under CERCLA: Soil Vapor Extraction. Washington, DC: Office of Emergency and Remedial Response. EPA/540/2-91/019A.

U.S. Environmental Protection Agency (EPA). 1993. Decision-Support Software for Soil Vapor Extraction Technology Application: HyperVentilate. Cincinnati, OH: Office of Research and Development. EPA/600/R-93/028.

Wisconsin Department of Natural Resources (DNR). 1993. Guidance for Design, Installation and Operation of Soil Venting Systems. Madison, WI: Emergency and Remedial Response Section. PUBL-SW185-93.

Phytoremediation

The Phytoremediation Bibliography (http://www.rtdf.org/phytodoc.htm): >1,000 citations on phytoremediation and related topics.

Prokaryotes (2006) 1:864–903
DOI: 10.1007/0-387-30741-9_27

CHAPTER 3.9

Biodeterioration

JI-DONG GU AND RALPH MITCHELL

Introduction

Bacteria were the first life-forms to appear on Earth and they have a long history of association with mineral surfaces. Surfaces of clay minerals played an important role in the initial evolutionary processes of bacteria and the diversification of their physiology (Kluyver and van Niel, 1956; Mayr, 1998; Wächtershäuser, 1988; Woese, 1987; Woese, 1990; Woese, 1998; Woese and Olsen, 1986). Bacteria also adapt themselves to a mode of living on physical surfaces and at interfaces (Angles et al., 1993; Bitton, 1980; Brune et al., 2000; Caldwell et al., 1997; Fenchel and Finlay, 1995; Fletcher, 1980; Glagolev, 1984; Hugenholtz et al., 1998; Margulis, 1981; Marshall, 1992; Moat and Foster, 1988; Pace, 1997; Wolfaardt et al., 1994; Zavarzin et al., 1994; Zinder, 1993). In addition, they are capable of degrading a wide range of pollutants (Gibson, 1984; Gu and Berry, 1991; Gu and Berry, 1992a; Gu et al., 1992b; Young and Cerniglia, 1995). It is well recognized that degradation of organic chemicals and nutrient cycling are more rapid on surfaces. Unfortunately, microbial association with surfaces also has a potential negative economic impact, when it accelerates degradation and deterioration of a wide range of materials, including inorganic minerals (Gu et al., 2000b; Mitchell and Gu, 2000), concrete and stone (Gebers and Hirsch, 1978; Gu et al., 2000b; Moosavi et al., 1986; Padival et al., 1995; Piervittori et al., 1994; Prieto et al., 1995); metals (Ford and Mitchell, 1990b; Gu et al., 2000a; Miller, 1970); and natural and synthetic polymers (Gu et al., 2000d; Guezennec et al., 1998; Swift et al., 1979). In all cases, the essential ingredient is the close association between the microflora and the material surface.

Under natural conditions, all surfaces are covered with microorganisms except for extremely clean rooms. Biofilm formation is the process by which a complex community of microorganisms becomes established on a surface. Biofilms can exist in many different forms and have many different compositions (Fig. 1). They are ubiquitous on surfaces in soil and aquatic environments (Ford, 1993) and are also present on materials exposed to humidity, particularly in tropical and subtropical climates. For example, microbial biofilms are common on surfaces of ancient archaeological limestone in Southern Mexico (Fig. 2). This process of biofilm formation is prerequisite to substantial corrosion and/or deterioration of the underlying materials (Arino et al., 1997; Saiz-Jimenez, 1995; Saiz-Jimenez, 1997; Walch, 1992).

Biofilms on surfaces are highly structured (Breznak, 1984; Costerton et al., 1978; Costerton et al., 1994; Dalton et al., 1994; Davey and O'Toole, 2000; Kelly-Wintenberg and Montie, 1994; Lappin-Scott et al., 1992; L'Hostis et al., 1997; O'Toole et al., 2000; Wimpenny and Colasanti, 1997). Architectural structure and the organization of microorganisms on a particular surface are generally material specific, dependent on surface properties (Fletcher and Leob, 1979; van Loosdrecht et al., 1987; van Loosdrecht et al., 1990; Wiencek and Fletcher, 1995) and ambient conditions, including externally supplied electrical current, cation concentration, solution chemistry and hydrodynamic conditions (Caldwell and Lawrence, 1986; Korber et al., 1989; Lawrence et al., 1987; Lewandowski et al., 1995; Leyden and Basiulis, 1989; Little et al., 1986b; Marshall et al., 1971; Martrhamuthu et al., 1995; Neu, 1996; Pendyala et al., 1996; Power and Marshall, 1988; Rijnaarts et al., 1993; Schmidt, 1997; Sneider et al., 1994; Stoodley et al., 1997). All surfaces may act as substrata for bacterial adhesion and biofilm formation (Busscher et al., 1990; Costerton et al., 1995; Geesey and White, 1990; Geesey et al., 1996; Marshall, 1980), and microbial attack on materials can take place either directly or indirectly, depending on the specific microorganism and on the biology, chemistry and physical properties of the materials and their environment (Gu et al., 2000c). Specifically, these factors may include material composition (Bos et al., 1999; Busscher et al., 1990; Wiencek and Fletcher, 1995), the nature of the surface (Becker et al., 1994; Caldwell et al., 1997; Callow and Fletcher, 1994), and the indigenous microflora. In addition, other factors affecting the physical environ-

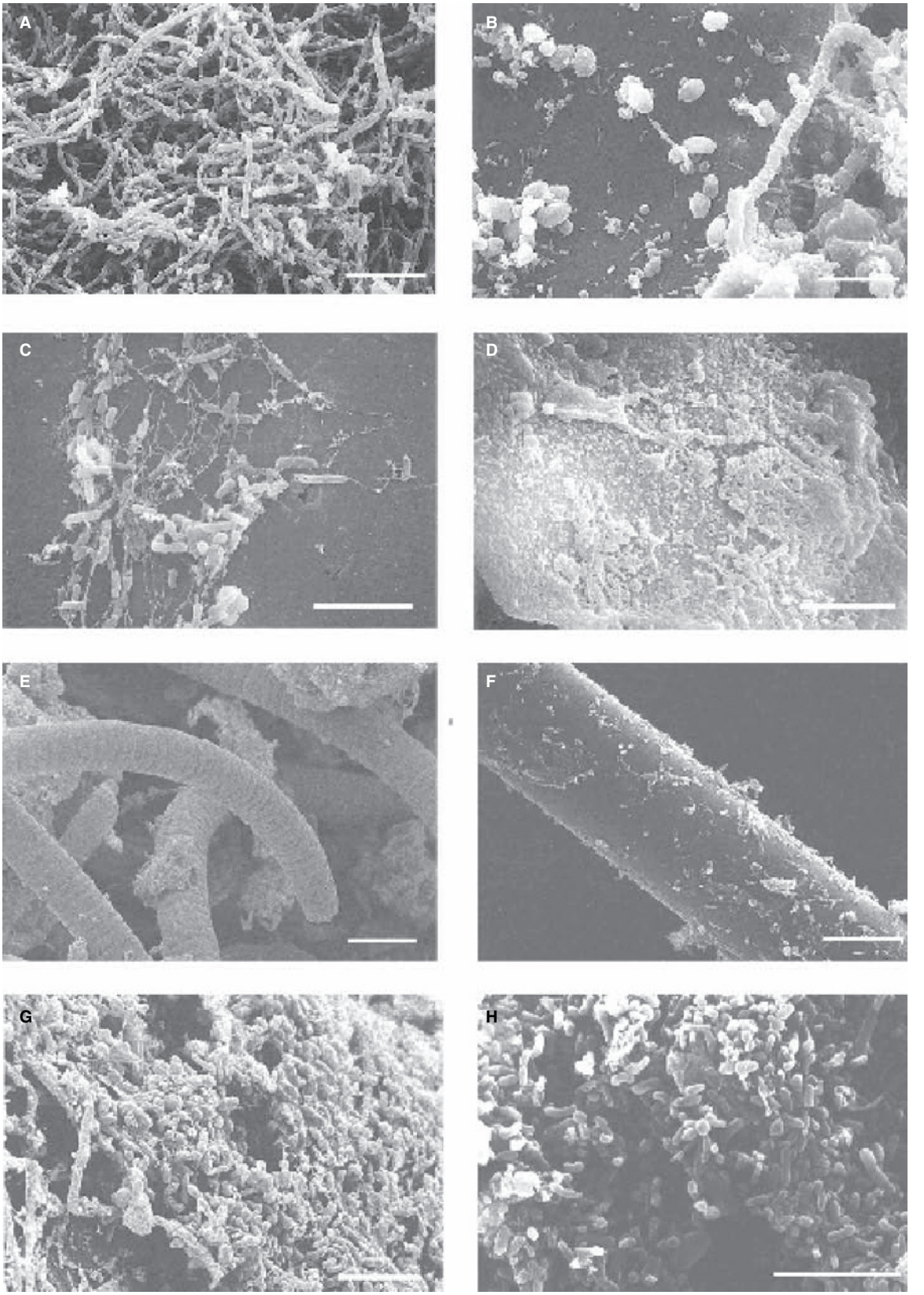

Fig. 1. Scanning electron micrographs of biofilms on A) a stainless steel coupon after a one-month incubation in the Merrimack River, Massachusetts, United States (bar = 10 μm), B) contaminated air-conditioner condenser (bar = 10 μm), C) urinary tract catheter after use (bar = 5 μm), D) dental suture after 3 days in a patient's mouth (bar = 5 μm), E) phototrophic organisms on stone surfaces from a tropical region (bar = 5 μm), F) fiber (from a bioreactor) used for immobilization of pollutant-degrading microorganisms (bar = 10 μm), G) an artificial carrier of fiber after immersion for 20 days in a wastewater treatment plant (bar = 5 μm), and H) surface of a sewer's concrete pipe (bar = 5 μm).

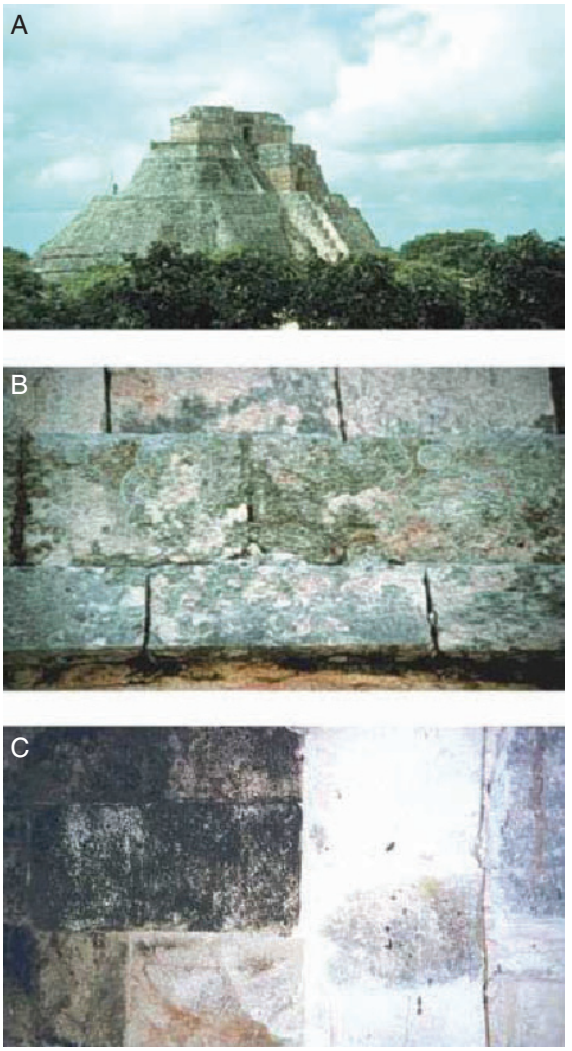

Fig. 2. Photographs showing A) an ancient Mayan temple made of limestone covered by microbial biofilms, B) a close-up view near the base, and C) the contrast between newly replaced and original stone.

ment influence the extent of bacterial adhesion, including ionic strength of the solution, type of cation, hydrodynamic force, and surface properties (e.g., hydrophobicity or hydrophilicity; Bos et al., 1999; Fletcher, 1996; Marshall, 1976). Recently, chemical signaling has been found to play a role in bacterial attachment on surfaces (Davies et al., 1998; McLean et al., 1997; Reynolds and Fink, 2001).

Microbial biofilms immobilized on solid materials are exploited in pollutant biodegradation, wastewater treatment and bioleaching (Bryers, 1990a; Bryers, 1994; Osswald et al., 1995; Sharp et al., 1998). In contrast, biofilms are undesirable in food processing, energy production and on submerged mechanical equipment surfaces. Biofouling is generally defined as the undesirable accumulation of microorganisms, their products, deposits such as minerals and organic matter, and macroorganisms on a substratum surface (Novikova and Zalogoyev, 1985; Nefedov et al., 1988; Solomin, 1985; Sunesson et al., 1995; Viktorov, 1994; Viktorov and Novikova, 1985; Viktorov and Ilyin, 1992; Viktorov et al., 1993; Zaloguyev, 1985). The thin film on fouled surfaces usually consists of microorganisms embedded in an organic matrix of biopolymers, which are produced by the microorganisms under natural conditions. In addition, microbial precipitates, minerals, and corrosion products may be present (Beveridge et al., 1997; Konhauser et al., 1994; Liken, 1981; Lovley, 1991; Pierson and Parenteau, 2000; Zehnder and Stumm, 1988). Industrial fouling is a complex phenomenon involving interactions between chemical, biological and physical processes. Materials immersed in aqueous environments or under high humidity conditions are susceptible to biofouling (Characklis, 1990; Gu et al., 1998c; Jones-Meehan et al., 1994a; Jones-Meehan et al., 1994b; Knyazev et al., 1986; Little et al., 1990; Thorp et al., 1994). These include medical implants (Dobbins et al., 1989; Gu et al., 2001b; McLean et al., 1995; Mittelman, 1996), water pipes (Rogers et al., 1994), artificial coatings (Edwards et al., 1994; Gu et al., 1998b; Jones-Meehan et al., 1994b; Stern and Howard, 2000; Thorp et al., 1997), rubber (Berekaa et al., 2000), ultrapure systems (Flemming et al., 1994; Mittelman, 1995a), porous media (Bouwer, 1992; Cunningham et al., 1990; Cunningham et al., 1991; Mills and Powelson, 1996; Rittman, 1993; Vandevivere, 1995; Vandevivere and Kirchman, 1993; Williams and Fletcher, 1996), water and wastewater treatment equipment (Bryers and Characklis, 1990b; Gillis and Gillis, 1996; Rethke, 1994; Tall et al., 1995), oilfield water systems (Lynch and Edyvean, 1988), equipment on the space station (Gazenko et al., 1990; Meshkov, 1994; Novikova et al., 1986; Pierson and Mishra, 1992; Stranger-Joannesen et al., 1993; Zaloguyev, 1985) and magnetic diskettes (McCain and Mirocha, 1995).

In general, biodeterioration is described as the undesirable degradation of materials by microorganisms. The term "biodeterioration" also implicitly includes biocorrosion and biodegradation. All three terms, "corrosion," "degradation" and "deterioration," will be used in this review. In the following sections, microbial deterioration of metals, polymeric materials, concrete and stone will be discussed.

Corrosion of Metals

Biodeterioration of materials includes the corrosion of metals, a process commonly called

"microbiological induced/influenced corrosion" (MIC) by corrosion engineers. This process affects a wide range of industrial materials, including those in oil fields, offshore drilling platforms, pipelines, pulp and paper factories, armaments, nuclear and fossil fuel power plants, chemical manufacturing facilities, and food processing plants (Corbett et al., 1987; Evans, 1948; Hill et al., 1987; Kobrin, 1993; Pope et al., 1989; Sequeira and Tiller, 1988; Widdel, 1992; Zachary et al., 1980). Significant economic loss has resulted from undesirable processes caused by the growth of microorganisms and subsequent accumulation of fouling organisms (Jensen, 1992; Ross, 1994). Corrosion has severe economic consequences. It was estimated that 70% of the corrosion in gas transmission lines is due to problems caused by microorganisms. The American oil refining industry loses $1.4 billion a year from microbial corrosion (Knudsen, 1981). The terminology of microbiological corrosion and the term "microfouling" have frequently been used interchangeably. The term "MIC" is not clearly defined and is commonly misused.

Biocorrosion of metals was first reported by von Wolzogen Kuhr and van der Vlugt (1934) more than sixty years ago. A wide variety of microorganisms are capable of degradation of metal alloys: the causative microorganisms include both aerobic and anaerobic bacteria. In the early studies, most attention was given to the strictly anaerobic sulfate-reducing bacteria (SRB; see the reviews by Dexter, 1993; Dowling and Guezennec, 1997; Dowling et al., 1992; Eashwar et al., 1995; Evans, 1948; Ford and Mitchell, 1990b; Ford and Mitchell, 1990a; Gu et al., 2000a; Hamilton, 1985; Lee et al., 1995). In addition to SRBs, thermophilic bacteria, iron-oxidizing bacteria, and exopolymer- and acid-producing bacteria were found to participate actively in corrosion processes by mechanisms in which metal ions are either transformed by or complexed with functional groups of the exopolymers, resulting in release of metallic species into solution (Chen et al., 1995; Chen et al., 1996c; Clayton et al., 1994; Ford and Mitchell, 1990b; Little et al., 1986b; Paradies, 1995; Schmidtt, 1986; Siedlarek et al., 1994).

Corrosion of metals is a result of both electrochemical and biological processes occurring on or near material surfaces, initiated and accelerated by microbial activity (the formation of complex microbial biofilms and metabolism of microorganisms on surfaces; Breslin et al., 1997; Ford and Mitchell, 1990b; Gu et al., 1998a; Gu et al., 1998c; Walch, 1992). In the process, surface-attached microorganisms alter the chemical and biological environments on the substratum. The microbial communities, in turn, form differential aeration zones under aerobic conditions because dissolved oxygen is consumed beneath microbial colonies (Uhlig and Revie, 1985).

The difference in oxygen concentrations on two localized and adjacent areas generates an electrochemical potential and electron flow (Fig. 3). The area more exposed to oxygen serves as a cathode, whereas the area underneath the microbial biofilm serves as an anode, resulting in dissolution of metallic matrices, crevice corrosion and pitting (Ford and Mitchell, 1990b; Gu et al., 2000a; Vaidya et al., 1997; Videla, 1996; Walch et al., 1989; Wang, 1996). Subsequently, the decrease in oxygen levels provides an opportunity for anaerobic microorganisms to become established within biofilms. In particular, activity of anaerobic sulfate-reducing bacteria has been shown to directly cause corrosion of underlying metals by a process of cathodic depolarization. In addition, methanogenic microorganisms may participate in the corrosion process (Daniels et al., 1987; Kim et al., 1996). Overall, the interactions between chemistry and biology create unique niches for the propagation of corrosion. Methods used to study biocorrosion are available in the literature (Hungate, 1969; Murray et al., 1993; Peng and Park, 1994; Tatnall, 1986; Wagner and Ray, 1994). The participating microorganisms and their corrosive processes under different environmental conditions are discussed below.

Microorganisms Involved in Corrosion

Aerobic Microorganisms Several groups of aerobic microorganisms play an important role in corrosion, including the sulfur bacteria, the iron- and manganese-depositing and exopolymer-producing bacteria, and fungi and algae. Figure 4 shows bacteria and corrosion products on the surface of a stainless steel cold water pipe. The "iron bacteria" include *Sphaerotilus*, *Leptothrix*, *Gallionella* and *Siderocapsa* (Ehrlich, 1996). Ghiorse and Hirsch (Ghiorse and Hirsch, 1978; Ghiorse and Hirsch, 1979) also observed that two *Pedomicrobium*-like budding bacteria deposit Fe and Mn ions on their cell walls. Most

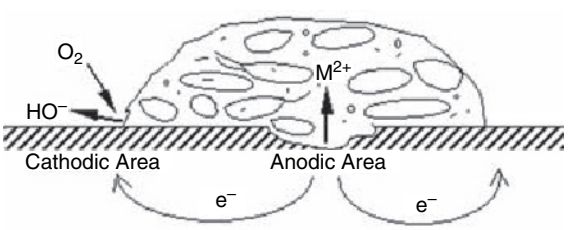

Fig. 3. Schematic of an aeration zone created by a biofilm, with resultant corrosion. Metallic cations (M^{2+}) are released from the anodic area.

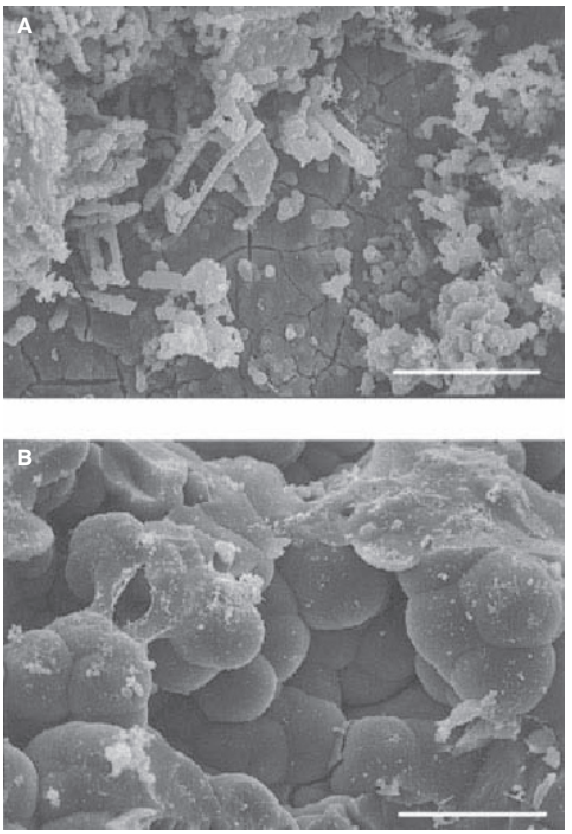

Fig. 4. Scanning electron micrographs of A) an aerobic microbial biofilm on a stainless steel surface (bar = 10 μm) and (b) the associated corrosion products (bar = 5 μm).

of these bacteria are difficult to culture under laboratory conditions (Hanert, 1981). Elucidation of their impact on corrosion is still limited by availability of culturing techniques. At neutral pH, Fe^{2+} is not stable in the presence of O_2 and is rapidly oxidized to the insoluble Fe^{3+} state. In fully aerated freshwater at pH 7, the half-life of Fe^{2+} oxidation is less than 15 minutes (Ghiorse, 1989; Stumm and Morgan, 1996). Because of the rapidity of this reaction, the only neutral pH environments where Fe^{2+} is present are interfaces between anoxic and oxic conditions.

Improved microbiological techniques permit the isolation of new Fe^{2+}-oxidizing bacteria under microaerophilic conditions at neutral pH (Emerson and Moyer, 1997). Ferric oxides may be enzymatically deposited by *Gallionella ferruginea* and nonenzymatically deposited by *Leptothrix* sp., *Siderocapsa*, *Naumanniella*, *Ochrobium*, *Siderococcus*, *Pedomicrobium*, *Herpetosyphon*, *Seliberia*, *Toxothrix*, *Acinetobacter* and *Archangium* (Ehrlich, 1996; Ghiorse and Hirsch, 1978). Iron-depositing bacteria include *Acholeplasma*, *Actinomyces* spp., *Arthrobacter*, *Caulococcus*, *Clonothrix*, *Crenothrix*, *Ferrobacillus*, *Gallionella*, *Hypomicrobium*, *Leptospirillum*, *Leptothrix*, *Lieskeela*, *Metallogenium*, *Naumaniella*, *Ochrobium*, *Peloploca*, *Pedomicrobium*, *Planctomyces*, *Seliberia*, *Siderococcus*, *Sphaeotilus*, *Sulfolobus*, *Thiobacillus*, *Thiopedia* and *Toxothrix* (Ford and Mitchell, 1990b). However, questions remain as to the extent of microbial involvement in specific processes of corrosion involving iron oxidation. Further investigation requires integrated approaches including microbiology, materials science, and electrochemistry.

Microorganisms in the genus *Thiobacillus* are also responsible for oxidative corrosion. Because they oxidize sulfur compounds to sulfuric acid, the acid surrounding the cells may attack alloys. Similarly, organic acid-producing microorganisms, including bacteria and fungi, are of concern. A number of acid-tolerant microorganisms are capable of Fe^0 oxidation. *Thiobacillus* is the most common. *Thiobacillus ferrooxidans* oxidizes Fe^{2+} to Fe^{3+}. However, the product limits growth of the organisms (Ehrlich, 1996; Kuenen and Tuovinen, 1981). Sulfate (SO_4^{2-}) is required by the Fe-oxidizing system in *T. ferrooxidans*. The role of sulfur is probably to stabilize the hexa-aquated complex of Fe^{2+} as a substrate for the Fe-oxidizing enzyme system, with the Fe^{2+} being oxidized at the surface of the bacterium. The electrons removed from Fe^{2+} are passed to periplasmic cytochrome *c*. The reduced cytochrome *c* binds to the outer plasma membrane of the cell, allowing transport of electrons across the membrane to cytochrome oxidase located in the inner membrane.

Most microorganisms accumulate Fe^{3+} on their outer surface by reacting with acidic polymeric materials. Such mechanisms have important implications not only for the corrosion of metals, but also for the accumulation of heavy metals in natural habitats. *Aquaspirillum magnetotacticum* is capable of taking up complexed Fe^{3+} and transforms it into magnetite (Fe_3O_4) by reduction and partial oxidation (Blakemore, 1982; Schüler and Frankel, 1999). The magnetite crystals are single-domain magnets. They play an important role in bacterial orientation to the two magnetic poles of the Earth in natural environments. However, magnetite can also be formed extracellularly by some nonmagnetactic bacteria (Lovley et al., 1987). The role of these bacteria in metal corrosion is unkown.

Manganese deposition by microorganisms also affects the corrosion behavior of alloys. Growth of Leptothrix discophora resulted in ennoblement of stainless steel by elevating the open circuit potential to +375 mV (Dickinson et al., 1996; Dickinson et al., 1997). Further examination of the deposits on surfaces of coupons using X-ray photoelectron spectroscopy (XPS) confirmed that the product was MnO_2. The MnO_2 can also

be reduced to Mn^{2+} by accepting two electrons generated by metal dissolution. The intermediate product is MnOOH (Olesen et al., 1998). Manganese-depositing bacteria may include *Aeromonas*, *Bacillus*, *Caulobacter*, *Caulococcus*, *Citrobacter*, *Clonothrix*, *Cytophaga*, *Enterobacter*, *Flavobacterium*, *Hypomicrobium*, *Kuznetsovia*, *Lepothrix*, *Metallogenium*, *Micrococcus*, *Nocardia*, *Oceanspirillum*, *Pedomicrobium*, *Pseudomonas*, *Siderocapsa*, *Streptomyces* and *Vibrio* spp.

Strictly Anaerobic Microorganisms The sulfate-reducing bacteria are mostly responsible for corrosion under anaerobic conditions, as described earlier (and in Fig. 5). Currently, eighteen genera of dissimilatory sulfate-reducing bacteria have been recognized (Balow et al., 1992; Campaignolle and Crolet, 1997; Clapp, 1948; Enos and Taylor, 1996; Holland et al., 1986; Krieg and Holt, 1984). They are further divided into two physiological groups (Madigan et al., 2000; Odom, 1993a; Odom and Singleton, 1993b; Postgate, 1984). One group utilizes lactate, pyruvate, or ethanol as carbon and energy sources and reduces sulfate to sulfide. Examples are *Desulfovibrio*, *Desulfomonas*, *Desulfotomaculum* and *Desulfobulbus*. The other group oxidizes fatty acids, particularly acetate, and reduces sulfate to sulfide. This group includes *Desulfobacter*, *Desulfococcus*, *Desulfosarcina* and *Desulfonema*. Some species of *Desulfovibrio* lack hydrogenase. For example, *D. desulfuricans* is hydrogenase negative and *D. salexigens* is positive (Booth and Tiller, 1960; Booth and Tiller, 1962a). Booth et al. (Booth et al., 1962b; Booth et al., 1968) observed that the rate of corrosion by these bacteria correlated with their hydrogenase activity. Hydrogenase negative SRBs were completely inactive in corrosion. Apparently, hydrogenase-positive organisms utilize cathodic hydrogen, depolarizing the cathodic reaction, which controls the kinetics. In contrast to this theory, it has been suggested that ferrous sulfide (FeS) is the primary catalyst (Lee et al., 1995; Sanders and Hamilton, 1986; Weimer et al., 1988; Westlake, 1986; White et al., 1986).

Other microorganisms should be noted for their role in anaerobic corrosion. They include methanogens (Daniels et al., 1987; Ferry, 1995), acetogens (Drake, 1994; Nozhevnikova et al., 1994), thermophilic bacteria (Ghassem and Adibi, 1995; Little et al., 1986a; Torres-Sanchez et al., 1997) and obligate proton reducers (Tomei et al., 1985). More work is needed to elucidate the role of their contributions to corrosion.

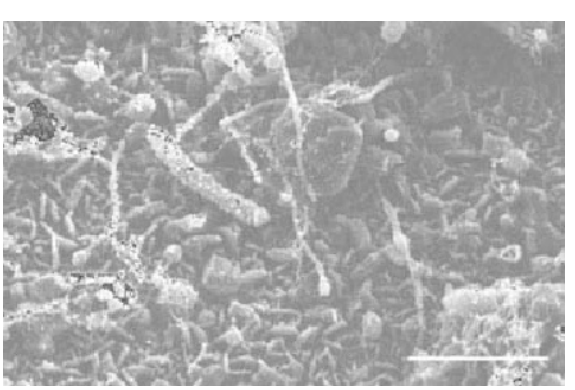

Fig. 5. Scanning electron micrograph of an anaerobic microbial biofilm together with corrosion products (bar = 5 μm).

Mechanisms of Microbial Corrosion

Aerobic Conditions Iron is the most abundant element in the Earth's crust and is present in two oxidative states, ferrous (Fe^{2+}) and ferric (Fe^{3+}). Metallic iron is a product of human activity by smelting. When molecular oxygen (O_2) is available as an electron acceptor for oxidation of reduced organic compounds or metallic iron (Fe^0), the area beneath the microbial colonies acts as an anode, whereas the area further away from the colonies, where oxygen concentrations are relatively higher, serves as a cathodic site. Electrons flow from anode to cathode and the corrosion process is initiated, resulting in the dissolution of metal. Depending on the species of bacteria present and the chemical conditions, dissociated metal ions form ferrous hydroxides, ferric hydroxide and a series of Fe-containing minerals in the solution phase. It should be noted that oxidation, reduction, and electron flow must all occur for corrosion to proceed. However, the electrochemical reactions never proceed at theoretical rates because the rate of oxygen supply to cathodes and removal of products from the anodes limit the overall reaction (Dowling and Guezennec, 1997; Lee et al., 1993a; Lee et al., 1993b; Little et al., 1990; Uhlig and Revie, 1985), although the corrosion reaction is thermodynamically favorable. Electrolytes in the adjacent environments affect the resultant distance between the anode and cathode, being shorter at low salt and longer at high salt concentrations. In addition, impurities and contaminants of the metal matrices also stimulate corrosion by initiating the formation of differential cells and accelerated electrochemical reactions.

Under aerobic conditions, corrosion products usually form a typical structure consisting of three layers called "tubercles." The inner green layer is almost entirely ferrous hydroxide ($Fe[OH]_2$). The outer one consists of orange ferric hydroxide ($Fe[OH]_3$). In between these two, magnetite (Fe_3O_4) forms a black layer (Lee et al., 1995). The most aggressive form of corrosion is

tuberculation caused by the formation of differential oxygen-concentration cells on material surfaces. The overall reactions are summarized as follows:

$$Fe^0 \rightarrow Fe^{2+} + 2e^- \quad \text{(anode)} \tag{1}$$

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \quad \text{(cathode)} \tag{2}$$

$$2Fe^{2+} + \tfrac{1}{2}O_2 + 5H_2O \rightarrow 2Fe(OH)_3 + 4H^+ \quad \text{(tubercle)} \tag{3}$$

Initial oxidation of Fe^0 of mild steel at near neutral pH is driven by dissolved O_2 (Uhlig, 1971). Subsequent oxidation of Fe^{2+} to Fe^{3+} is an energy producing process carried out by a few bacterial species including Gallionella, Leptothrix and Thiobacillus spp. Since the amount of free energy from this reaction is quite small for these microorganisms, approximately –31 kJ, large quantities of Fe^{2+} have to be oxidized to support the microbial growth. Because the Fe^{2+} oxidative reaction is rapid under natural conditions, microorganisms must compete with chemical processes for Fe^{2+}. As a result, biological involvement under aerobic conditions may be underestimated (Ford and Mitchell, 1990a; Ford and Mitchell, 1990b).

Anaerobic Conditions Adhesion to surfaces is a strategy microorganisms use for survival and multiplication (Marshall, 1992), and it provides an opportunity leading to corrosion. In all submerged environments including freshwater and marine, surfaces are covered with microorganisms and their exopolymeric layers. Within this gelatinous matrix of a biofilm, there are oxic and anoxic zones, permitting aerobic and anaerobic processes to take place simultaneously within the biofilm layer. Aerobic processes consume oxygen, which is toxic to the anaerobic microflora, whereas anaerobes benefit from the decrease in oxygen tension. In the absence of oxygen, anaerobic bacteria, including methanogens, sulfate-reducing bacteria, acetogens and fermentative bacteria are actively involved in corrosion processes. Interactions between these microbial species allow them to coexist under conditions where nutrients are limited.

Sulfate-reducing bacteria (SRBs) are among the most intensely investigated groups of microorganisms causing biological corrosion (Angell et al., 1995; Audouard et al., 1995; Hadley, 1948; Iverson, 1984a; Little et al., 1994; Pope et al., 1989; Starkey, 1986; Walch and Mitchell, 1986; Widdel, 1988). Characteristic corrosion by SRBs on metal surfaces results in pitting corrosion. Since molecular oxygen is not available to accept electrons under anaerobic conditions, SO_4^{2-} or other compounds (CO_2, H_2 and organic acids) are used as electron acceptors. Each type of electron acceptor is unique in the pathway of microbial metabolism. When corrosion begins, the following reactions take place:

$$4Fe^0 \rightarrow 4Fe^{2+} + 8e^- \quad \text{(anodic reaction)} \tag{4}$$

$$8H_2O \rightarrow 8H^+ + 8OH^- \quad \text{(water dissociation)} \tag{5}$$

$$8H^+ + 8e^- \rightarrow 8H \quad \text{(adsorbed)} \quad \text{(cathodic reaction)} \tag{6}$$

$$SO_4^{2-} + 8H \rightarrow S^{2-} + 4H_2O \quad \text{(bacterial consumption)} \tag{7}$$

$$Fe^{2+} + S^{2-} \rightarrow FeS\downarrow \quad \text{(corrosion products)} \tag{8}$$

$$4Fe + SO_4^{2-} + 4H_2O \longrightarrow 3Fe(OH)_2\downarrow + FeS\downarrow + 2OH^- \tag{9}$$

Von Wolzogen Kuhr and van der Vlugt (1934) suggested that the above set of reactions is caused by SRBs. This electrochemical generalization has been accepted and is still prevalent. During corrosion, the redox potential of the bacterial growth medium is –52 mv (Hadley, 1948). After inoculation of a corrosion testing cell with SRBs, the electrochemical potential decreases from the initial value of –470 mv to approximately –538 mv (Fig. 6).

Several changes take place in the electrical potential of steel after inoculation with the SRBs. Before inoculation, the value is determined by the concentration of hydrogen ions in the medium. A film of hydrogen forms on surfaces of Fe^0 and steel, inducing polarization. Immediately after inoculation, SRBs begin growth and depolarization occurs, resulting in a drop in the anodic direction. The SRBs, by means of their hydrogenase system, remove the adsorbed hydrogen, depolarizing the system. The overall process was described as depolarization, based on the theory that these bacteria remove hydrogen that accumulates on the surfaces of iron. The electron removal as a result of hydrogen utilization results in cathodic depolarization and forces more iron to be dissolved at the anode.

The direct removal of hydrogen from the surface is equivalent to lowering the activation energy for hydrogen removal by providing a depolarization reaction. The enzyme, hydrogenase, synthesized by many species of *Desulfovibrio* spp., is involved in this specific depolarization process (Starkey, 1986). Under aerobic conditions, the presence of molecular oxygen serves as an electron sink; under anaerobic conditions, particularly in the presence of SRBs, SO_4^{2-} in the aqueous phase can be reduced to S_2^- by the action of the microflora. The biogenically produced S_2^- reacts with Fe^{2+} to form a

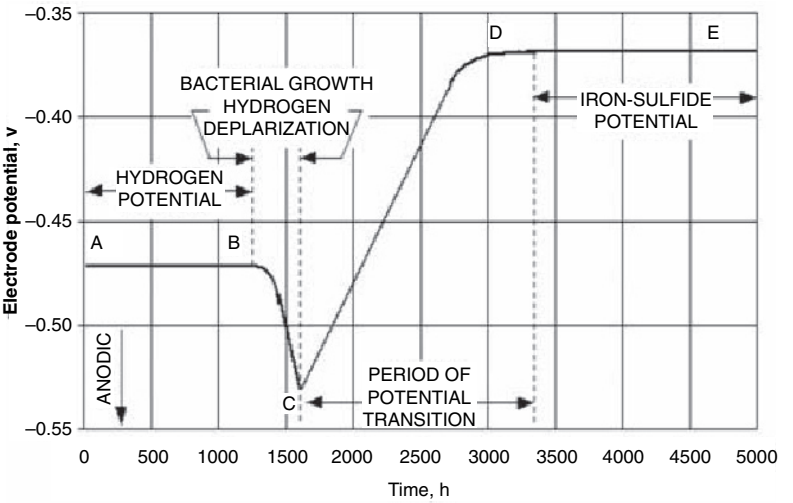

Fig. 6. Cathodic depolarization potential after inoculation of sulfate-reducing bacteria.

precipitate of FeS. Controversy surrounding the mechanisms of corrosion includes more complex mechanisms involving both sulfide and phosphide (Iverson, 1981; Iverson, 1984a; Iverson and Olson, 1983; Iverson and Olson, 1984b; Iverson et al., 1986) and processes related to hydrogenase activity (Li and Lü, 1990; Starkey, 1986). The addition of chemically prepared Fe_2S and fumarate as electron acceptors also depolarizes the system. However, higher rates are always observed in the presence of SRBs.

As a result of the electrochemical reactions, the cathode always tends to be alkaline with an excess of OH^-. These hydroxyl groups also react with ferrous irons to form precipitates of hydroxy iron. Precipitated iron sulfites are frequently transformed into minerals, such as mackinawite, greigite, pyrrhotite, marcasite and pyrite. Lee et al. (Lee et al., 1993a; Lee et al., 1995) suggest that biogenic iron sulfides are identical to those produced by purely inorganic processes under the same conditions. Little et al. (1994) showed evidence that biogenic minerals are microbiological signature markers.

Alternation Between Aerobic and Anaerobic Conditions Constant oxic or anoxic conditions are rare in natural or industrial environments. It is more common that the two alternate, depending on oxygen gradient and diffusivity in a specific environment. Microbial corrosion under such conditions is quite complex, involving two different groups of microorganisms and an interface that serves as a transition boundary between the two conditions. Resultant corrosion rates are often higher than those observed under either oxic or anoxic conditions. Microbial activity reduces the oxygen level at interfaces, facilitating anaerobic metabolism. The corrosion products (such as FeS, FeS_2 and S^0) resulting from anaerobic processes can be oxidized when free oxygen is available (Nielsen et al., 1993).

During oxidation of reduced sulfur compounds, more corrosive sulfides are produced under anoxic conditions, causing cathodic reactions. The corrosion rate increases as the reduced and oxidized FeS concentrations increase (Lee et al., 1993a; Lee et al., 1993b). Cathodic depolarization processes also can yield free O_2 which reacts with polarized hydrogen on metal surfaces.

Other Processes Contributing to Corrosion Bacteria produce copious quantities of exopolymers which appear to be implicated in corrosion (Ford et al., 1986; Ford et al., 1987b; Ford et al., 1988; Ford et al., 1990d; Ford et al., 1990c; Ford et al., 1991; Little and Depalma, 1988; Paradies, 1995; Roe et al., 1996; Whitfield, 1988). The process is shown in Fig. 7. These exopolymers are acidic and contain functional groups capable of binding to metal ions. Paradies (1995) recently reviewed this subject. The exopolymers faciliate adhesion of bacteria to surfaces. They are involved in severe corrosion of copper pipes and water supplies in large buildings and hospitals (Fischer et al., 1987; Paradies et al., 1990). Some materials also play an important role in cueing the settlement of invertebrate larvae and others in repelling larvae from surfaces (Gu et al., 1997b; Holmström et al., 1992; Maki et al., 1989; Maki et al., 1990a; Maki et al., 1990b; Mitchell and Maki, 1989; Rittschof et al., 1986). They primarily consist of polysaccharides and proteins and influence the electrochemical potential of metals (Chen et al., 1995; Chen et al., 1996c). Surface analysis using XPS showed that these functionality-rich materials can complex metal ions from the surface, releasing them into aqueous solution. As a result, corrosion is initiated.

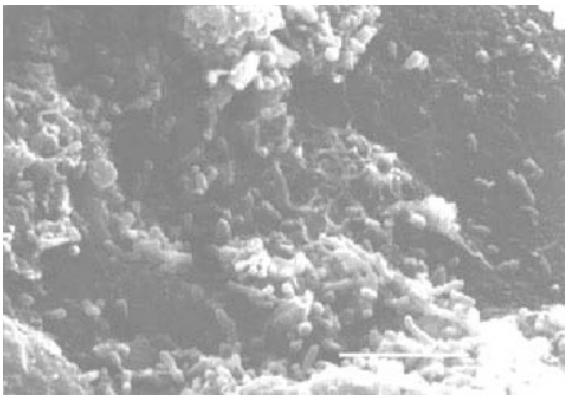

Fig. 7. Scanning electron micrograph showing a biofilm and large quantities of exopolysaccharide on the surface of stainless steel (bar = 5 μm).

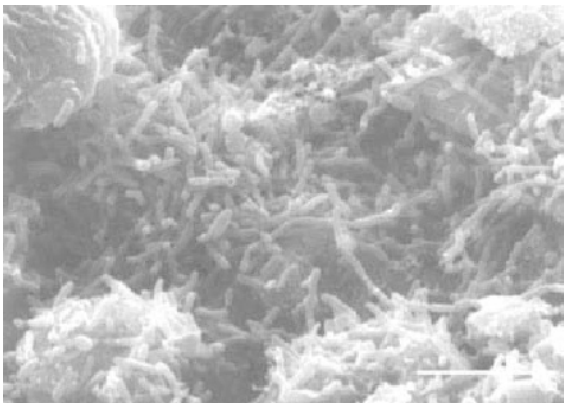

Fig. 8. Scanning electron micrograph of a bacterial biofilm of the type that is inhibiting corrosion of underlying stainless steel (bar = 5 μm).

Proteins in polymeric materials use their disulfide-rich bonds to induce corrosion.

Bacterial polymers were recently found to promote corrosion of copper pipes in water supplies (Fischer et al., 1987; Mittelman and Geesey, 1985; Paradies et al., 1990), owing to the high affinity of the polymeric materials for copper ions (Mittelman and Geesey, 1985; Ford et al., 1988). The corrosion processes are accelerated when the pipes are filled with stagnant soft water. Cations influence the production of bacterial exopolymers. Polysaccharide production by *Enterobacter aerogenes* is stimulated by the presence of Mg, K and Ca ions (Wilkinson and Stark, 1956). Toxic metal ions (e.g., Cr^{6+}) also enhance polysaccharide production. Synthesis is positively correlated with Cr concentration. These bacteria also can be used in the mining and recovery of precious metals, a process called "bioleaching" (Clark and Ehrlich, 1992; Davidson et al., 1996; Dunn et al., 1995).

The role of bacteria in embrittlement of metallic materials by hydrogen is not fully understood. During the growth of bacteria, fermentation processes produce organic acids and molecular hydrogen. This hydrogen can be adsorbed to material surfaces, causing polarization. Some bacteria, particularly the methanogens, sulfidogens, and acetogens, can also utilize hydrogen (Gottschalk, 1986). Walch and Mitchell (1986) proposed a possible role for microbial hydrogen in hydrogen embrittlement. They measured permeation of microbial hydrogen into metal, using a modified Devanathan cell (Devanathan and Stachurski, 1962). In a mixed microbial community commonly found in natural conditions, hydrogen production and consumption occur simultaneously. Competition for hydrogen between microbial species determines the ability of hydrogen to permeate metal matrices, causing crack initiation.

Microbial hydrogen involved in material failure may be explained by two distinct hypotheses: pressure and surface energy change (Borenstein, 1994; Gangloff and Kelly, 1994). The kinetic nature of hydrogen embrittlement of cathodically charged mild steel is determined by the competition between diffusion and plasticity. The greater the strength of the alloy, the more susceptible it is to embrittlement. However, microstructures were also proposed to be the more critical determinant of material susceptibility. Hydrogen permeation may increase the mobility of screw dislocations, but not the mobility of edge dislocations (Luu et al., 1997; Wang, 1996). On the other hand, corrosion may also be inhibited by the presence of biofilms on surfaces (Hernandez et al., 1994; Jayaraman et al., 1997; Jayaraman et al., 1999; Mattila et al., 1997; Potekhina et al., 1999).

Inhibition of Corrosion by Microorganisms

The mechanisms involved during microbial corrosion of metals are: 1) stimulation of an anodic or cathodic process by bacterial metabolites, 2) breakdown of the protective layers, and 3) enhanced conductivity near the surface liquid environment. However, bacteria may also inhibit corrosion processes (Fig. 8) by electrochemical processes (Hernandez et al., 1994; Jayaraman et al., 1997; Jayaraman et al., 1999; Mattila et al., 1997; Potekhina et al., 1999). Bacteria may also 1) neutralize the corrosive substances, 2) form protective layers on materials, or 3) decrease the corrosiveness of the aqueous environment.

Nonferrous Metals

Metals other than Fe are commonly used in alloys to inhibit corrosion and enhance mechanical properties. They include Mo, Cr, Ni, Cu, Zn and Cd. The selection of metal species and the quantities in the iron matrices are based on the engineering properties of the materials. Pure metals in common use are limited to Fe, Al, Cu and Ti. We know very little about biocorrosion of Al and Ti (Gu et al., 2000a). Alumininum (Al) reacts with molecular oxygen (O_2) under ambient conditions, forming an oxidized layer of protective aluminum oxide on the outer surface of the material matrix. When Al ions are released, the free Al^{3+} is toxic to both the microflora (Appanna and Piperre, 1996; Illmer and Schinner, 1999) and animals (Nieboer et al., 1995). Because of their corrosion resistance, titanium (Ti) alloys are used in water cooling systems on ships and in water recycling systems in space. Biofilm formation on these materials has been documented (Gu et al., 1998c). However, the extent of attack by microorganiams is unknown.

Recent research on microbial interactions with metals has focused on the precipitation (Cunningham and Lundie, 1993; Fortin et al., 1994), mineral formation (Bazylinski et al., 1993; Douglas and Beveridge, 1998; Klaus et al., 1999), and oxidation/reduction processes (Kessi et al., 1999; Lovley and Phillips, 1994; Santini et al., 2000; Stoltz and Oremland, 1999; Sugio et al., 1988; Sugio et al., 1992; Tebo and Obraztsova, 1998; Wang et al., 1989). Surprisingly, information on Zn, one of the most widely used metals, is very limited.

Microorganisms may affect transitional metals in several ways, including precipitation by metabolic products (Fortin et al., 1994), cellular complexation (Schembri et al., 1999; Schultzen-Lam et al., 1992), and concentration and mineral formation of internal cellular structures. Sulfate-reducing bacteria can effectively immobilize a wide range of soluble metals by forming sulfide precipitates (Sakaguchi et al., 1993). Recently, bacterial exopolymers have been found to be capable of complexing metals, leading to accumulation at the cell surface. This ability is not restricted to a specific group of microorganisms and has been documented in the aerobic bacterium *Deleya marina* and an anaerobe *Desulfovibrio desulfuricans* (Chen, 1996a). Since many transitional metals exist in several oxidation/reduction states, both bacterial oxidation and reduction are possible.

Chromium (Cr) can exist in either the hexavalent or trivalent form. Reduction of Cr^{6+} to Cr^{3+} is mediated by both aerobic and anaerobic microorganisms. A change of phenotypic expression in *Pseudomonas indigoferas* (*Vogesella indigofura*) has been observed in the presence of Cr^{6+} (Gu and Cheung, 2001a). Intracellular partition of Cr was reported using bacteria from a subsurface environment. Reduction of Cr^{6+} is a process in which the toxicity of the metal is greatly reduced. Bacteria possessing this ability include *Achromobacter eurydice*, *Aeromonas dechromatica*, *Agrobacterium radiobacter*, *Arthrobacter* spp., *Bacillus subtilis*, *B. cereus*, *Desulfovibrio vulgaris*, *Escherichia coli*, *Enterobacter cloacae*, *Flavobacterium devorans*, *Sarcina flava*, *Micrococcus roseus* and *Pseudomonas* spp. (Ehrlich, 1996).

Similarly, Mo exists in a number of oxidation states, with Mo^{4+} and Mo^{6+} being most common. *Thiobacillus ferrooxidans* is capable of oxidizing Mo^{5+} to Mo^{6+}, whereas *Enterobacter cloacae*, *Sulfolobus* sp. and *Thiobacillus ferrooxidans* can reduce Mo^{6+} to Mo^{5+} (Sugio et al., 1988; Sugio et al., 1992). Microbiological oxidation or reduction of other metals, including Cd, Ni, and Zn, has not been fully established. Microbial exopolymers have a significant effect on the solubilization of metals from material matrices through complexation and chelation. Because of this property, wastewater containing these metals ions can be purified through biomass adsorption, a process in which metal ions are concentrated on a biosorbent (Gelmi et al., 1994; Matis et al., 1996; Sánchez et al., 1999).

Our knowledge of microbial transformation of metals is very limited. Recent developments in isolation of Bacteria and Archaea may provide new tools to investigate metal transformations in natural habitats (Amann et al., 1995). Molecular techniques, including DNA probes and in situ hybridization, permit the identification of physiologically unique bacteria without the need to culture the organisms. Microbial resistance to metals is widespread in nature (Lin and Olsen, 1995; Mergeay, 1991; Sandaa et al., 1999). Elucidation of the genetic structure of these bacteria should provide new insights into the processes involved in resistance.

Biodeterioration of Polymeric Materials

Microorganisms also contribute to the deterioration and degradation of synthetic and natural polymers (Lee, 1948; Gu et al., 2000d). Very little is known about the biodegradation of synthetic polymeric materials, probably because of their relatively recent widespread use and very slow rate of degradation in natural habitats. Since chemically synthesized polymeric materials have become an indispensable part of human activities and have more diversified applications than

metals, issues related to polymer deterioration will receive more attention in the future.

Polymeric materials are very diversified in their chemical composition, physical forms, mechanical properties and applications. Variations in their chemical structure result from the versatility of the hydrocarbon (C—C, C—R and C—H) bonds and substituent groups and their possible configurations, stereochemistry and orientation (Odian, 1991). These small variations in strucure result in large differences in biodegradability. Because of their versatility, they are widely used in product packaging, insulation, structural components, protective coatings, medical implants, drug delivery carriers, slow-release capsules, electronic insulation, telecommunications equipment, aviation and space industry applications, sporting and recreational equipment, and building consolidants. In service, they are constantly exposed to a range of natural and artificial conditions often involving microbial contamination, resulting in aging, disintegration and deterioration over time (Lemaire et al., 1992; Pitt, 1992).

Microorganisms and Degradation of Synthetic Polymers

Polymers are potential substrates for heterotrophic microorganisms. Microbial utilization of polymers depends on their chemical structure, molecular weight, crystallinity and physical form (Gu et al., 2000d). Generally, an increase in molecular weight results in a decreased degradation rate of the polymer. By contrast, monomers, dimers, and oligomers of a polymer's repeating units are easily degraded and mineralized. High molecular weights result in a decrease in solubility and are unfavorable for microbial attack because bacteria require that the substrate be assimilated through the cellular membrane and then further degraded by intercellular enzymes. Both abiological and biological processes may faciltiate degradation of polymers.

At least two types of enzymes are active in biological degradation of polymers: extracellular and intracellular depolymerases (Doi, 1990). During degradation, exoenzymes from microorganisms break complex polymers to yield short units including oligomers, dimers and monomers that are small enough to pass through permeable outer bacterial membranes and subsequently be utilized as carbon and energy sources (Fig. 9). The more similar a polymeric structure is to a natural analog, the more easily it is degraded and mineralized. Cellulose, chitin, pullulan and poly β-hydroxybutyrate (PHB) are all biologically synthesized and can be completely and rapidly biodegraded by heterotrophic microorganisms

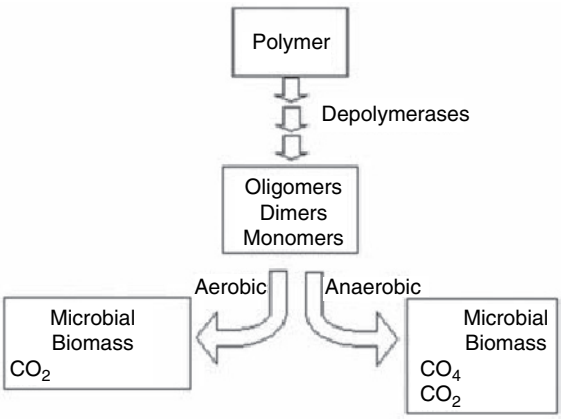

Fig. 9. A schematic of polymer degradation under aerobic and anaerobic conditions.

(Bérenger et al., 1985; Byrom, 1991; Chahal et al., 1992; Frazer, 1994; Gamerith et al., 1992; Gujer and Zehnder, 1983; Gunjala and Sulflita, 1993; Hamilton et al., 1995; Hass et al., 1992; Hespell and O'Bryan-Shah, 1988; Kormelink and Voragen, 1993; Lee et al., 1985; Lee et al., 1987a; Lee et al., 1987b; Lee et al., 1993c; Lüthi et al., 1990a; Lüthi et al., 1990b; MacDonald et al., 1985; MacKenzie et al., 1987; Nakanishi et al., 1992; Sonne-Hansen et al., 1993; Sternberg et al., 1977; Törrönen et al., 1993; Wong et al., 1988; Yoshizako et al., 1992). The complete decomposition of a polymer to CO_2 and H_2O under aerobic conditions or to organic acids, CO_2, and CH_4 under anaerobic conditions is rare. Degradation and mineralization of a polymer substrate rarely are complete, because of the utilization of a portion of the partially degraded polymer to form humus and other natural products (Alexander, 1977; Narayan, 1993).

Environmental conditions may determine the dominant groups of microorganisms and the degradative pathways associated with polymer degradation. Under high redox conditions, aerobic microorganisms are mostly responsible for destruction of complex materials, with microbial biomass, CO_2, and H_2O as the final products (Fig. 9). In contrast, under low redox conditions, anaerobic consortia of microorganisms are involved in polymer deterioration, and the primary products will be microbial biomass, CO_2, CH_4 and H_2O (Barlaz et al., 1989a; Barlaz et al., 1989b; Gu et al., 2000d; Gu et al., 2000e; Fig. 9). These conditions are widely found in natural environments and can be simulated in the laboratory with appropriate inocula. Both aerobic and strictly anaerobic microorganisms also coexist in natural environments.

We can divide synthetic polymers into three groups: 1) relatively degradable, 2) recalcitrant, and 3) completely resistant. We have excluded

natural polymers, e.g., cellulose, chitin, chitosan, lignin and polysaccharides.

Degradable Polymers

Microbial degradability of polymers depends on their molecular composition and molecular weight. Some can be almost completely utilized as a source of carbon and energy, whereas others are only partially degraded. Examples of the former include the polyhydroxyalkanoates (PHAs; Anderson and Dowes, 1990; Brandl et al., 1988; Choi and Yoon, 1994; Doi, 1990; Nakayama et al., 1985; Stenbüchel, 1991; Stuart et al., 1995; Tanio et al., 1982), γ-polyglutamates (Cromwick and Gross, 1995), cellulose acetates (Buchanan et al., 1993; Gross et al., 1993; Gross et al., 1995; Gu et al., 1992c; Gu et al., 1992d; Gu et al., 1993a; Gu et al., 1993b; Gu et al., 1993c; Gu et al., 1994b), polyethers (Kawai, 1987; Kawai and Moriya, 1991; Kawai and Yamanaka, 1986), polylactides (Gu et al., 1992c; Gu et al., 1992d), polyurethanes (Blake et al., 1998; Crabbe et al., 1994; El-Sayed et al., 1996; Filip, 1978; Gillatt, 1990; Gu et al., 1998c; Mitchell et al., 1996; Nakajima-Kambe et al., 1995; Szycher, 1989), and rubbers (Berekaa et al., 2000; Heisey and Papadatos, 1995). A general rule is that biologically synthesized polymers are readily biodegradable in natural environments, whereas synthetic polymers are either less biodegradable or degrade very slowly, depending on their chemical composition, structural complexity, and molecular weights. However, the rate of degradation is largely determined by the chemical structure, e.g., the C—C and other types of bonds, molecular weight, structure and configuration, as well as the participating microorganisms. It is also determined by the environment, specifically the presence of an active microbial population capable of utilizing all or part of the polymer.

Chemical structure of a polymer determines its biodegradability. As a general rule, high molecular weight synthetic polymers are less biodegradable or degrade at a slower rate than those with low molecular weights. The same principal may also apply to the polymers mentioned above. In addition, the rate of hydrolytic chain cleavage is dependent on the copolymer composition. For example, Doi (1990) determined that the most susceptible polyhydroxybutyrates to hydrolysis (from highest to lowest hydrolysis rate) are: poly(3-hydroxybutyrate-co-27% 4-hydroxybutyrate), also known as (P[3HB-co-27% 4HB]) > (P[3HB-co-17% 4HB]) > (P[3HB-co-10% 4HB]) > poly(3-hydroxybutyrate-co-45% 3-hydroxyvalerate), also known as (P[3HB-co-45% 3HV]) > (P[3HB-co-71% 3HV]). Similarly, Parikh et al. (1993) in another series arranged PHBs in the order of their rates (from highest to lowest) of enzymatic hydrolysis as follows: (P[HB-co-16% HV]) > (P[HB-co-32% HV]) > PHB (Fig. 10). The crystallinity of the polymer also affects the rate of degradation but is rarely taken into account (Budwill et al., 1992).

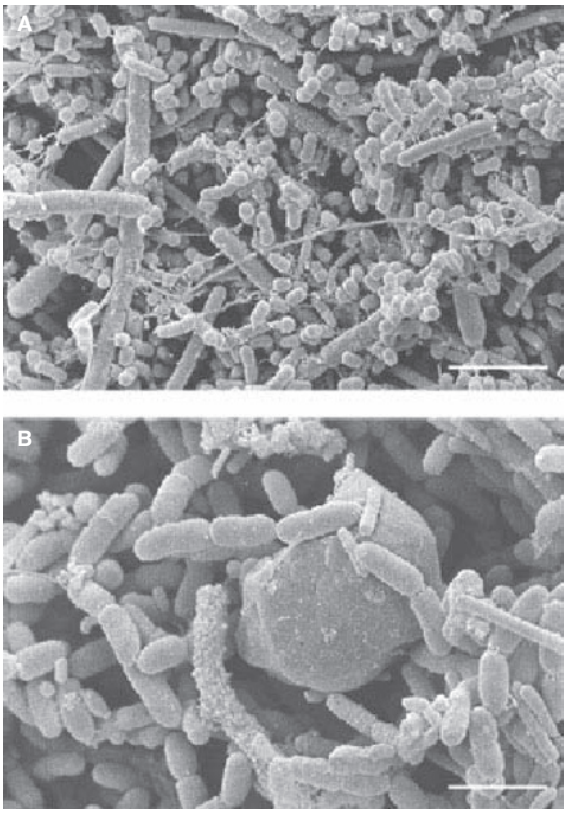

Fig. 10. Scanning electron micrographs of (a) an aerobic microbial biofilm on the surface of poly-β-hydroxybutyrate (PHB; bar = 10 μm) and (b) bacteria surrounding a PHB granule (bar = 5 μm).

BIOPOLYMERS Bacterial poly(β-hydroxyalkanoates) are formed as energy storage materials during nutrient-limited growth when the carbon source is in excess, e.g., high C/N ratio (Anderson and Dawes, 1990; Brandl et al., 1988; Doi, 1990; Holmes et al., 1985; Kim et al., 1995; Lemoigne, 1926; Stenbüchel, 1991). They consist of homo- or copolymers of [R]-β-hydroxyalkanoic acids. The polymer forms intracellular inclusions (granules 0.3–1.0 μm in diameter) in the bacterial cytoplasm, and it can comprise as much as 30–80% of cellular biomass. The polymer is isolated from *Bacillus megaterium* by extraction in chloroform and has a molecular weight of approximately 10^5–10^6 with more than 50% in crystalline form (J-D. Gu et al., unpublished observation). Unlike other biopolymers,

such as polysaccharides, proteins and DNAs, P(3HB) is a thermoplastic with a melting temperature around 180°C, making it a good candidate for thermoprocessing. Poly-β-hydroxyalkanoates (PHAs) and copolymers have also been produced through genetic engineering in plants (John and Keller, 1996) and by chemical synthesis (Kemnitzer et al., 1992; Kemnitzer et al., 1993).

Homopolymers and copolymers can be degraded in biologically active environments, e.g., soil (Albertsson et al., 1987; Mas-Castellà et al., 1995; Tsao et al., 1993), sludge, compost (Gilmore et al., 1992; Gilmore et al., 1993; Gross et al., 1993; Gross et al., 1995; Gu et al., 1993b), river water (Andrady et al., 1993; Iman et al., 1992) and seawater (Andrady et al., 1993; Sullivan et al., 1993; Wirsen and Jannasch, 1993). Extracellular P(HB) depolymerases have been isolated from Pseudomonas lemoignei (Lusty and Doudoroff, 1966) and A. faecalis (Saito et al., 1989; Tanio et al., 1982). Other bacteria capable of degrading these polymers include *Acidovorax facilis*, *Variovorax paradoxus*, *Pseudomonas syringae* subsp. *savastanoi*, *Comamonas testosteroni*, *Cytophaga johnsonae*, *Bacillus megaterium*, *B. polymyxa* and *Streptomyces* spp. (Mergaert et al., 1993). The enzymatic degradation occurs at the surfaces of the polyester film (Fig. 11), and the rate of surface erosion is highly dependent on the molecular weight (degree of polymerization), composition of the polyester, crystallinity and the dominant species of bacteria.

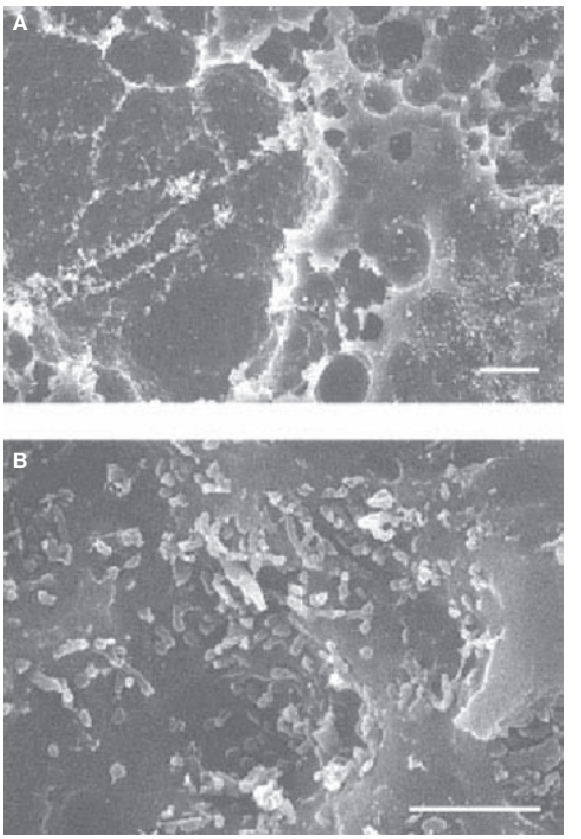

Fig. 11. Scanning electron micrographs showing A) extensive surface erosion by surface colonizing bacteria in soil (bar = 1 μm) and B) a close-up of the eroded area (bar = 5 μm).

Chemically Modified Biopolymers Cellulose acetates (CAs) are a class of chemically modified natural polymers designed to improve their processibility and mechanical properties for different applications (Bogan and Brewer, 1985). Theoretically, they carry substitution values from as low as near zero to 3.0. Generally, CAs with a degree of substitution less than 2.5 have been shown to be degraded in thermophilic compost (Gross et al., 1993; Gross et al., 1995; Gu et al., 1992c; Gu et al., 1992d; Gu et al., 1993a; Gu et al., 1993b; Gu et al., 1993c; Gu et al., 1994b) or transformed through biologically catalyzed reactions (Downing et al., 1987). Increasing the degree of substitution (DS) value on a repeating unit makes the CA less degradable. It is also apparent that deviation from the natural structure increases resistance of degradation.

Cellulose acetate (CA) degradation occurs more rapidly under aerobic conditions. The mechanisms of degradation are deacetylation, which releases the substituted groups, followed by cleavage of the C–C backbone. It is believed that the molecular weight decrease and deacetylation proceed simultaneously during degradation, after CA reaches a critical value of substitution. Structural substitution groups, and their number per repeating unit, also affect the degradation kinetics. For example, cellulose acetates (CAs) with lower degree of substitution (DS) values are more quickly degraded than more substituted ones under both aerobic and anaerobic conditions (Buchanan et al., 1993; Gross et al., 1993; Gross et al., 1995; Gu et al., 1992c; Gu et al., 1992d; Gu et al., 1992c; Gu et al., 1993a; Gu et al., 1993b; Gu et al., 1993c; Gu et al., 1994b). Cellulose acetates (CAs) with lower substitution values also show relatively higher solubility. During degradation of CA, both molecular weight and degree of substitution decreased, suggesting that deacetylation and decomposition of the polymer backbone proceed simultaneously (Gu et al., 1993c). Earlier data suggested that CA with DS values greater than 0.82 are recalcitrant to biodegradation and that the limiting step is deacetylation, followed by breaking of the polymer carbon-carbon bonds (Reese, 1957). Obviously, degradation of CA can proceed at DS values higher than 0.82.

Chemically Synthesized Polymers The most common group of synthetic polymers are polyethers. These polymers include polyethylene glycols (PEGs), polypropylene glycols (PPGs) and polytetramethylene glycols (PTMGs). They are widely used in pharmaceuticals, cosmetics, lubricants, inks and surfactants. They frequently contaminate natural waters, including coastal waters and streams where wastewater is discharged.

Degradability of this class of polymers has been studied under both oxic (Kawai and Moriya, 1991; Kawai and Yamanaka, 1986; Kawai, 1987) and anoxic conditions (Frings et al., 1992; Schink and Stieb, 1983; Dwyer and Tiedje, 1983). Their degradability is dependent on molecular weight. Molecules with molecular weights higher than 1,000 have been considered resistant to biodegradation (Kawai, 1987). However, degradation of PEGs with molecular weights up to 20,000 has been reported. The ability of a microflora to degrade larger PEG molecules is dependent primarily on the ability of a syntrophic association of bacteria to metabolize the chemicals. For example, *Flavobacterium* and *Pseudomonas* (acting in concert) can degrade PEG. After each oxidation cycle, PEG molecules are reduced by a glycol unit.

The central theme of PEG degradation is cleavage of an aliphatic ether linkage. In a coculture of aerobic *Flavobacterium* and *Pseudomonas* species, PEG degradation proceeds through dehydroxylation to form an aldehyde and continues through a dehydrogenation to a carboxylic acid derivative (Kawai, 1987; Kawai and Yamanaka, 1986). Either of these bacteria in pure culture cannot degrade PEG alone. Cellular contact between them seems to be essential for effective activity.

In the *Flavobacterium* and *Pseudomonas* system, three enzymes (PEG dehydrogenase, PEG-aldehyde dehydrogenase and PEG-carboxylate dehydrogenase) are involved in the complete degradation of PEG (Kawai, 1987). All three were found in *Flavobacterium*, whereas only PEG-carboxylate dehydrogenase was present in *Pseudomonas*. The polymer PEG 6000 cannot be degraded by either bacterial species alone. The ether cleavage is extremely sensitive to the presence of glyoxylic acid. However, *Pseudomonas*, though not directly involved in the degradation, utilizes the toxic metabolite that inhibits the activity of the *Flavobacterium*. This appears to be the essential link for their syntrophic association in the degradation of PEG.

Recalcitrant Polymers

Electronic Insulation Polymers Generally, polymers in this category are chemically synthesized with the objective of high strength and resistance to degradation. They include thermosetting polyimides (Brown, 1982; Ford et al., 1995; Gu et al., 1994a; Gu et al., 1995b; Gu et al., 1996b; Gu et al., 1996e; Gu et al., 1998b; Gu et al., 1998c; Mitton et al., 1993; Mitton et al., 1996; Mitton et al., 1998), corrosion protective coatings (Mitchell et al., 1996), and fiber reinforced polymeric composites (Gu et al., 1994a; Gu et al., 1995b; Gu et al., 1995c; Gu et al., 1995d; Gu et al., 1995e; Gu et al., 1996c; Gu et al., 1996a; Gu et al., 1996e; Gu et al., 1997a; Gu et al., 1997b; Wagner, 1995; Wagner et al., 1996). Wide acceptance of polyimides in the electronics industry (Brown, 1982; Jensen, 1987; Lai, 1989; Verbicky, 1988; Verbiest et al., 1995) has drawn attention to the stability of these materials. The National Research Council in 1987 (NRC, 1987) emphasized the need to apply these polymers in the electronics industries because data acquisition, information processing and communication are critically dependent on materials performance. The interlayering of polyimides and electronics in integrated circuits prompted several studies on the interactions between these two materials (Hahn et al., 1985; Kelley et al., 1987).

Polyimides are also widely used in load bearing applications (e.g., struts, chassis, and brackets in automotive and aircraft structures), owing to their flexibility and compressive strength. They are also used in appliance construction, cookware, and food packaging because of their chemical resistance (to oils, greases, and fats), microwave transparency, and thermal resistance. Their electrical properties are ideally suited for use in the electrical and electronics markets, especially as high temperature insulation materials and passivation layers in the fabrication of integrated circuits and flexible circuitry. In addition, the flammability resistance of this class of polymers may provide a halogen-free flame-retardant material for aircraft interiors, furnishings, and wire insulation. Other possible uses may include fibers for protective clothing, advanced composite structures, adhesives, insulation tapes, foam, and optics operating at high temperatures (Verbiest et al., 1995).

Electronic packaging polyimides are particularly useful because of their outstanding performance and engineering properties. However, they are susceptible to degradation by fungi (Ford et al., 1995; Gu et al., 1994a; Gu et al., 1995b; Gu et al., 1996b; Mitton et al., 1993; Mitton et al., 1998; Fig. 12).

Polyimide degradation occurs through biofilm formation and subsequent physical changes in the polymer. Using electrochemical impedance spectroscopy (EIS) (Mansfeld, 1995; van Westing et al., 1994), fungi growing on polyimides have been shown to yield distinctive EIS spectra, indicative of failing resistivity. Two steps are

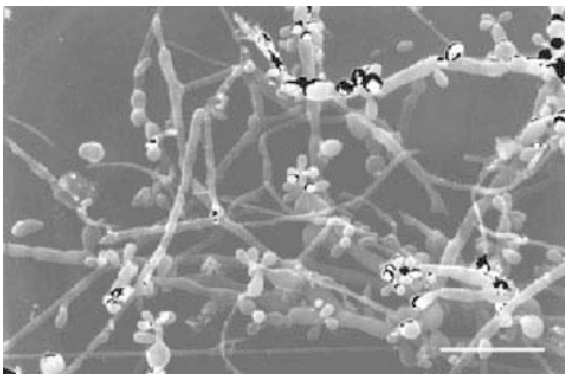

Fig. 12. Scanning electron micrograph showing a fungal community growing on the surface of electronic insulation polyimides (bar = 10 μm).

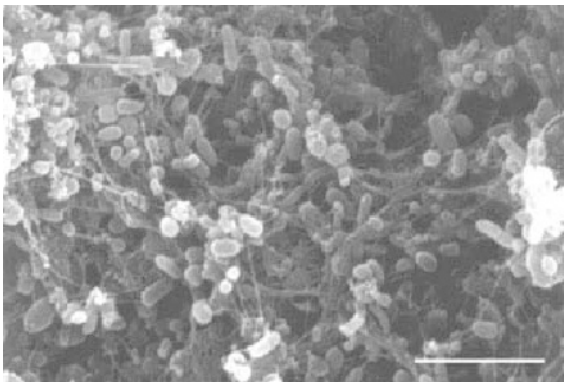

Fig. 13. Scanning electron micrograph of microorganisms colonizing the surface of a fiber-reinforced polymeric composite (bar = 10 μm).

involved during degradation: an initial decline in coating resistance is related to the partial ingress of water and ionic species into the polymer matrices. This is followed by further deterioration of the polymer by activity of the fungi, resulting in a large decrease in resistivity. The data support the hypothesis that polyimides are susceptible to microbial degradation resulting in the corrosion of underlying metal. They also confirm the versatility of EIS as a method in evaluation of the biosusceptibility of polymers.

Our studies showed that the dielectric properties of polyimides could be altered drastically following growth of a microbial biofilm (Ford et al., 1995; Gu et al., 1995b; Gu et al., 1996b; Mitton et al., 1993; Mitton et al., 1998). This form of deterioration may be slow under ambient conditions. However, the deterioration processes can be accelerated in humid conditions or in enclosed environments, e.g., submarines, space vehicles, aircraft and other closed facilities. Very small changes in material properties may have serious consequences.

Fiber-Reinforced Polymeric Composite Materials

Fiber-reinforced polymeric composite materials (FRPCMs) are also susceptible to biological attack. Impurities and additives that can promote microbial growth are potential sources of carbon and energy for the microorganisms (Fig. 13). Recently, two groups reported microbial degradation of FRPCMs (Gu and Mitchell, 1995a; Gu et al., 1995c; Gu et al., 1995d; Gu et al., 1995e; Gu et al., 1996c; Gu et al., 1996e; Gu et al., 1997a; Gu et al., 1997b; Wagner et al., 1996). Wagner and her collaborators (Wagner et al., 1996) used a mixed culture of microorganisms including a sulfate-reducing bacterium. Gu and colleagues (Gu et al., 1994a; Gu et al., 1996b; Gu et al., 1996c; Gu et al., 1996e; Gu et al., 1997a; Gu et al., 1997b) used a fungal consortium originally isolated from degraded polymers. This consortium consisted of *Aspergillus versicolor*, *Cladosporium cladosorioides*, and a *Chaetomium* sp. Physical and mechanical tests were not sufficiently sensitive to detect any significant physical changes in the materials (Gu et al., 1997a; Thorp et al., 1994). However, the resins were actively degraded, indicating that the materials were at risk of failure.

The increasing usage of FRPCMs as structural components of public structures and aerospace applications has generated an urgent need to evaluate the biodegradability of this class of new material. It has become clear that FRPCMs are not immune to adhesion by microorganisms (Ezeonu et al., 1994b; Gu et al., 1998c; Mitchell et al., 1996).

Recently, natural populations of microorganisms were found capable of growth on surfaces of FRCPM coupons at both relatively high (65–70%) and lower humidity (55–65%; Gu et al., 1998c). The accumulation of bacteria on surfaces of composites develops into a biofilm layer, providing some initial resistance to further environmental changes. However, the resistivity of composite materials was found to decline significantly during a year of monitoring using EIS (Gu et al., 1996e; Gu et al., 1997a). Clear differences resulting from biofilm development were detected on FRCPMs used in aerospace applications (Gu et al., 1997a). Further study indicated that microorganisms utilize chemicals introduced during composite manufacture as carbon and energy sources (Gu et al., 1996c). Lignopolystyrene graft copolymers were also susceptible to attack by fungi (Milstein et al., 1992).

A critical question remains about the effect of FRCPM degradation on the mechanical properties of the composite materials. Thorp et al. (1994) attempted to determine mechanical changes in composite coupons after exposure to a fungal culture. No mechanical changes could be measured after 120 days of exposure. They suggested that methodologies sufficiently sensitive to detect surface changes need to be utilized. Acoustic techniques also have been proposed as a means of detecting changes in the physical properties of the composite (Wagner et al., 1996).

Corrosion Protective Coatings

Polymeric coatings are designed to prevent contact of the underlying materials with corrosive chemicals and microorganisms. However, microbial degradation of coatings may accelerate and severely damage the underlying metals. Natural bacterial populations were found to readily form microbial biofilms on surfaces of coating materials, including epoxy and polyamide primers and aliphatic polyurethanes (Blake et al., 1998; Gu et al., 1998c; Thorp et al., 1997; Fig. 14). Surprisingly, the addition of biocides to polyurethane coatings may not inhibit bacterial attachment or growth of bacteria (Gu et al., 1998c; Mitchell et al., 1996).

Using EIS, both primers and top-coatings were monitored for their response to biodegradation by fungi. Results indicated that primers are more susceptible to degradation than are top-coats (Gu et al., 1998c).

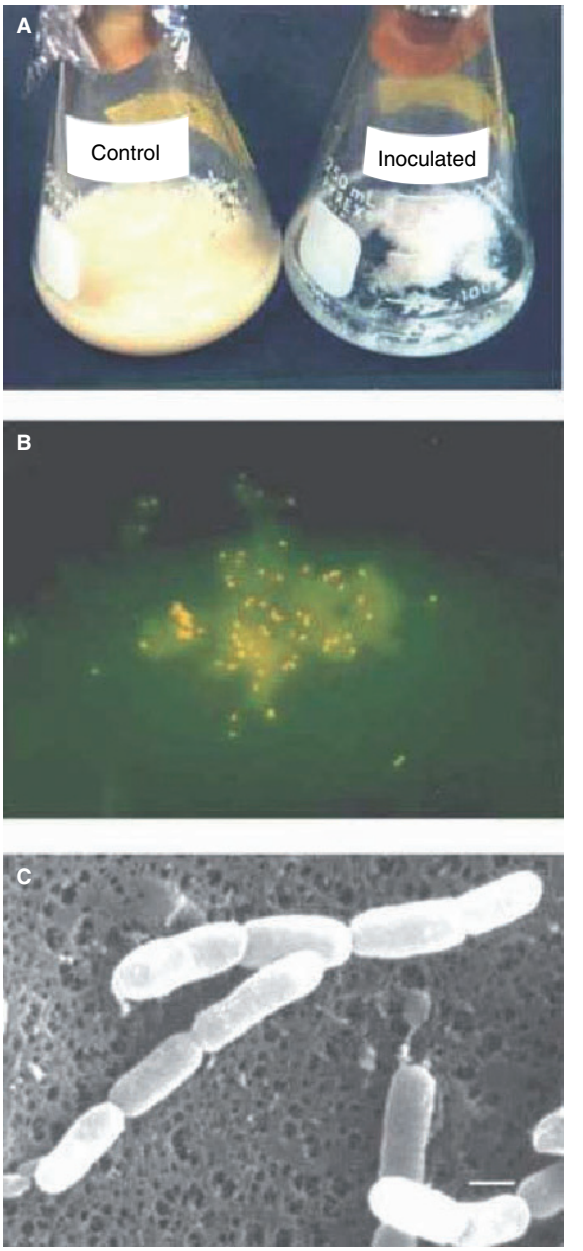

Fig. 14. A polyurethane-degrading bacterium isolated and in pure culture. A) Photograph shows clearing in the inoculated flask and a homogeneous emulsion in the control flask. B) An epifluorescence photomicrograph of the precipitated materials from the inoculated flask shows surface-associated bacteria (magnification 1,300×), and C) a scanning electron micrograph shows a bacterium capable of degrading polyurethane in pure culture (bar = 1 μm).

Nondegradable Polymers

Polyethylenes (PEs) of high and low density are primarily used in product packaging as sheets and thin films. Their degradability in natural environments poses serious environmental concerns, owing to their slow degradation rate under natural conditions and the hazard they present to freshwater and marine animals. Biodegradation of PEs has been studied extensively (Albertsson, 1980; Breslin, 1993a; Breslin and Swanson, 1993b; Iman and Gould, 1990). Prior exposure of PEs to UV promotes polymer degradation. It is believed that polymer additives, such as starch, antioxidants, coloring agents, sensitizers and plasticizers, may significantly alter the biodegradability of the parent polymers (Karlsson et al., 1988). Degradation rates may be increased by 2–4% following photosensitizer addition. However, degradation is very slow (estimated in decades). Crystallinity, surface treatment, additives, molecular weight and surfactants are all factors affecting the fate and rate of PE degradation, and they may accelerate the process.

In one study, extracellular culture concentrates of three *Streptomyces* species were inoculated to starch-containing PE films (Pometto et al., 1992; Pometto et al., 1993). It was claimed

that the PE was degraded. However, degradation may have been minimal. Other data describing degradation of PE-containing starch are questionable. Microbial metabolites may contaminate the PE surfaces and could be interpreted as degradation products of the parent PE. Abiotic degradation of PE is evident by the appearance of carbonyl functional groups in abiotic environments. In contrast, an increase of double bonds was observed when polymers showed weight loss resulting from biodegradation (Albertsson et al., 1994). It was proposed that microbial PE degradation is a two-step process: an initial abiotic photo-oxidation, followed by a cleavage of the polymer carbon backbone. However, the mechanism of the second step needs extensive analysis before plausible conclusions can be drawn. Lower molecular weight PEs including paraffin can be biodegraded. Paraffin undergoes hydroxylation oxidatively to form an alcohol group, followed by formation of carboxylic acid. At higher temperatures, ketones, alcohols, aldehydes, lactones, and carboxylic acids are formed abiotically in six weeks (Albertsson et al., 1994). Polyethylene (PE) pipes used in gas distribution systems may fail because of cracking. It is unlikely that biological processes are involved (Zhou and Brown, 1995).

Polypropylenes (PPs) are also widely utilized in engineering pipes and containers. Degradation of PPs results in a decrease of their tensile strength and molecular weight. The mechanism may involve the formation of hydroperoxides which destabilize the polymeric carbon chain to form a carbonyl group (Cacciari et al., 1993; Severini et al., 1988). Degradability of pure and high molecular weight PPs is still an open question.

Corrosion of Concrete and Stone

Concrete and stone are the most widely used materials in construction and infrastructures. Biodeterioration of these materials has important economic consequences, especially when replacement or repair of infrastructures such as bridges or municipal sewer systems is involved (Biczók, 1968; Diercks et al., 1991; Ford, 1993; Mansfeld et al., 1990; Sand and Bock, 1984; Sand et al., 1983; Sand et al., 1987b; Sand et al., 1991; Yao and Li, 1995). In addition, biodeterioration plays an important role in deterioration of stone in historic buildings, monuments and archeological sites (Arino et al., 1997; Bianchi et al., 1980; Bock et al., 1989; Cariola et al., 1987; Danin, 1993; Feddema and Mererding, 1991; Garcia de Miguel et al., 1995; Kumar and Venkataraman, 1996; Mitchell and Gu, 2000; Ortega-Calvo et al., 1993; Ortega-Calvo et al., 1995; Tiano, 1993; Tomaselli et al., 2000; Urzì and Realini, 1998). Degradation of these materials is a complex phenomenon involving chemical, physicochemical, electrochemical, and biological processes (Ascaso et al., 1998). Microorganisms have been implicated for many years (Dutton and Evans, 1996; Ezeonu et al., 1994a; Parker, 1945a; Parker, 1945b), but their involvement is poorly understood. Bacteria in the genus *Thiobacillus* were initially identified as the major culprits through biologically produced sulfuric acid, resulting in dissolution of materials. Other chemolithotrophs have been implicated in the deterioration process, including inorganic acid-producing (Atlas and Bartha, 1997; Jazsa et al., 1996a; Jazsa et al., 1996b; Sand et al., 1991), organic acid-producing (Gu et al., 1996a; Gu et al., 1998a) and exopolymer-producing bacteria (Freeman and Lock, 1995; Gehrke et al., 1998; Gu et al., 1996a; Gu et al., 1998a). Macroscopic organisms, invertebrates in particular, also participate in degradation of concrete in submerged structures, but no detailed study has been reported.

As early as 1900, corrosion of concrete sewer pipes was detected by Olmstead and Hamlin (1900). Hydrogen sulfide, an anaerobic decomposition product of sulfur-containing organic compounds and reduction of SO_4^{2-} in wastewater during microbial metabolism, was identified as the cause of corrosion (Fig. 15). The corrosion reaction was initially regarded as a purely chemical process (Lea and Desch, 1936), in which hydrogen sulfite produced under anaerobic conditions in wastewater is oxidized chemically to sulfuric acid in the presence of oxygen (Biczók, 1968). The sulfuric acid then reacts with calcium in concrete to form $CaSO_4$ or gypsum. Parker and his coworkers established the relationship

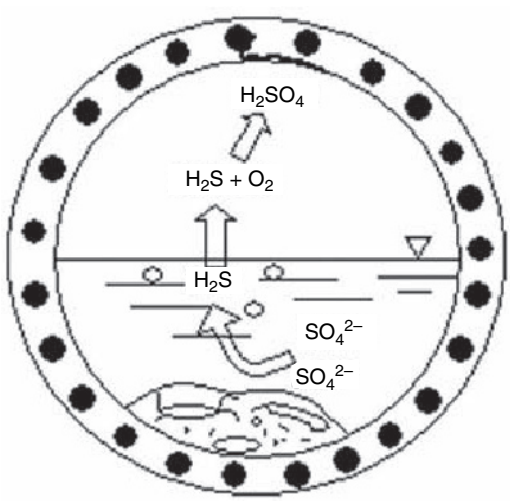

Fig. 15. A schematic of concrete corrosion by bacteria.

between acidophilic thiobacilli and concrete degradation by isolating *Thiobacillus concretivorus* (renamed "*Thiobacillus thiooxidans*") and another *Thiobacillus* species from corroded concrete (*T. neopolitabus*; Parker, 1945a; Parker, 1945b; Parker, 1947; Parker and Jackson, 1965; Parker and Prisk, 1953). The extent of corrosion correlates positively with the population of these microorganisms.

In the early 80s, rapid deterioration of newly replaced sewer systems in Hamburg, Germany, renewed interest in the corrosion processes (Milde et al., 1983; Sand and Bock, 1984; Sand et al., 1983; Sand et al., 1987b; Sand et al., 1991). In these investigations, positive correlations were observed between the extent of concrete corrosion and the numbers of *T. thiooxidans*. Sand (Sand, 1987a; Sand, 1997) also observed that *T. ferrooxidans* was associated with oxidative activity of H_2S. In the presence of sodium thiosulfate, the dominant microorganisms were *T. neapolitanus*, *T. intermedius* and *T. novellus*.

Biological corrosion of sewer pipes can be a serious problem in coastal cities, owing to the abundance of SO_4^{2-} in the wastewater. At the time of the Hamburg failure, coastal cities in the United States faced similar problems with newly installed concrete sewer systems, especially the city of Los Angeles (Mansfeld et al., 1990; Morton et al., 1991). A reason for the reemergence of the problem was the advent of the National Pollution Discharge Elimination Systems (NPDES) in 1972, which bans discharge of toxic metals and chemicals into sewers. As a result of this legislation, inorganic and organic toxic wastes were no longer permitted to be discharged directly to sewers, with a resultant increased activity of microorganisms producing large quantities of H_2S (Bitton, 1994; Somlev and Tishkov, 1994; Widdel, 1988; Fig. 15). In addition to sewers, concrete corrosion problems today involve highway bridges, historic buildings and monuments (Eckhardt, 1978; Islam et al., 1995; Jain et al., 1993; May et al., 1993), river dams (Mittelman and Danko, 1995b) and nuclear depositories (Pedersen, 1996; Stroes-Gascoyne et al., 1996) where corrosion rates are unacceptably high.

Microorganisms Responsible for Deterioration

Recent findings suggest that microorganisms participate actively in the degradation of buildings by utilization of pollutants deposited from the atmosphere as primary substrates (Corvo et al., 1997; Hutchinson et al., 1993; Lefebvre-Drouet and Rousseau, 1995; Mitchell and Gu, 2000; Ortega-Calvo, 1991; Saiz-Jimenez, 1995; Warcheid et al., 1991; Fig. 16). The predominant groups of microorganisms are those capable of sulfur oxidation (Kulpa and Baker, 1990; Sand, 1987a) and nitrification (Bock et al., 1986; Hirsch et al., 1995a; Hirsch et al., 1995b; Jazsa et al., 1996a; Sand et al., 1983; Sand et al., 1987b; Sand et al., 1991). Reported microorganisms associated with biodeterioration of building stone include *Thiobacillus*, *Desulfovibrio*, *Nitrosomonas*, *Nitrosococcus*, *Nitrobacter*, *Bacillus*, *Pseudomonas*, *Micrococcus* and *Staphylococcus*. Actinomyces include *Nocardia*, *Micropolyspora*,

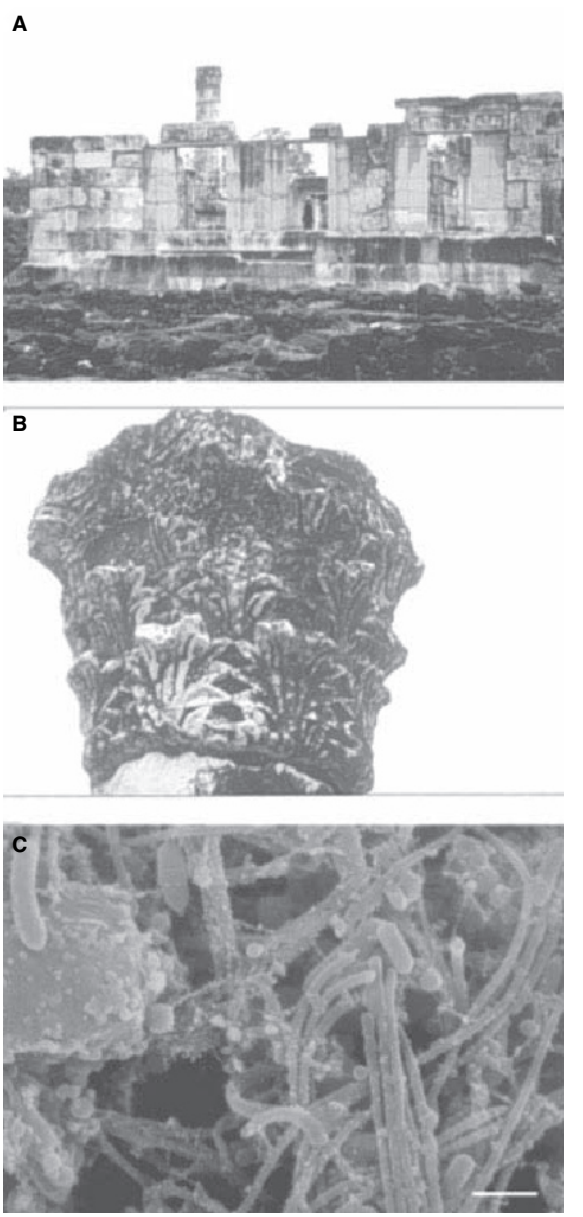

Fig. 16. Deterioration of an ancient building by microorganisms. A) A view of the building, B) a close-up of a black layer of microorganisms on the stone, and C) a scanning electron micrograph illustrating the dense complex microbial community (bar = 1 μm).

Micromonospora, *Microbispora* and *Streptomyces*. *Cyanobacteria* include *Anabaena*, *Anacystis*, *Aphanothece*, *Aulosira*, *Calothrix*, *Chlorogloea*, *Chorococcus*, *Entophysalis*, *Gloecocapsa*, *Gomphosphaeria*, *Heterohormogonium*, *Lyngsa*, *Microcystis*, *Nostoc*, *Oscillatoria*, *Phormidium*, *Porphyrosiphon*, *Schizothrix*, *Scytonema*, *Stignoema*, *Synechocystis*, and *Tolypothrix*. Organic acids, particularly from fungi, were also found to contribute to building and concrete corrosion (Gu et al., 1996a). Bacterial exopolysaccharides may also be involved in degradation of building materials (Ford et al., 1986, 1987a, 1995; Gu et al., 1998b).

Chemical and biological processes interact, resulting in the corrosion of the materials (Chen et al., 1995; Chen et al., 1996c). However, a systematic approach that integrates the role of both acid deposition and microbial activity with degradation of concrete and stone has not been attempted. Data describing stone degradation in the presence of various natural microbial populations have been made available in recent years (Flores et al., 1997; Hirsch et al., 1995b; Krumbein, 1968; Tayler and May, 1991; Tayler and May, 1994; Tiano et al., 1995; Torre et al., 1991; Torre et al., 1993a; Torre et al., 1993b). Pollutants in the air may become a source of microbial substrates on building materials and monuments (Mitchell and Gu, 2000), in the form of not only sulfurous and nitrogenous oxides but also hydrocarbons and other sulfur- and nitrogen-containing compounds (Gómez-Alarcón et al., 1995a; Gómez-Alarcón et al., 1995b; Jorgensen, 1988; Jazsa et al., 1996a; Kelley, 1981; Kelley, 1987; MacDonald, 1986; Ortega-Morales et al., 1999; Saiz-Jimenez, 1995; Schmidt, 1982). Information regarding the interaction of the natural microflora with these chemicals on concrete surfaces is still very limited.

Bock and Sand (1990) found that the nitrifying bacteria play an important role in the degradation of concrete as a result of nitric acid production during nitrification (Jazsa et al., 1996a). Nitrifying bacteria were also found to be the predominant contributors to the deterioration of other stone materials (Ehrich and Bock, 1996; Gómez-Alarcón et al., 1995a; Gómez-Alarcón et al., 1995b). These bacteria differ from the thiobacilli in that the former are capable of growth on nonimmersed surfaces (such as buildings), whereas the latter require an aqueous environment in the presence of sulfate.

Microbial Exopolymers

Microorganisms produce large quantities of exopolymeric materials in late growth phase and/or in high carbon/nitrogen (C/N) ratio environments or during adhesion to surfaces. These polymeric materials are important carbon and energy reserves and are utilized during periods of nutrient deficiency. Bacterial exopolymeric materials also play an important role in the formation of microbial biofilms (Bonet et al., 1993; Davies et al., 1998), subsequent corrosion of metals, and transport of metal ions in porous media (Chen et al., 1995; Chen et al., 1996c; Ford and Mitchell, 1992; Ford et al., 1986; Ford et al., 1987a; Ford et al., 1995). They are multifunctional group molecules (Ford et al., 1991; Paradies, 1995). The activity of these molecules and their functional groups in chelation and dissolution of calcium from concrete is still not understood. Ford and Mitchell (1992) and Geesey et al. (1986) proposed that bacterial exopolymers bind metals and promote formation of ionic concentration cells, accelerating dissolution and corrosion of metallic materials. Similarly, negatively charged carboxylic and hydroxyl groups of exopolymeric materials from thiobacilli (Fig. 17) may form complexes with calcium and leach the calcium from concrete matrices. This process may contribute to concrete degradation, particularly when biofilms grow in close proximity to the surface (Fig. 18). Further research is needed to identify a possible role for these exopolymers in concrete degradation.

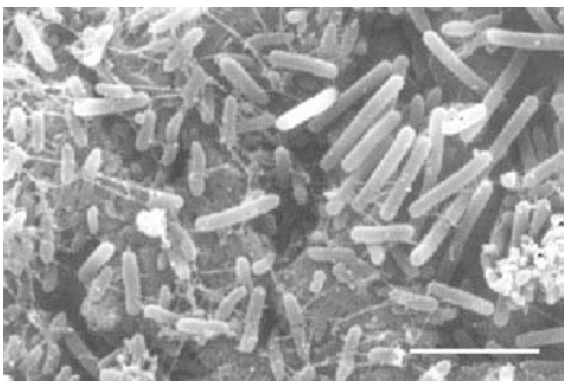

Fig. 17. Scanning electron micrograph of *Thiobacillus ferrooxidans* on the surface of corroding concrete (bar = 5 μm).

Corrosion of Reinforcing Materials

Concrete is often formulated with steel reinforcement for a wide range of applications. Reinforcement of concrete with steel bars may accelerate concrete corrosion because of the growth of SRBs and the resultant production of H_2S in submerged environments. Both SRBs and the H_2S are detrimental to steel (Gu et al., 2000a). The corrosion products expand in volume, generating stress for surrounding concrete materials and resulting in cracks in the concrete

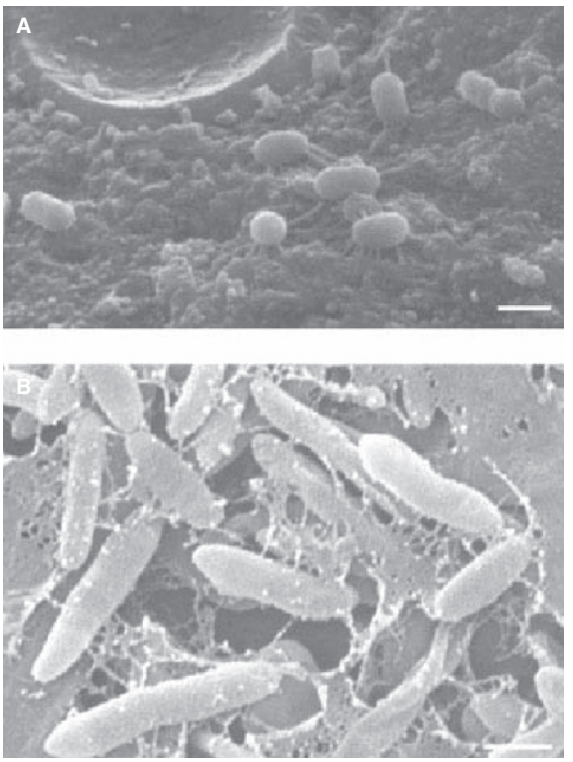

Fig. 18. Scanning electron micrographs of A) a bacterial attachment on a concrete surface (bar = 1 μm) and B) bacteria and their exopolymers (bar = 5 μm).

(Broomfield, 2000; Millard et al., 1995; Nakagawa et al., 1993; Saito et al., 1995). In this case, biological and physicochemical processes act in concert. Preventive measures involve the use of polymeric coating or reinforced polymeric sheets to prevent moisture reaching the steel reinforcing bars. Microbial activity is significantly reduced at low moisture levels (Gu et al., 1998c).

Hydrogen-producing bacteria may also contribute to the stress cracking of high strength steel. Ford and Mitchell (1990b) reported the presence of bacteria in the cracking areas of a high strength steel bar under loading conditions. They suggest that metabolites of these bacteria, particularly molecular hydrogen, would significantly weaken the strength of steel, owing to permeation of the alloy with microbially produced hydrogen (Ford et al., 1990d).

Biodeterioriation of Cultural Heritage Materials

Additional materials of interest and importance to society for protection from biodeterioration are objects with historical and cultural value. Examples of these materials are bronze (Wang et al., 1991; Wang et al., 1993; Wu et al., 1992; Zuo et al., 1994), jade, ceramic and glass (Fuchs et al., 1991), lacquer, silk, papers (Adamo et al., 1998; Arai, 2000; Fabbri et al., 1997; Florian, 1996; Zyska, 1996), paintings (Fabbri et al., 1997; Lauwers and Heinen, 1974; Rölleke et al., 1998), animal bones and shells, wood (Blanchette, 1995; King and Eggins, 1972), and mummified bodies. Figure 19 shows ancient script on paper, a textile and a bronze object, and a modern book from a library in the tropics. These materials suffer from potential biodeterioration due to the growth of microorganisms. Preservation of a variety of historic cultural materials presents a major challenge, especially where microbial activities are involved.

Wood is susceptible to a wide range of biodeteriogens including rodents, insects and microorganisms (Blanchette, 1995). Common treatment involves deep freezing and biocides. Maintenance of low humidity and low temperature are important factors in the inhibition of microbial deterioration.

Staining of historic paper manuscripts, known as "foxing," appears to have a microbial origin. Though several mechanisms of foxing found in museums and libraries (Fabbri et al., 1997; Florian, 1996) have been proposed, the cause is still not understood. In one proposed mechanism, fungal spores are believed to contribute to the development of color, and melanin synthesis may be involved (Williamson et al., 1998). Treatment using biocides is widely debated on the basis of their effectiveness, potential adverse effects on the materials, and the environmental impact of the chemicals (Hugo, 1995).

Leather and mummified materials are polymers that were dehydrated for long-term preservation. When they are being kept under low humidity and sealed conditions, long-term preservation is feasible. However, deterioration may be due to oxidation by atmospheric oxygen and adsorption of moisture. When the moisture level is increased, further growth of microorganisms occurs. One example is the deterioration of recovered mummies after their archaeological excavation.

Organic polymers are widely used in consolidation of monuments (Selwitz, 1992). Utilization of these materials by microorganisms has been documented (J-D. Gu and R. Mitchell, unpublished data) and guidelines are needed for systematic evaluation of candidate polymers and their suitability in specific applications. Polymer additives are also probable sources of carbon and energy for microbial growth (Gu et al., 1998c; Gu et al., 2000d; Tilstra and Johnsonbaugh, 1993). Physical conditions for biodeterio-

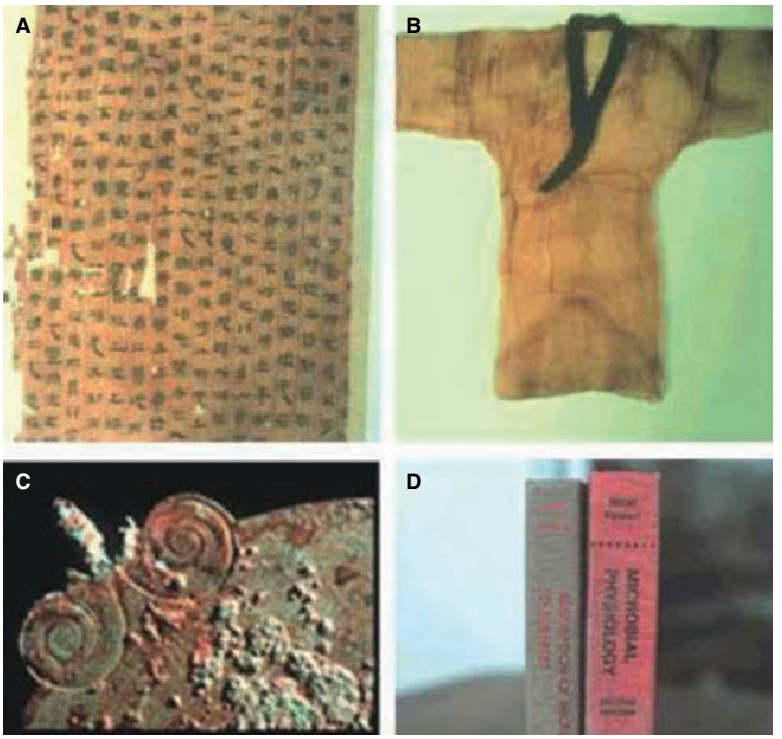

Fig. 19. Photographs showing A) an ancient script, B) textile, C) bronze, and D) a moldy book from a library in the tropics.

ration are favorable, particularly in tropical and subtropical developing countries.

The application of biocides has become a routine practice in the conservation of cultural heritage materials (Fig. 20). However, environmental issues have severely limited the number of available effective biocidal chemicals for use in conservation (Bingaman and Willingham, 1994).

Detection and Preventive Strategies

Microbial growth and propagation on material surfaces can be controlled by physical or chemical manipulation of the material or the environment. Prevention includes surface engineering to prevent microbial adhesion (Mansfeld, 1994; Matamala et al., 1994; Scamans et al., 1989; Williamson, 1994; Young, 1948). Methods of detection of growth on surfaces are well described in the literature (e.g., Madigan et al., 2000; Balow et al., 1992; Krieg and Holt, 1984; Sneath et al., 1986; Staley et al., 1989; Williams et al., 1989). Humidity control is commonly used to inhibit growth of microorganisms on surfaces in enclosed environments (Gu et al., 1998c).

Control measures require information about the biofilm population. Molecular methods permit early detection of growth on surfaces (Amann et al., 1995). Using the principle of

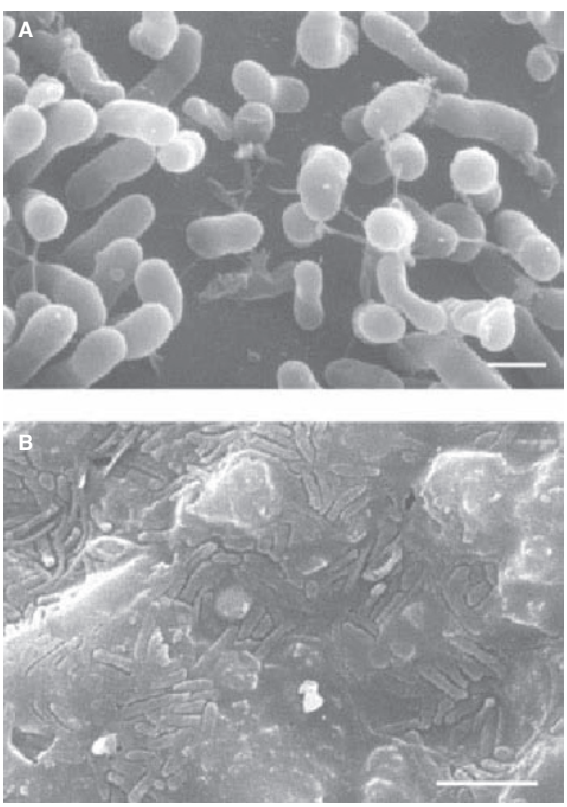

Fig. 20. Scanning electron micrographs showing A) bacterial biofilm developed on the surface of a protective coating (bar = 5 μm) and B) biofilm on the same coating, containing a biocide, but with the bacteria killed (bar = 5 μm).

modification of the microbial community, Sand et al. (1991) proposed oxygenation as a means of alleviating the propagation of SRBs. Alternatively, biocides can be effective in controlling biofilms and subsequent deterioration of materials (Bell and Chadwick, 1994; Bell et al., 1992; Wakefield, 1997). Other attempts at community modification include precipitation of microbially produced H_2S by ferrous chloride (Morton et al., 1991) and displacement of *Thiobacillus* by heterotrophic bacteria (Padival et al., 1995). All of these efforts have met with limited success.

Biocides are commonly applied in repairing, cleaning and maintenance of artworks. Chlorine and iodine compounds are used widely and routinely in controlling biofilms that cause corrosion of a wide range of industrial materials (Bloomfield and Megid, 1994; Cargill et al., 1992; Chen and Stewart, 1996b). These chemicals have been shown to be ineffective as means of killing biofilm bacteria (Huang et al., 1996; Keevil and Mackerness, 1990; Koenig et al., 1995; Liu et al., 1998; Lü et al., 1984; Lü et al., 1989; McFeters, 1991; McFeters et al., 1995; Moore and Postle, 1994; Myers, 1988; Pyle et al., 1992; Reinsel et al., 1996; Rossmoore and Rossmoore, 1993; Srinivasan et al., 1995; Stewart, 1996a; Stewart et al., 1996b; Suci et al., 1998; Wakefield, 1997; Xu et al., 1996; Yu and McFeters, 1994). In addition to their environmental unacceptability, biocides induce the development of biofilms that are highly resistant to the levels of biocide normally utilized to prevent biocorrosion. Organic biocides, used to prevent bacterial growth in industrial systems, may selectively enrich populations of microorganisms capable of biocide resistance (Fig. 21). No solution to these problems is currently available. Alternative biocides have been screened from natural products (Abdel-Hafez and El-Said, 1997; Bell and Chadwisk, 1994; Bell et al., 1992; Brözel and Cloete, 1993). Current research by materials scientists is focused on the prevention of adhesion of corrosive microorganisms to surfaces through surface treatment and modification (Costerton et al., 1988).

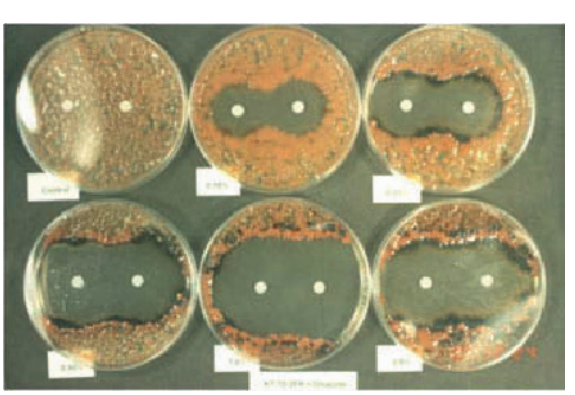

Fig. 21. At low concentrations of biocide, resistant bacteria are selected.

Since bacteria are capable of forming biofilms on most surfaces, future tests should be focused on biofilm quantification. In assaying biocide efficacy, tests should be conducted based on biofilm populations rather than on liquid culture efficacy (Gu et al., 1998c; Gu et al., 2000c). Planktonic cells are not representative of conditions on surfaces of materials.

Unfortunately routine analysis is not effective in the development of control strategies. Simulation testing of microbial growth on materials frequently includes only selected species of fungi (ASTM, 1993a; ASTM, 1993b; ASTM, 1993c; ASTM, 1993d; ASTM, 1993e). In addition, biodeterioration assessment is rarely quantitative. Modern methods are available to determine biodeterioration kinetics (Gross et al., 1993; Gross et al., 1995; Gu et al., 2000a). Recently, a sensitive method was introduced to evaluate polymer integrity using electrochemical impedance spectroscopy (Gu et al., 1998b).

Surface engineering also provides a means of preventing adhesion of microorganisms and subsequent material deterioration (Mansfeld, 1994; Matamala et al., 1994; Scamans et al., 1989; Williamson, 1994; Young, 1948; Fig. 22). Protection can be enhanced by early detection (Li et al., 1997). New detection technologies include optical fibers (Bacci, 1995). DNA probes and microarrays (Raychaudhuri et al., 2001; Salama et al., 2000) are being increasingly utilized to control biodeterioration of materials.

Conclusions

Microorganisms are involved in the corrosion of metal and the degradation and deterioration of polymers, concrete and stone under both aerobic and anaerobic conditions. They may influence the surface electrochemical properties, resulting in corrosion of metals under aerobic conditions, and induce hydrogen embrittlement by microbial production of hydrogen. Indirectly, degradation and deterioration of metals, stone and concrete are often associated with complexation mechanisms of microbial exopolysaccharides with substratum materials. We have only recently begun to understand the complex nature of interactions between the microflora and metals leading to corrosion. Modern methods in molecular biology, specifically gene technology, combined with recently developed techniques in materials science, such as laser confocal scanning microscopy and atomic force microscopy, should permit us to understand more fully the role of microorganisms in metal corrosion.

Fig. 22. Microbial adhesion and consequent deterioration only occurred at the top (dark) part of the building. The lower, protected portion was free of bacteria and deterioration.

Protection of materials can be achieved to some extent through surface engineering and control of the physical, chemical and biological environments. Application of biocides presents major challenges. A better understanding of the microbial ecology on material surfaces will be needed before a suitable control strategy can be identified. Utilization of molecular techniques to detect specific groups of microorganisms involved in the degradation process will permit a more complete view of the organization of the microbial community involved in the attack of materials. Passivation mechanisms of metals by bacteria should be further elucidated. Control methods should be developed based on combined information about both the material characteristics and the microbial community.

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Literature Cited

Abdel-Hafez, S. I. I., and A. H. M. El-Said. 1997. Effect of garlic, onion and sodium benzoate on the mycoflora of pepper, cinnamon and rosemary in Egypt. Int. Biodeter. Biodegr. 39:67–97.

Adamo, A. M., M. Giovannotti, G. Magaudda, M. Plossi Zappalà, F. Rocchetti, and G. Rossi. 1998. Effect of gamma rays on pure cellulose paper. Restaurator 19:41–59.

Albertsson, A.-C. 1980. The shape of the biodegradation curve for low and high density polyethylenes in prolonged series of experiments. Eur. Polym. J. 16:623–630.

Albertsson, A.-C., S. O. Andersson, and S. Karlsson. 1987. The mechanism of biodegradation of polyethylene. Polym. Degr. Stab. 18:73–87.

Albertsson, A.-C., C. Barenstedt, and S. Karlsson. 1994. Abiotic degradation products from enhanced environmentally degradable polyethylene. Acta Polym. 45:97–103.

Alexander, M. 1977. Ecological interrelationships. Introduction to Soil Microbiology (2nd ed.). John Wiley. New York, NY. 405–456.

Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.

Anderson, A. J., and E. A. Dowes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.

Andrady, A. L., J. E. Pegram, and S. Nakatsuka. 1993. Studies on enhanced degradable plastics. 1: The geographic variability in outdoor lifetimes of enhanced photodegrable polyethelenes. J. Environ. Polym. Degr. 1:31–43.

Angell, P., J.-S. Luo, and D. C. White. 1995. Studies of the reproducible pitting of 304 stainless steel by a consortium containing sulphate-reducing bacteria. *In:* P. Agell, S. W. Borenstein, R. A. Buchanan, S. C. Dexter, N. J. E. Dowling, B. J. Little, C. D. Lundin, M. B. McNeil, D. H. Pope, R. E. Tatnall, D. C. White, and H. G. Ziegenfuss (Eds.) International Conference on Microbial Induced Corrosion. NACE International. Houston, TX. 1/1–10.

Angles, M. L., K. C. Marshall, and A. E. Goodman. 1993. Plasmid transfer between marine bacteria in the aqueous phase and biofilms in reactor microcosms. Appl. Environ. Microbiol. 59:843–850.

Appanna, V. D., and M. S. Piperre. 1996. Aluminum elicits exocellular phosphatidylethanolamine production in Pseudomonas fluorescens. Appl. Environ. Microbiol. 62:2778–2782.

Arai, H. 2000. Foxing caused by fungi: Twenty-five years of study. Int. Biodeter. Biodegr. 46:181–188.

Arino, X., M. Hernandez-Marine, and C. Saiz-Jimenez. 1997. Colonization of Roman tombs by calcifying cyanobacteria. Phycologia 36:366–373.

Ascaso, C., J. Wierzchos, and R. Castello. 1998. Study of the biogenic weathering of calcareous litharenite stones caused by lichen and endolithic microorganisms. Int. Biodeter. Biodegr. 42:29–38.

ASTM (American Society for Testing, and Materials). 1993a. Standard practice for determining resistance of synthetic

polymeric materials to fungi. 1993 Annual Book of ASTM Standards. ASTM. Philadelphia, PA. 08.03, G21-90:527–529.

ASTM (American Society for Testing, and Materials). 1993b. Standard practice for determining resistance of plastics to bacteria. 1993 Annual Book of ASTM Standards. ASTM. Philadelphia, PA. 08.03, G22-76:531–533.

ASTM (American Society for Testing, and Materials). 1993c. Standard test method for determining the aerobic biodegradability of degradable plastics by specific microorganisms. 1993 Annual Book of ASTM Standards. ASTM. Philadelphia, PA. 08.03, D5247-92:401–404.

ASTM (American Society for Testing, and Materials). 1993d. Standard test method for assessing the aerobic biodegradation of plastic materials in an activated-sludge-wastewater-treatment system. 1993 Annual Book of ASTM Standards. ASTM. Philadelphia, PA. 08.03, D5247-92:411–416.

ASTM (American Society for Testing, and Materials). 1993e. Standard test method for determining aerobic biodegradation of plastic materials under controlled composting conditions. 1993 Annual Book of ASTM Standards. ASTM. Philadelphia, PA. 08.03, D5338-92:444–449.

Atlas, R. M., and, R. Bartha. 1997. Physiological ecology of microorganisms: adaptations to environmental conditions. Microbial Ecology: Fundamentals and Applications (4th ed.). Benjamin/Cummings Publishing. Menlo Park, CA. 281–331.

Audouard, J. P., C. Compère, N. J. E. Dowling, D. Feron, D. Festy, A. Mollica, T. Rogne, V. Scotto, U. Steinsmo, K. Taxen, and D. Thierry. 1995. Effect of marine biofilms on high performance stainless steel exposed in European coastal waters. *In:* Microbial Corrosion: Proceedings of the 3rd International European Federation of Corrosion (EFC) Workshop. European Federation of Corrosion. EFC Publication no. 15:198–210.

Bacci, M. 1995. Fibre optics applications to works of art. Sensors and Actuators B29:190–196.

Balow, A., H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer. 1992. The Prokaryotes. I, II, III, and IV:Springer-Verlag. New York, NY.

Barlaz, M. A., R. K. Ham, and D. M. Schaefer. 1989a. Mass-balance analysis of anaerobically decomposed refuse. J. Environ. Eng. 115:1088–1102.

Barlaz, M. A., D. M. Schaefer, and R. K. Ham. 1989b. Bacterial population development and chemical characterization of refuse decomposition in a simulated sanitary landfill. Appl. Environ. Microbiol. 55:55–65.

Bazylinski, D. A., A. J. Garrett-Reed, A. Abedi, and R. B. Frankel. 1993. Copper association with iron sulfide magnetosomes in a magnetotactic bacterium. Arch. Microbiol. 160:35–42.

Becker, T. W., W. E. Feumbein, T. Warscheid, and M. A. Resende. 1994. Investigation into microbiology. *In:* G. M. Herkenrath (Ed.) Investigations into Devices Against Environmental Attack on Stone. GKSS-Forschungszentrum Geesthacht, GmbH. Geesthacht, Germany. 147–190.

Bell, E., P. Dowding, and T. P. Cooper. 1992. The effect of a biocide treatment and a silicone treatment on the weathering of limestone. Environ. Technol. 13:687–693.

Bell, G. M., and J. Chadwick. 1994. Regulatory controls on biocides in the United Kingdom and restrictions on the use of triorganotin-containing antifouling products. Int. Biodeter. Biodegr. 34:375–386.

Berekaa, M. M., A. Lino, R. Reichelt, U. Keller, and A. Steinbüchel. 2000. Effect of pretreatment of rubber material on its biodegradability by various rubber degrading bacteria. FEMS Microbiol. Lett. 184:199–206.

Bérenger, J.-F., C. Frixon, J. Bigliardi, and N. Creuzet. 1985. Production, purification and properties of thermostable xylanase from Clostridium stercorarium. Can. J. Microbiol. 31:635–643.

Beveridge, T. J., S. A. Makin, J. L. Kadurugamuwa, and Z. Li. 1997. Interactions between biofilms and the environment. FEMS Microbiol. Rev. 20:291–303.

Bianchi, A., M. A. Favali, N. Barbieri, and M. Bassi. 1980. The use of fungicides on mold-covered frescoes in S. Eusebio in Pavia. Int. Biodeter. Bull. 16:45–51.

Biczok, I. 1968. Betonkorrosion, Betoschutz. Wiesbaden. Berlin, Germany. 28–30.

Bingaman, W. W., and G. L. Willingham. 1994. The changing regulatory environment: EPA registration of a new marine antifoulant active ingredient. Int. Biodeter. Biodegr. 34:387–399.

Bitton, G. 1980. Adsorption of viruses to surfaces: Technological and ecological implications. *In:* G. Bitton and K. C. Marshall (Eds.) Adsorption of Microorganisms to Surfaces. John Wiley. New York, NY. 331–374.

Bitton, G. 1994. Processes based on attached microbial growth. Wastewater Microbiology. John Wiley. New York, NY. 189–198.

Blake II, R. C., W. N. Norton, and G. T. Howard. 1998. Adherence and growth of a Bacillus species on an insoluble polyester polyurethane. Int. Biodeter. Biodegr. 42:63–73.

Blakemore, R. P. 1982. Magnetotactic bacteria. Ann. Rev. Microbiol. 36:217–238.

Blanchette, R. A. 1995. Biodeterioration of archaeological wood. Biodeter. Abstr. 9:113–127.

Bloomfield, S. F., and R. Megid. 1994. Interaction of iodine with Bacillus subtilis spores and spore forms. J. Appl. Bacteriol. 76:492–499.

Bock, E., H.-P. Koops, and H. Harms. 1986. Cell biology of nitrifying bacteria. *In:* J. I. Prosser (Ed.) Nitrification. IRL Press. Oxford, UK. 17–38.

Bock, E., B. Ahlers, and C. Myer. 1989. Biogene Korrosion von Beton- und Natursteinen durch salpetersäure bildende Bakterien. Bauphysik 11:141–144.

Bock, E., and W. Sand. 1990. Microbially influenced corrosion of concrete and natural sandstone. *In:* N. J. Dowling, M. W. Mittelman, and J. C. Danko (Eds.) Microbially Influenced Corrosion and Biodeterioration. National Association of Corrosion Engineers. Houston, TX. 3/29–3/33.

Bogan, R. T., and R. T. Brewer. 1985. Cellulose esters, organic. *In:* J. I. KroschwitzEncyclopedia of Polymer Science and Engineering (2nd ed.). John Wiley. New York, NY. 158–181.

Bonet, R., M. D. Simon-Pujol, and F. Congregado. 1993. Effects of nutrients on exopolysacchaaride production and surface properties of Aeromonas salmonicida. Appl. Environ. Microbiol. 59:2437–2441.

Booth, G. H., and A. K. Tiller. 1960. Polarization studies of mild steel in cultures of sulphate-reducing bacteria. Trans. Faraday Soc. 56:1689–1696.

Booth, G. H., and A. K. Tiller. 1962a. Polarization studies of mild steel in cultures of sulphate-reducing bacteria. Part 2: Thermophilic organisms. Trans. Faraday Soc. 58:110–115.

Booth, G. H., A. K. Tiller, and F. Wormwell. 1962b. Ancient iron nails well preserved from apparently corrosive soils. Nature (Lond.) 195:376–377.

Booth, G. H., L. Elford, and D. J. Wakerly. 1968. Corrosion of mild steel by sulfate-reducing bacteria: An alternative mechanism. Br. Corrosion. J. 3:242–245.

Borenstein, S. 1994. Microbiologically Influenced Corrosion Handbook. Woodhead Publishing. Cambridge, MA. 56.

Bos, R., H. C. van der Mei, and H. J. Busscher. 1999. Physico-chemistry of initial microbial adhesion interactions—its mechanisms and methods for study. FEMS Microbiol. Rev. 23:179–230.

Bouwer, E. J. 1992. Bioremediation of organic contaminants in the subsurface. *In:* R. Mitchell (Ed.) Environmental Microbiology. John Wiley. New York, NY. 319–333.

Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller. 1988. Pseudomonas oleovorans as a source of poly(β-hydroxy-alkanoates) for potential application as biodegradable polyesters. Appl. Environ. Microbiol. 54:1977–1982.

Breslin, V. T. 1993a. Degradation of starch-plastic composites in a muinicipal solid waste landfill. J. Environ. Polym. Degr. 1:127–141.

Breslin, V. T., R. L. Swanson. 1993b. Deterioration of starch-plastic composite in the environment. J. Air Waste Manag. Assoc. 43:325–335.

Breslin, C. B., C. Chen, and F. Mansfeld. 1997. The electrochemical behaviour of stainless steels following surface modification in cerium-containing solutions. Corrosion Sci. 39:1061–1073.

Breznak, J. A. 1984. Activity on surfaces. *In:* K. C. Marshall (Ed.) Microbial Adhesion and Aggregation. Dahlem Konferenzen, Springer-Verlag. Berlin, Germany. 203–221.

Broomfield, J. P. 2000. Corrosion of steel in concrete. *In:* R. W. Revie (Ed.) Uhlig's Corrosion Handbook (2nd ed.). John Wiley. New York, NY. 581–600.

Brown, G. A. 1982. Implications of electronic and ionic conductivities of polyimide films in integrated circuit fabrication. *In:* E. D. Feit and C. W. Wilkins (Eds.) Polymer Materials for Electronic Applications. American Chemical Society. Washington DC, ACS Symposium Ser. 184:151–169.

Brözel, V. S., and T. E. Cloete. 1993. Bacterial resistance to conventional water treatment biocides. Biodeter. Abstr. 7:387–395.

Brune, A., P. Frenzel, and H. Cypionka. 2000. Life at the oxic-anoxic interface: Microbial activities and adaptations. FEMS Microbiol Rev. 24:691–710.

Bryers, J. D. 1990a. Biofilms in biotechnology. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley. New York, NY. 733–773.

Bryers, J. D., and W. G. Characklis. 1990b. Biofilms in water and wastewater treatment. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley. New York, NY. 671–696.

Bryers, J. D. 1994. Biofilms and the technological implications of microbial cell adhesion. Colloid Surf. B: Biointerf. 2:9–23.

Buchanan, C. M., R. M. Gardner, and R. J. Komarek. 1993. Aerobic biodegradation of cellulose acetate. J. Appl. Polym. Sci. 47:1709–1719.

Budwill, K., P. M. Fedorak, and W. J. Page. 1992. Methanogenic degradation of poly(3-hydroxyalkanoates). Appl. Environ. Microbiol. 58:1398–1401.

Busscher, H. J., J. Sjollema, and H. C. van der Mei. 1990. Relative importance of surface free energy as a measure of hydrophobicity in bacterial adhesion to solid surfaces. *In:* R. J. Doyle and M. Rosenberg (Eds.) Microbial Cell Surface Hydrophobicity. American Society for Microbiology. Washington DC, 335–339.

Byrom, D. 1991. Miscellaneous biomaterials. *In:* D. Byrom (Ed.) Biomaterials: Novel Materials From Biological Sources. Macmillan. New York, NY. 335–359.

Cacciari, I., P. Quatrini, G. Zirletta, E. Mincione, V. Vinciguerra, P. Lupattelli, and G. G. Sermanni. 1993. Isotactic polyproplene biodegradation by a microbial community: Physicochemical chraracterization of metabolites produced. Appl. Environ. Microbiol. 59:3695–3700.

Caldwell, D. E., and J. R. Lawrence. 1986. Growth kinetics of Pseudomonas fluorescens microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. 12:299–312.

Caldwell, D. E., G. M. Wolfaaedt, D. R. Korber, and J. R. Lawrence. 1997. Do bacterial communities transcend Darwinism? Adv. Microb. Ecol. 15:105–191.

Callow, M. E., and R. L. Fletcher. 1994. The influence of low surface energy materials on bioadhension—a review. Int. Biodeter. Biodegr. 34:333–348.

Campaignolle, X., J.-L. Crolet. 1997. Method for studying stablization of localized corrosion on carbon steel by sulfate-reducing bacteria. Corrosion 53:440–447.

Cargill, K. L., B. H. Pyle, and G. A. McFeters. 1992. Effects of culture conditions and biofilm formation on the iodine susceptibility of Legionella pneumophila. Can. J. Microbiol. 38:423–429.

Cariola, M. G., C. Forni, and P. Albertano. 1987. Characterization of the algal flora growing on ancient Roman frescoes. Phycologia 26:387–397.

Chahal, P. S., D. S. Chahal, and G. André. 1992. Cellulase production profile of Trichoderma reesei on different celllulosic substrates at various pH levels. J. Ferment. Bioeng. 74:126–128.

Characklis, W. G. 1990. Microbial fouling. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley. New York, NY. 523–584.

Chen, G., C. R. Clayton, R. A. Sadowski, J. B. Gillow, and A. J. Francis. 1995. Influence of sulfate-reducing bacteria on the passive film formed on austenitic stainless steel, AISI 304. NACE International. Houston, TX. Corrosion.

Chen, G. 1996a. An XPS Study of the Passivity of Stainless Steels Influenced by Sulfate-reducing Bacteria (PhD dissertation). State University of New York at Stony Brook. New York, NY. 279.

Chen, X., and Stewart, P. S. 1996b. Chlorine penetration into artificial biofilm is limited by a reaction-diffusion interaction. Environ. Sci. Technol. 30:2078–2083.

Chen, G., S. V. Kagwade, G. E. French, T. E. Ford, R. Mitchell, and C. R. Clayton. 1996c. Metal ion and expolymer interaction: A surface analytical study. Corrosion 52:891–899.

Choi, M. H., and S. C. Yoon. 1994. Polyester biosynthesis characteristics of Pseudomonas citronellolis grown on various carbon sources, including 3-methyl-branched substrates. Appl. Environ. Microbiol. 60:3245–3254.

Clapp, W. P. 1948. Macro-organisms in sea water and their effect on corrosion. *In:* H. H. Uhlig (Ed.) The Corrosion Handbook. John Wiley. New York, NY. 433–441.

Clark, T. R., and H. L. Ehrlich. 1992. Copper removal from an industrial waste by bioleaching. J. Ind. Microbiol. 9:213–218.

Clayton, C. R., G. P. Halada, J. R. Kearns, J. B. Gillow, and A. J. Francis. 1994. Spectroscopic study of sulfate reducing bacteria-metal ion interactions related to microbiologically influenced corrosion (MIC). *In:* J. R. Kearns and B. J. Little (Eds.) Microbiologically Influenced Corrosion Testing. American Society for Testing and Materials. Philadelphia, PA. ASTM STP 1232:141–152.

Corbett, R. A., W. S. Morrison, and D. F. Bickford. 1987. Corrosion evaluation of alloys for nuclear waste processing. Mat. Perf. 2:40–45.

Corvo, F., C. Haces, N. Betancourt, L. Maldonado, L. Véleca, M. Echeverria, O. T. de Rincón, and A. Rincón. 1997. Atmospheric corrosivity in the Caribbean area. Corrosion Sci. 39:823–833.

Costerton, J. W., G. G. Geesey, and K.-J. Cheng. 1978. How bacteria stick. Scient. Amer. 238:86–95.

Costerton, J. W., G. G. Geesey, and P. A. Jones. 1988. Bacterial biofilms in relation to internal corrosion monitoring and biocide strategies. Mat. Perf. 4:49–53.

Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber, and G. James. 1994. Biofilms, the customized microniche. J. Bacteriol. 176:2137–2142.

Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott. 1995. Microbial biofilms. Ann. Rev. Microbiol. 49:711–745.

Crabbe, J. R., J. R. Campbell, L. Thompson, S. L. Walz, and W. W. Scultz. 1994. Biodegradation of a colloidal ester-based polyurethane by soil fungi. Int. Biodeter. Biodegr. 33:103–113.

Cromwick, A.-M., and R. A. Gross. 1995. Investigation by NMR of metabolic routes to bacterial γ-poly(glutamic acid) using ^{13}C-labelled citrate and glutamate as media carbon source. Can. J. Microbiol. 41:902–909.

Cunningham, A. B., E. J. Bouwer, and W. G. Characklis. 1990. Biofilms in poroud media. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley. New York, NY. 697–732.

Cunningham, A. M. W. G. Characklis, F. Abedeen, and D. Crawford. 1991. Influence of biofilm accumulation on porous media hydrodynamics. Environ. Sci. Technol. 25:1305–1311.

Cunningham, D. P., and L. L. Lundie Jr. 1993. Precipitation of cadmium by Clostridium thermoaceticum. Appl. Environ. Microbiol. 59:7–14.

Dalton, H. M., L. K. Poulsen, P. Halasz, M. L. Angles, A. E. Goodman, and K. C. Marshall. 1994. Substratum-induced morphological changes in a marine bacterium and their relevance to biofilm structure. J. Bacteriol. 176:6900–6906.

Daniels, L., N. Belay, B. Rajagopal, and P. Weimer. 1987. Bacterial methanogenesis and growth from CO_2 with elemental ion as the sole source of electrons. Science 237:509–511.

Danin, A. 1993. Biogenic weathering of marble monuments in Didim, Turkey and in Trajan Column, Rome. Water Sci. Technol. 27:557–563.

Davey, M. E., and G. A. O'Toole. 2000. Microbial biofilms: From ecology to molecular genetics. Microbiol. Molec. Biol. Rev. 64:847–867.

Davidson, D., B. Beheshti, and M. W. Mittelman. 1996. Effects of Arthrobacter sp., Acidovorax delafieldii, and Bacillus megaterium colonisation on copper solvency in a laboratory reactor. Biofouling 9:279–292.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298.

Devanathan, M. A. V., and Z. Stachurski. 1962. Adsorption and diffusion of electrolytic hydrogen in palladium. Proc. R. Soc. Lond. A.270:90–110.

Dexter, S. C. 1993. Role of microfouling organisms in marine corrosion. Biofouling 7:97–127.

Dickinson, W. H., F. Caccavo Jr., and Z. Lewandowski. 1996. The ennoblement of stainless steel by manganic oxide biofouling. Corrosion Sci. 38:1407–1422.

Dickinson, W. H., F. Caccavo, Jr., B. Olesen, and Z. Lewandowski. 1997. Enoblement of stainless steel by the manganese-depositing bacterium Leptothrix discophora. Appl. Environ. Microbiol. 63:2502–2506.

Diercks, M., W. Sand, and E. Bock. 1991. Microbial corrosion of concrete. Experientia 47:514–516.

Dobbins, J. J., B. L. Giammara, J. S. Hanker, P. E. Yates, and W. C. DeVries. 1989. Demonstration of the bacterial-biomaterial interface in implant specimens. *In:* J. S. Hanker and B. L. Giammara (Eds.) Biomedical Materials and Devices. Materials Research Society. Philadelphia, PA. 110:337–348.

Doi, Y. 1990. Biodegradation of microbial polyesters. Microbial Polyesters. VCH Publishers. New York, NY. 135–152.

Douglas, S., and T. J. Beveridge. 1998. Mineral formation by bacteria in natural microbial communities. FEMS Microbiol. Ecol. 26:79–88.

Dowling, N. J. E., S. A. Brooks, T. J. Phelps, and D. C. White. 1992. Effects of selection and fate of substrate supplied to anaerobic bacteria involved in the corrosion of pipeline steel. J. Ind. Microbiol. 10:207–215.

Dowling, N. J. E., and J. Guezenec. 1997. Microbially influenced corrosion. *In:* C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter (Eds.) Manual of Environmental Microbiology. ASM Press. Washington DC, 842–855.

Downing, K. M., C. S. Ho, and D. W. Zabriskie. 1987. Enzymatic production of ethanol from cellulose using soluble cellulose acetate as an intermediate. Biotech. Bioeng. 29:1086–1096.

Drake, H. 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: past and current perspectives. Acetogenesis. Chapman and Hall. New York, NY. 1–60.

Dunn, D. S., N. Sridhar, and G. A. Cragnolino. 1995. Effects of surface chromium depletion on localized corrosion of alloy 825 as a high-level nuclear waste container material. Corrosion 51:618–624.

Dutton, M. V., and C. S. Evans. 1996. Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. Can. J. Microbiol. 42:881–895.

Dwyer, D., and J. M. Tiedje. 1983. Degradation of ethylene glycol and polyethylene glycols by methanogenic consortia. Appl. Environ. Microbiol. 46:185–190.

Eashwar, M., S. Martrhamuthu, S. Palanichamy, and K. Balakrishnan. 1995. Sunlight irradiation of seawater eliminates ennoblement-causation by biofilms. Biofouling 8:215–221.

Eckhardt, F. E. W. 1978. Microorganisms and weathering of a sandstone monument. *In:* W. E. Krumbein (Ed.) Environmental Biogeochemistry and Geomicrobiology, Vol. 2: The terrestrial Environment. Ann Arbor Science Publishers. Ann Arbor, MI. 675–586.

Edwards, D. P., T. G. Nevell, B. A. Plunkett, and B. C. Ochiltree. 1994. Resistance to marine fouling of elasto-

meric coatings of some poly(dimethylsiloxanes) and poly(dimethyldiphenylsiloanes). Int. Biodeter. Biodegr. 34:349–359.

Ehrich, S., and E. Bock. 1996. Biogenic sulfuric acid corrosion test procedure for cement bound materials. *In:* W. Sand (Ed.) DECHEMA Monographs, Vol. 133: Biodeterioration and Biodegradation. VCH Verlagesellschäft. Frankfurt, Germany. 193–198.

Ehrlich, H. L. 1996. Geomicrobial processes: a physiological and biochemical overview. Geomicrobiology (3rd ed.). Marcel Dekker. New York, NY. 108–142.

El-Sayed, A. H. M. M., W. M. Mohmoud, E. M. Davis, and R. W. Coughlin. 1996. Biodegradation of polyurethane coatings by hydrocarbon-degrading bacteria. Int. Biodeter. Biodegr. 37:69–79.

Emerson, A., and C. Moyer. 1997. Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. Appl. Environ. Microbiol. 63:4784–4792.

Enos, D. G., and S. R. Taylor. 1996. Influence of sulfate-reducing bacteria on alloy 625 and austenitic stainless steel wedments. Corrosion 52:831–842.

Evans, U. R. 1948. An outline of corrosion mechanisms, including the electrochemical theory. *In:* H. H. Uhlig (Ed.) The Corrosion Handbook. John Wiley. New York, NY. 3–11.

Ezeonu, I. M., J. A. Noble, R. B. Simmons, D. L. Price, S. A. Crow, and D. G. Ahearn. 1994a. Effect of relative humidity on fungal colonization of fiberglass insulation. Appl. Environ. Microbiol. 60:2149–2151.

Ezeonu, I. M., D. L. Price, R. B. Simmons, S. A. Crow, and D. G. Ahearn. 1994b. Fungal production of volatiles during growth on fiberglass. Appl. Environ. Microbiol. 60:4172–4173.

Fabbri, A. A., A. Ricelli, S. Brasini, and C. Fanelli. 1997. Effect of different antifungals on the control of paper biodeterioration caused by fungi. Int. Biodeter. Biodegr. 39:61–65.

Feddema, J. J., and T. C. Mererding. 1991. Marble deterioration in the urban atmosphere. *In:* N. S. Baer, C. Sabbioni, and A. I. Sors (Eds.) Science, Technology and European Cultural Heritage. Butterworth-Heinemann. Oxford, England. 443–446.

Fenchel, T., and B. J. Finlay. 1995. Anaerobic environments. Ecology and Evolution in Anoxic Worlds. Oxford University Press. New York, NY. 1–31.

Ferry, J. G. 1995. Physiological ecology of methanogens. *In:* J. G. Ferry (ed.) Methanogenesis: Ecology, Physiology, Biochemistry and Genetics. Chapman and Hall. New York, NY. 128–206.

Filip, Z. 1978. Decomposition of polyurethane in a garbage landfill leakage water and by soil microorganisms. Eur. J. Appl. Microbiol. 5:225–231.

Fischer, W., I. Haenßel, and H. H. Paradies. 1987. Gutachten: Schadensanalyse von Korrosionshäden an Brauchwasserleitungen aus Kupferrohren im Krieskrankenhaus Lüdenscheid/Hellersen, FRG. Märkischer Kreis 17 March.

Flemming, H.-C., G. Schaule, R. McDonogh, and H. F. Ridgway. 1994. Effects and extent of biofilm accumulation in membrane systems. *In:* G. G. Geesey, Z. Lewandowski, and H.-C. Flemming (Eds.) Biofouling and Biocorrosion in Industrial Water Systems. Lewis Publishers. Boca Raton, FL. 63–89.

Fletcher, M., and G. I. Loeb. 1979. Influence of substratum characteristics on the attachment of a marine Pseudomonad to solid surfaces. Appl. Environ. Microbiol. 37:67–72.

Fletcher, M. 1980. Adherence of marine micro-organisms to smooth surfaces. *In:* E. H. Beachey (Ed.) Bacterial Adherence. Chapman and Hall. London, Receptors and Recognition, Series B, Vol. 6:345–374.

Fletcher, M. 1996. Bacterial attachment in aquatic environments: A diversity of surfaces and adhesion strategies. *In:* M. Fletcher (Ed.) Bacterial Adhesion: Molecular and Ecological Diversity. Wiley-Liss. New York, NY. 1–24.

Flores, M., J. Lorenzo, and G. Gomez-Alarcon. 1997. Algae and bacteria on historic monuments at Alcala de Henares, Spain. Int. Biodeter. Biodegr. 40:241–246.

Florian, M.-L. E. 1996. The role of the conidia of fungi in fox spots. Studies Conserv. 41:65–75.

Ford, T., J. P. Black, and R. Mitchell. 1986. Relationship between bacterial exopolymers and corroding metal surfaces. National Association of Corrosion Engineers. Houston, TX. Corrosion/90 110:

Ford, T., J. Maki, and R. Mitchell. 1987a. The role of metal-binding bacterial exopolymers in corrosion processes. National Association of Corrosion Engineers. Houston, TX. Corrosion/87 380:

Ford, T. E., M. Walch, and R. Mitchell. 1987b. Corrosion of metals by thermophilic microorganisms. Mat. Perf. 26:35–39.

Ford, T., J. Maki, and R. Mitchell. 1988. Involvement of bacterial exopolymers in biodeterioration of metals. *In:* D. R. Houghton, R. N. Smith, and H. O. W. Eggins (Eds.) Biodeterioration. Elsevier Science Publishing. New York, NY. 7:378–384.

Ford, T., and R. Mitchell. 1990a. Metal embrittlement by bacterial hydrogen—an overview. Marine Technol. Soc. J. 24:29–35.

Ford, T., and R. Mitchell. 1990b. The ecology of microbial corrosion. *In:* K. C. Marshall (Ed.) Advances in Microbial Ecology. Plenum Press. New York, NY. 11:231–262.

Ford, T. E., P. C. Seaison, T. Harris, and R. Mitchell. 1990c. Investigation of microbiologically produced hydrogen permeation through palladium. J. Electrochem. Soc. 137:1175–1179.

Ford, T. E., J. P. Black, and R. Mitchell. 1990d. Relationship between bacterial exopolymers and corroding metal surfaces. NACE International. Houston, TX. Corrosion.

Ford, T., E. Sacco, J. Black, T. Kelley, R. Goodacre, R. C. W. Berkley, and R. Mitchell. 1991. Characterization of exopolymers of aquatic bacteria by pyrolysis-mass spectroscopy. Appl. Environ. Microbiol. 57:1595–1601.

Ford, T., and R. Mitchell. 1992. Microbial transport of toxic metals. *In:* R. Mitchell (Ed.) Environmental Microbiology. John Wiley. New York, NY. 83–101.

Ford, T. E. 1993. The microbial ecology of water distribution and outfall systems. *In:* T. E. Ford (Ed.) Aquatic Microbiology: An Ecological Approach. Blackwell Scientific Publishers. Boston, MA. 455–482.

Ford, T., J. Maki, and R. Mitchell. 1995. Metal-microbe interactions. *In:* C. C. Gaylarde and H. A. Videla (Eds.) Bioextraction and Biodeterioration of Metals. Cambridge University Press. New York, NY. 1–23.

Fortin, D., G. Southam, and T. J. Beveridge. 1994. Nickel sulfide, iron-nickel silfide and iron sulfide precipitation by a newly isolated Desulfotomaculum species and its relation to nickel resistance. FEMS Microbiol. Ecol. 14:121–132.

Frazer, A. C. 1994. O-methyylation and other transformations of aromatic compounds by acetogenic bacteria. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 445–483.

Freeman, C., and M. A. Lock. 1995. The biofilm polysaccharide matrix: A buffer against chaging organic substrate supply? Limnol. Oceanogr. 40:273–278.

Frings, J., E. Schramm, and B. Schink. 1992. Enzymes involved in anaerobic polyethylene glycol degradation by Pelobacter venetianus and Bacteroides Strain PG1. Appl. Environ. Microbiol. 58:2164–2167.

Fuchs, D. R., M. Popall, H. Römich, and H. Schmidt. 1991. Preservation of stained glass windows: New materials and techniques. *In:* N. S. Baer, C. Sabbioni, and A. I. Sors (Eds.) Science, Technology and European Cultural Heritage. Butterworth-Heinemann. Oxford, England. 679–683.

Gamerith, G., R. Groicher, S. Zeilinger, P. Herzog, C. P. Kubicek. 1992. Cellulase-poor xylanases produced by Trichoderma reesei RUT C-30 on hemicellulose substrates. Appl. Microbiol. Biotechnol. 38:315–322.

Gangloff, R. P., and R. G. Kelly. 1994. Microbe-enhanced environmental fatigue crack propagation in HY130 steel. Corrosion 50:345–354.

Garcia de Miguel, J. M., L. Sanchez-Castillo, J. J. Ortega-Calvo, J. A. Gil, and C. Saiz-Jimenez. 1995. Deterioration of building materials from the Great Jaguar Pyramid at Tikal, Guatemala. Building and Environment 30:591–598.

Gazenko, O. G., A. I. Grigoryev, S. A. Bugrov, V. V. Yegorov, V. V. Bogomolov, I. B. Kozlovskaya, and I. K. Tarasov. 1990. Review of the major results of medical research during the flight of the second prime crew of the Mir space station [in Russian]. Kosmicheskaya Biologiya i Aviakosmicheskaya Medistina 23:3–11.

Gebers, R., and P. Hirsch. 1978. Isolation and investigation of Pedomicrobium spp., heavy metal-depositing bacteria from soil habitats. *In:* W. E. Krumbein (Ed.) Environmental Biogeochemistry and Geomicrobiology: Methods, Metals and Assessment. Ann Arbor Science Publishers. Ann Arbor, MI. 3:911–922.

Geesey, G. G., M. W. Mittelman, T. Iwaoka, and P. R. Griffiths. 1986. Role of bacterial exopolymers in the deterioration of copper surfaces. Mat. Perf. 25:37–40.

Geesey, G. G., and D. C. White. 1990. Determination of bacterial growth and activity at solid-liquid interfaces. Ann. Rev. Microbiol. 44:579–602.

Geesey, G. G., R. J. Gills, R. Avci, D. Daly, M. Hamilton, P. Shope, and G. Harkon. 1996. The influence of surface features on bacterial colonization and subsequent substratum chemical changed of 316L stainless steel. Corrosion Sci. 38:73–95.

Gehrke, T., J. Telegdi, D. Thierry, and W. Sand. 1998. Importance of extracellular polymeric substances from Thiobacillus ferrooxidans for bioleaching. Appl. Environ. Microbiol. 64:2743–2747.

Gelmi, M., P. Apostoli, E. Cabibbo, S. Porrru, L. Alessio, and A. Turano. 1994. Resistance to cadmium salts and metal absorption by different microbial species. Curr. Microbiol. 29:335–341.

Ghassem, H., and N. Adibi. 1995. Bacterial corrosion of reformer heater tubes. Mat. Perf. 34:47–48.

Ghiorse, W. C., and P. Hirsch. 1978. Iron and manganse deposition by budding bacteria. *In:* W. E. Krumbein (Ed.) Environmental Biogeochemistry and Geomicrobiology, Vol. 3: Methods, Metals and Assessment. Ann Arbor Science Publishers. Ann Arbor, MI. 897–909.

Ghiorse, W. C., and P. Hirsch. 1979. An ultrastructural study of iron and manganese deposition associated with extracellular polymers of Pedomicrobium-like budding bacteria. Arch. Microbiol. 123:213–226.

Ghiorse, W. C. 1989. Manganese and iron as physiological electron donors and acceptors in aerobic-anaerobic transition zones. *In:* Y. Cohen and E. Rosenberg (Eds.) Microbial Mats: Physiological Ecology of Benthic Microbial Communities. American Society for Microbiology. Washington DC, 163–169.

Gibson, D. T., and Subramanian, V. 1984. Microbial degradation of aromatic hydrocarbons. *In:* D. T. Gibson (ed.) Microbial Degradation of Organic Compounds. Marcel Dekker. New York, NY. 181–252.

Gillatt, J. 1990. The biodeterioration of polymer emulsions and its prevention with biocides. Int. Biodeter. 26:205–216.

Gillis, R. J., and J. R. Gillis. 1996. A comparative study of bacterial attachment to high-purity water system surfaces. Ultrapure Water 9:27–36.

Gilmore, D. F., S. Antoun, R. W. Lenz, S. Goodwin, R. Austin, and R. C. Fuller. 1992. The fate of biodegradable plastics in municipal leaf compost. J. Indust. Microbiol. 10:199–206.

Gilmore, D. F., S. Antoun, R. W. Lenz, and R. C. Fuller. 1993. Degradation of poly(β-hydroxyalkanoates) and polyolefin blends in a municiple wastewater treatment facility. J. Environ. Polym. Degr. 1:269–274.

Glagolev, A. N. 1984. Motility and Taxis in Prokaryotes. *In:* V. P. Skulachev (Ed.) Soviet Scientific Reviews Supplement Series: Physiocochemical Biology. Harwood Academic Publishers. Zurich, Switzerland. 59–64.

Gómez-Alarcón, G., B., Cilleros. M. Flores, and J. Lorenzo. 1995a. Microbial communities and alteration processes in monuments at Alcala de Henares, Spain. Sci. Total Environ. 167:231–239.

Gómez-Alarcón, G., M. Munoz, X. Arino, and J. J. Ortega-Calvo. 1995b. Microbial communities in weathered sandstone: The case of Carrascosa del Campo church, Spain. Sci. Total Environ. 169:249–254.

Gottschalk, G. 1986. Regulation of bacterial metabolism. Bacterial Metabolism (2nd ed.). Springer-Verlag. New York, NY. 178–282.

Gross, R. A., J.-D. Gu, D. Eberiel, M. Nelson, and S. P. McCarthy. 1993. Cellulose acetate biodegradability in simulated aerobic composting and anaerobic bioreactors as well as by a bacterial isolate derived from compost. *In:* D. Kaplan, E. Thomas, and C Ching (Eds.) Fundamentals of Biodegradable Materials and Packaging. Technomic Publishing. Lancaster, PA. 257–279.

Gross, R. A., J.-D. Gu, D. Eberiel, and S. P. McCarthy. 1995. Laboratory scale composting test methods to determine polymer degradability: Model studies on cellulose acetate. *In:* A. Albertson and S. Huang (Eds.) Degradable Polymers, Recycling and Plastics Waste Management. Marcel Dekker. New York, NY. 21–36.

Gu, J.-D., and D. F. Berry. 1991. Degradation of substituted indoles by an indole-degrading methanogenic consortium. Appl. Environ. Microbiol. 57:2622–2627.

Gu, J.-D., and D. F. Berry. 1992a. Metabolism of 3-methylindole by a methanogenic consortium. Appl. Environ. Microbiol. 58:2667–2669.

Gu, J.-D., D. F. Berry, R. H. Taraban, D. C. Martens, H. L. Walker Jr., and W. J. Edmonds. 1992b. Biodegradability of atrazine, cyanazine, and dicamba in wetland soils. Virginia Water Resource Research Center, Bull.

Gu, J.-D., M. Gada, G. Kharas, D. Eberiel, S. P. McCarty, and R. A. Gross. 1992c. Degradability of cellulose acetate (1. 7 and 2. 5, d. s.) and poly(lactide) in simulated composting bioreactors. Polymer Materials: Science and Engineering 67:351–352.

Gu, J.-D., S. P. McCarty, G. P. Smith, D. Eberiel, and R. A. Gross. 1992d. Degradability of cellulose aceteate (1.7, d.s.) and cellophane in anaerobic bioreactors. Polymer Materials: Science and Engineering 67:230–231.

Gu, J.-D., S. Coulter, D. Eberiel, S. P. McCarthy, and R. A. Gross. 1993a. A respirometric method to measure mineralization of polymeric materials in a matured compost environment. J. Environ. Polym. Degr. 1:293–299.

Gu, J.-D., D. T. Eberiel, S. P. McCarthy, and R. A. Gross. 1993b. Cellulose acetate biodegradability upon exposure to simulated aerobic composting and anaerobic bioreactor environments. J. Environ. Polym. Degr. 1:143–153.

Gu, J.-D., D. Eberiel, S. P. McCarthy, and R. A. Gross. 1993c. Degradation and mineralization of cellulose acetate in simulated thermophilic composting environment. J. Environ. Polym. Degr. 1:281–291.

Gu, J.-D., T. E. Ford, K. E. G. Thorp, and R. Mitchell. 1994a. Microbial degradation of polymeric materials. *In:* T. Naguy (Ed.) Proceedings of the Tri-Service Conference on Corrosion., June 21–23, Orlando, Florida. US Government Printing House. Washington DC, 291–302.

Gu, J.-D., S. Yang, R. Welton, D. Eberiel, S. P. McCarthy, and R. A. Gross. 1994b. Effects of environmental parametrs on the degradability of polymer films in laboratory-scale composting reactors. J. Environ. Polym. Degr. 2:129–135.

Gu, J.-D., and R. Mitchell. 1995a. Microbiological influenced corrosion of metal, degradationa and deterioration of polymeric materials of space application [in English]. Chinese J. Mat. Res. 9 (Suppl.):473–489.

Gu, J.-D., T. E. Ford, B. Mitton, and R. Mitchell. 1995b. Microbial degradation of complex polymeric materials used as insulattion in electronic packaging materials. National Association of Corrosion Engineers. Houston, TX. Corrosion.

Gu, J.-D., T. E. Ford, K. E. G. Thorp, and R. Mitchell. 1995c. Effects of microorganisms on stability of fiber reinforced polymeric composites. *In:* D. Hui (ed.) Second International Conference on composites engineering, August 21–24, New Orleans. University of New Orleans. New Orleans, LA. 279–280.

Gu, J.-D., T. E. Ford, K. E. G. Thorp, and R. Mitchell. 1995d. Microbial biodeterioration of fiber reinforced composite materials. *In:* P. Angell, S. W. Borenstein, R. A. Buchanan, S. C. Dexter, N. J. E. Dowling, B. J. Little, C. D. Lundin, M. B. McNeil, D. H. Pope, R. E. Tatnall, D. C. White, and H. G. Zigenfuss (Eds.) 1995 International Conference on Microbial Influenced Corrosion. NACE International. Houston, TX. 25/1–7.

Gu, J.-D., T. E. Ford, K. E. G. Thorp, and R. Mitchell. 1995e. Microbial deterioration of fiber reinforced polymeric materials. *In:* J. Scully (Ed.) Corrosion/95, Research in Progress Symposium. NACE International. Houston, TX. 16–17.

Gu, J.-D., T. E. Ford, N. S. Berke, and R. Mitchell. 1996a. Fungal degradation of concrete. *In:* W. Sand (Ed.) DECHEMA Monographs, Vol. 133: Biodeterioration and Biodegradation. VCH Publishers. Frankfurt, Germany. 135–142.

Gu, J.-D., T. E. Ford, and R. Mitchell. 1996b. Susceptibility of electronic insulating polyimides to microbial degradation. J. Appl. Polym. Sci. 62:1029–1034.

Gu, J.-D., T. Ford, K. Thorp, and R. Mitchell. 1996c. Microbial growth on fiber reinforced composite materials. Int. Biodeter. Degr. 39:197–204.

Gu, J.-D., C. Lu, K. Thorp, A. Crasto, and R. Mitchell. 1996d. Susceptibility of polymeric coatings to microbial degradation. NACE International. Houston, TX. Corrosion.

Gu, J.-D., K. Thorp, A. Crasto, and R. Mitchell. 1996e. Microbiological degradation of fiber-reinforced polymeric composites. *In:*The Electrochemical Society Spring Meeting, May 5–10, Los Angeles. The Electrochemical Society. Pennington, NJ. 143–144.

Gu, J.-D., C. Lu, K. Thorp, A. Crasto, and R. Mitchell. 1997a. Fiber-reinforced polymeric composite materials are susceptible to microbial degradation. J. Indust. Microbiol. Biotechnol. 18:364–369.

Gu, J.-D., C. Lu, K. Thorp, A. Crasto, and R. Mitchell. 1997b. Fungal degradation of fiber-reinforced composite materials. Mat. Perf. 36:37–42.

Gu, J.-D., J. S. Maki, and R. Mitchell. 1997c. Microbial biofilms and their role in the induction and inhibition of invertebrate settlement. *In:* F. M. D'Itri (Ed.) Zebra Mussels and Aquatic Nuisance Species. Ann Arbor Press. Chelsea, MI. 343–357.

Gu, J.-D., T. E. Ford, N. S. Berke, and R. Mitchell. 1998a. Biodeterioration of concrete by the fungus Fusarium. Int. Biodeter. Biodegr. 41:101–109.

Gu, J.-D., D. B. Mitton, T. E. Ford, and R. Mitchell. 1998b. Microbial degradation of polymeric coatings measured by electrochemical impedance spectroscopy. Biodegr. 9:35–39.

Gu, J.-D., M. Roman, T. Esselman, and R. Mitchell. 1998c. The role of microbial biofilms in deterioration of space station candidate materials. Int. Biodeter. Biodegr. 41:25–33.

Gu, J.-D., T. E. Ford, and R. Mitchell. 2000a. Microbial corrosion of metals. *In:* W. Revie (Ed.) The H. H. Uhlig Corrosion Handbook (2nd ed.). John Wiley. New York, NY. 915–927.

Gu, J.-D., T. E. Ford, and R. Mitchell. 2000b. Microbial corrosion of concrete. *In:* W. Revie (Ed.) The H. H. Uhlig Corrosion Handbook (2nd ed.). John Wiley. New York, NY. 477–491.

Gu, J.-D., T. E. Ford, and R. Mitchell. 2000c. Microbial degradation of materials: General processes. *In:* W. Revie (Ed.) The H. H. Uhlig Corrosion Handbook (2nd ed.). John Wiley. New York, NY. 349–365.

Gu, J.-D., T. E. Ford, D. B. Mitton, and R. Mitchell. 2000d. Microbial degrdation and deterioration of polymeric materials. *In:* W. Revie (Ed.) The H. H. Uhlig Corrosion Handbook (2nd ed.). John Wiley. New York, NY. 439–460.

Gu, J.-D., J.-G. Gu, and X. Y. Li. 2000e. Degradation of poly(hydroxybutyrate-co-16% valerate) and cellulose acetate (DS 1. 7 and 2. 5) under simulated landfill condition. *In:* Bao, X. and Englande, A. J. (eds.) Critical Technologies to the World in 21th Century: Pollution Control and Reclamation in Process Industries, September 18–20, 2000. International Water Association (IWA). Beijing, P.R. China. 564–572.

Gu, J-D., and K. H. Cheung. 2001a. Phenotypic expression of Vogesella indigofura upon exposure to hexavalent chromium, Cr^{6+}. World J. Microbiol. Biotechnol. [in press] 17:475–480.

Gu, J.-D., B. Belay, and R. Mitchell. 2001b. Protection of catheter surfaces from adhesion of Pseudomonas aeruginosa by a combination of silver ions and lectins. World J. Microbiol. Biotechnol. 17:173–179.

Gu, J.-D., J.-G. Gu, H. C. Shi, and X. Y. Li. 2001c. Simulating anaerobic landfill conditions in bioreactors and testing polymer degradability using poly(β-hydroxybutyrate-co-16% valerate) and cellulose acetates (DS 1.7 and 2.5). Water Sci. Technol. in press:in press.

Guezennec, J., O. Ortega-Morales, G. Raguenes, G. Geesey. 1998. Bacterial colonization of artificial substrate in the vicinity of deep-sea hydrothermal vents. FEMS Microbiol. Ecol. 26:89–99.

Gujer, W., and A. J. B. Zehnder. 1983. Conversion processes in anaerobic digestion. Water Sci. Technol. 15:127–167.

Gunjala, K. R., and J. M. Sulflita. 1993. Environmental factors influencing methanogenesis from refuse in landfill samples. Environ. Sci. Technol. 27:1176–1181.

Hadley, R. F. 1948. Corrosion by micro-organisms in aqueous and soil environments. *In:* H. H. Uhlig (Ed.) The Corrosion Handbook. John Wiley. New York, NY. 466–481.

Hahn, P. O., G. W. Rubloff, J. W. Bartha, F. Legoues, R. Tromp, and P. S. Ho. 1985. Chemical interactions at metal-polymer interfaces. Mat. Res. Soc. Symp. Proc. 40:251–263.

Hamilton, W. A. 1985. Sulphate-reducing bacteria and anaerobic corrosion. Ann. Rev. Microbiol. 39:195–217.

Hamilton, J. D., K. H. Reinert, J. V. Hogan, and W. V. Lord. 1995. Polymers as solid waste in municipal landfills. J. Air Waste Manag. Assoc. 43:247–251.

Hanert, H. H. 1981. The Genus Siderocapsa (and other iron- or manganese-oxidizing eubacteria). *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balow, and H. G. Schlegel (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 1:1049–1060.

Hass, H., E. Herfurth, G. Stöffler, and B. Redl. 1992. Purification, characterization and partial amino acid sequences of a xylanase produed by Penicillium chrysogenum. Biochim. Biophys. Acta 1117:279–286.

Heisey, R. M., and S. Papadatos. 1995. Isolation of microorganisms able to metabolize purified natural rubber. Appl. Environ. Microbiol. 61:3092–3097.

Hernandez, G., V. Kucera, D. Thierry, A. pedersen, and M. Hermansson. 1994. Corrosion inhibition of steel by bacteria. Corrosion 50:603–608.

Hespell, R. B., and P. J. O'Bryan-Shah. 1988. Esterase activities in Butyrivibrio fibrisolvens strains. Appl. Environ. Microbiol. 54:1917–1922.

Hill, E. C. 1987. Microbial Problems in the Off-Shore Oil Industry. Institute of Petroleum. London, 25–28.

Hirsch, P., F. Eckhardt, and R. J. Palmer. 1995a. Fungi active in weathering of rock and stone monuments. Can. J. Botany 73:1384–1390.

Hirsch, P., F. E. W. Eckhardt, and R. J. Palmer Jr. 1995b. Methods for the study of rock-inhabiting microorganisms—a mini review. J. Microbiol. Meth. 23:143–167.

Holland, K. T., J. S. Knapp, and J. G. Shoesmith. 1986. Industrial applications of anaerobes. Anaerobic Bacteria. Chapman and Hall. New York, NY. 165–188.

Holmes, P. A., L. F. Wright, and S. H. Colins. 1985. β-hydroxybutyrate polymers. European Patent Application. EP 52, 459:

Holmström, C., D. Rittschof, and S. Kjelleberg. 1992. Inhibition of settlement by larvae of Balanus amphitrite and Ciona intestinalis by a surface-colonizing marine bacterirum. Appl. Environ. Microbiol. 58:2111–2115.

Huang, C.-T., G. James, W. G. Pitt, and P. S. Stewart. 1996. Effects of ultrasonic treatment on the efficacy of gentamicin against established Pseudomonas aeruginosa biofilms. Colloid. Surf. B: Biointerf. 6:235–242.

Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace. 1998. Novel division level bacterial diversity in a Yellowstone Hot Spring. J. Bacteriol. 180:366–376.

Hugo, W. B. 1995. A brief history of heat, chemical and radiation preservation and disinfection. Int. Biodeter. Biodegr. 35:197–217.

Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes. *In:* J. R. Norris, and D. W. Ribbons (Eds.) Methods in Microbiology. Academic Press. New York, NY. 38:117–132.

Hutchinson, A. J., J. B. Johnson, G. E. Thompson, G. C. Wood, P. W. Sage, and M. J. Cooke. 1993. Stone degradation due to wet deposition of pollutants. Corrosion Sci. 34:1881–1898.

Illmer, P., and F. Schinner. 1999. Influence of nutrient solution on Al-tolerance of Pseudomonas sp. FEMS Microbiol. Lett. 170:187–190.

Iman, S. H., and J. M. Gould. 1990. Adhsion of an amyloltic Arthrobacter sp. to starch-containing plastic films. Appl. Environ. Microbiol. 56:872–876.

Iman, S. H., J. M. Gould, S. H. Gordon, M. P. Kinney, A. M. Ramsey, and T. R. Tosteson. 1992. Fate of starch-containing plastic films exposed in aquatic habitats. Curr. Microbiol. 25:1–8.

Islam, M., N. G. Thompson, D. R. Lankard, Y. P. Virmani. 1995. Quantitative Identification of Environmental Factors in Corrosion Induced Deterioration of Reinforced Bridge Structures. National Association of Corrosion Engineers. Houston, TX.

Iverson, W. P. 1981. An overview of the anaerobic corrosion of underground metallic structures, evidence for a new mechanism. *In:* E. Escalante (Ed.) Underground Corrosion. American Society for Testing and Materials. Philadelphia, PA. Technical Publication No. 741:33–52.

Iverson, W. P., and G. J. Olsen. 1983. Anaerobic corrosion by sulfate-reducing bacteria due to highly reactive volatile phosphorus compounds. Microbial Corrosion. The Metal Society. London, 46–53.

Iverson, W. P. 1984a. Mechanism of anaerobic corrosion of steel by sulfate reducing bacteria. Mat. Perf. 23:28–30.

Iverson, W. P., and G. J. Olsen. 1984b. Problems relating to sulfate-reducing bacteria in the petroleum industry. *In:* R. M. Atlas (Ed.) Petroleum Microbiology. Macmillan. New York, NY. 619–641.

Iverson, W. P., G. J. Olsen, and L. F. Heverly. 1986. The role of phosphorus and hydrogen sulfide in the anaerobic corrosion of iron and the possible detection of this corrosion by an electrochemical noise technique. *In:* S. C. Dexter (Ed.) Biological Influenced Corrosion. National Association of Corrosion Engineers. Houston, TX. 154–161.

Jain, K. K., A. K. Mishra, and T. Singh. 1993. Biodeterioration of stone: A review of mechanisms involved. *In:* K. L. Garg, N. Garg, and K. G. Mukerji (Eds.) Recent Advances in Biodeterioration and Biodegradation. Naya Prokash. Calcutta, India. 323–354.

Jayaraman, A., J. C. Earthman, and T. K. Wood. 1997. Corrosion inhibition by aerobic biofilms on SAE 1018 steel. Appl. Microbiol. Biotechnol. 47:62–68.

Jayaraman, A., D. Ornek, D. A. Duarte, C.-C. Lee, and T. K. Wood. 1999. Axenic aerobic biofilms inhibit corrosion of copper and aluminum. Appl. Microbiol. Biotechnol. 52:787–790.

Jazsa, P.-G., M. Kußmaul, and E. Bock. 1996a. Simulation of concrete corrosion by nitrifying bacteria. *In:* W. Sand (Ed.) DECHEMA Monographs, Vol. 133: Biodeterioration and Biodegradation. VCH Verlagesellschäft. Frankfurt, Germany. 127–134.

Jazsa, P.-G., R. Stüven, E. Bock, M. Kußmaul. 1996b. Statistical data analysis of microbially influenced deterioration of concrete. *In:* W. Sand (Ed.) DECHEMA monographs, Vol. 133: Biodeterioration and Biodegradation. VCH Verlagesellschäft. Frankfurt, Germany. 199–208.

Jensen, R. J. 1987. Polyimides as interlayer dielectrics for high-performance interconnections of integrated circuits. *In:* M. J. Bouwden and S. R. Turner (Eds.) Polymers for High Technology: Electronics and Phtonics. American Chemical Society. Washington DC, ACS Symp. Ser. 346:466–483.

Jensen, R. A. 1992. Marine bioadhensive: Role for chemosensory recognition in a marine invertebrate. Biofouling 5:177–193.

John, M. E., and G. Keller. 1996. Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. USA 93:12768–12773.

Jones-Meehan, J., K. L. Vasanth, R. K. Conrad, M. Fernandez, B. J. Little, and R. I. Ray. 1994a. Corrosion resistance of several conductive caulks and sealants from marine field tests and laboratory studies with marine, mixed communities containing sulfate-reducing bacteria (SRB). *In:* J. R. Kearns and B. J. Little (Eds.) Microbiologically Influenced Corrosion Testing. American Society for Testing and Materials. Philadelphia, PA. ASTM STP 1232:217–233.

Jones-Meehan, J., M. Walch, B. J. Little, R. I. Ray, and F. B. Mansfeld. 1994b. Effect of mixed sulfate-reducing bacterial communities on coatings. *In:* G. G. Geesey, Z. Lewandowski, and H.-C. Flemming (Eds.) Biofouling and Biocorrosion in Industrial Water Systems. Lewis Publishers. Boca Raton, FL. 107–135.

Jorgensen, B. B. 1988. Ecology of the sulphur cycle: Oxidative pathways in sediments. *In:* J. A. Cole and S. J. Ferguson (Eds.) The Nitrogen and Sulphur Cycles. Cambridge University Press. New York, NY. 31–63.

Karlsson, S., O. Ljungquist, and A.-C. Albertsson. 1988. Biodegradation of polyethylene and the influence of surfactants. Polym. Degr. Stab. 21:237–250.

Kawai, F., and H. Yamanaka. 1986. Biodegradation of polyethylene glycol by symbiotic mixed culture (obligate mutulism). Arch. Microbiol. 146:125–129.

Kawai, F. 1987. The biochemistry of degradation of polyethers. CRC Biotechnol 6:273–307.

Kawai, F., and F. Moriya. 1991. Bacterial assimilation of polytetramethylene glycol. J. Ferment. Bioeng. 71:1–5.

Keevil, C. W., and C. W. Mackerness. 1990. Biocide treatment of biofilms. Int. Biodeter. 26:169–179.

Kelley, D. P. 1981. Introduction to the chemolithotrophic bacteria. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balow, and, H. G. Schlegel (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 1023–1036.

Kelley, K., Y. Ishino, and H. Ishida. 1987. Fourier transform IR reflection techniques for characterization of polyimide films on copper substrates. Thin Solid Films 154:271–279.

Kelly, D. P. 1981. Oxidation of sulphur compounds. *In:* J. A. Cole and S. J. Ferguson (Eds.) The Nitrogen Cycle. Cambridge University Press. New York, NY. 997–1004.

Kelly-Wintenberg, K., and T. C. Montie. 1994. Chemotaxis to oligopeptides by Pseudomonas aeruginosa. Appl. Environ. Microbiol. 60:363–367.

Kemnitzer, J. E., S. P. McCarthy, and R. A. Gross. 1992. Poly(β-hydroxybutyrate) stereoisomers: A model study of the effects of sterechemical and morphological variables on polymer biological degradability. Macromol. 22:5927–5934.

Kemnitzer, J. E., S. P. McCarthy, and R. A. Gross. 1993. Syndiospecific ring-opening polymerization of β-butyrolactone to form predominantly syndiotactic poly(β-hydroxybutyrate) using Tin (IV) catalysts. Macromol. 23:6143–6150.

Kessi, J., M. Ramuz, E. Wehrli, M. Spycher, and R. Bachofen. 1999. Reduction of selenite and detoxification of elemental selenium by the phototrophic bacterium Rhodospirillum rubrum. Appl. Environ. Microbiol. 65:4734–4740.

Kim, O., R. A. Gross, and D. R. Rutherford. 1995. Bioengineering of poly(β-hydroxyalkanoates) for advanced material applications: Incorporation of cyano and nitrophenoxy side chain substituents. Can. J. Microbiol. 41(Suppl.):32–43.

Kim, B.-K., E. C. de Macario, J. Nölling, and L. Daniels. 1996. Isolation and characterization of a copper-resistant methanogen from a copper-mining soil samples. Appl. Environ. Microbiol. 62:2629–2635.

King, B., and H. O. W. Eggins. 1972. Some observations on decay mechanisms of microfungi deteriorating wood. *In:* A. H. Walters, and E. H. Hueck-van der Plas (Eds.) Biodeterioration of Materials. Halsted Press, John Wiley. New York, NY. 145–151.

Klaus, T., R. Joerger, E. Olsson, and C.-G. Granqvist. 1999. Silver-based crystalline nanoparticles, microbially fabricated. Proc. Natl. Acad. Sci. USA 96:13611–13614.

Kluyver, A. J., and C. B. van Niel. 1956. Life's flexibility, microbial adaptation. The Microbe's Contribution to Biology. Harvard University Press. Cambridge, MA. 93–129.

Knudsen, J. G. 1981. Fouling of heat transfer surfaces. Power Condenser Heat Transfer Technology. Hemisphere Publishing. New York, NY. 57–82.

Knyazev, V. M., V. I. Korolkov, A. N. Viktorov, G. O. Pozharskiy, L. N. Petrova, and V. P. Gorshkov. 1986. Sanitary and microbiological aspects of closed environment occupied by people and animals [in Russian]. Kosmicheskaya Biologiya i Aviakosmicheskaya Medistina 20:80–82.

Kobrin, G. 1993. R.E. Tatnall. Introduction. A Practical Manual on Microbiological Influenced Corrosion. NACE International. Houston, TX. 68–72.

Koenig, D. W., S. K. Mishra, and D. L. Pierson. 1995. Removal of Burkholderia Cepacia Biofilms with oxidants. Biofouling 9:51–62.

Kong, K., D. L. Johnstone, D. R. Yonge, J. N. Petersen, and T. M. Brouns. 1994. Long-term intracellular chromium partitioning with subsurface bacteria. Appl. Microbiol. Biotechnol. 42:403–407.

Konhauser, K. O., S. Schultze-Lam, F. G. Ferris, W. S. Fyfe, F. J. Longstaffe, and T. J. Beveridge. 1994. Mineral precipitation by epilithic biofilms in the Speed River, Ontario, Canada. Appl. Environ. Microbiol. 60:549–553.

Korber, D. R., J. R. Lawrence, B. Sutton, and D. E. Caldwell. 1989. Effect of laminar flow velocity on the kinetics of surface recolonization by Mot^+ and Mot^- Pseudomonas fluorescens. Microb. Ecol. 18:1–9.

Kormelink, F. J. M., and J. Voragen. 1993. Degradation of different [(glucurono)arabino]xylans by a combination of purified xylan-degrading enzymes. Appl. Microbiol. Biotechnol. 38:688–695.

Krieg, N. R., and J. G. Holt. 1984. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:

Krumbein, W. E. 1968. Inquiry on biological decomposition: The influence of microflora on the decomposition of building stone and its depedence on edaphic factors. Zeitschrift für Allgemeine Microbiologie 8:107–117.

Kuenen, J. G., and Tuovinen, O. H. 1981. The genera Thiobacillus and Thiomicrospira. *In:* M. P. Starr, H. Stolp, H. G. Trüoper, A. Balow, and H. G. Schlegel (Eds.) The Prokaryotes. Springer. New York, NY. 1023–1036.

Kulpa, C. F., and Baker, C. J. 1990. Involvement of sulfure-oxidizing bacteria in concrete deterioration. *In:* N. J. Dowling, M. W. Mittelman, and J. C. Danko (Eds.) Microbially Influenced Corrosion and Biodeterioration. National Association of Corrosion Engineers. Houston, TX. 4/7–4/9.

Kumar, R., and A. Venkataraman. 1996. Biodeterioration of Stone Monuments in Tropical Regions: A Review of Current Research Status. The Getty Conservation Institute. Marina del Rey, CA. 1–63.

Lai, J. H. 1989. Polymers for Electronic Applications. CRC Press. Boca Raton, FL. 23–24.

Lappin-Scott, H., J. W. Costerton, and T. J. Marrie. 1992. Biofilms and biofouling. Encyclopedia of Microbiology 1:277–284.

Lauwers, A. M., and W. Heinen. 1974. Biodegradation and utilization of silica and quartz. Arch. Microbiol. 95:67–78.

Lawrence, J. R., P. J. Delaquis, D. R. Korber, and D. E. Caldwell. 1987. Behavior of Pseudomonas fluorescens within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. 14:1–14.

Lea, F. M., and C. H. Desch. 1936. The Chemistry of Cement and Concrete. London. Edward Arnold, 16–17.

Lee, J. A. 1948. Plastics. *In:* H. H. Uhlig (Ed.) The Corrosion Handbook. John Wiley. New York, NY. 359–365.

Lee, S. F., C. W. Forsberg, and L. N. Gibbins. 1985. Xylanolytic activity of Clostridium acetobutylicum. Appl. Environ. Microbiol. 50:1068–1076.

Lee, S. F., C. W. Forsberg, and J. B. Rattray. 1987a. Purification and characterization of two endoxylanases from Clostridium acetobutylicum. ATCC 824. Appl. Environ. Microbiol. 53:644–650.

Lee, H., R. J. B. To, R. K. Latta, P. Biely, and H. Schneider. 1987b. Some properties of extacellular acetylxylan esterase produced by the yeast Rhodotorula mucilaginosa. Appl. Environ. Microbiol. 53:2831–2834.

Lee, W., Z. Lewandowski, S. Okabe, W. G. Characklis, and R. Avci. 1993a. Corrosion of mild steel underneath aerobic biofilms containing sulfate-reducing bacteria, part I: At low dissolved oxygen concentration. Biofouling 7:197–216.

Lee, W., Z. Lewandowski, M. Morrison, W. G. Characklis, R. Avci, and P. H. Nielsen. 1993b. Corrosion of mild steel underneath aerobic biofilms containing sulfate-reducing bacteria, part II: At high dissolved oxygen concentration. Biofouling 7:217–239.

Lee, Y.-E., S. E. Lowe, and J. G. Zeikus. 1993c. Regulation and characterization of xylanolytic enzymes of Thermoanaerobacterium saccharolyticum B6A-RI. Appl. Environ. Microbiol. 59:763–771.

Lee, W., Z. Lewandowski, P. H. Nielsen, and W. A. Hamilton. 1995. Role of sulfate-reducing bacteria in corrosion of mild steel: A review. Biofouling 8:165–194.

Lefebvre-Drouet, E., and M. F. Rousseau. 1995. Dissolution de différents oxyhydroydes de fer par voie chimique et par voie biologique: Importance des bactéries reductrices. Soil Biol. Biochem. 27:1041–1050.

Lemaire, J., P. Dabin, and R. Arnaud. 1992. Mechanisms of abiotic degradation of synthetic polymers. *In:* M. Vert, J. Feijen, A. Albertsson, G. Scott, and E. Chiellini (Eds.) Biodegradable Polymers and Plastics. Royal Society of Chemistry. Cambridge, UK. 30–39.

Lemoigne, M. 1926. Produits de déshydration et de polymérisation de l'acide β-oxybutyric. Bull. Soc. Chim. Biol. (Paris) 8:770–782.

Lewandowski, Z., P. Stoodley, and S. Altobelli. 1995. Experimental and conceptual studies on mass transport in biofilms. Water Sci. Technol. 31:153–162.

Leyden, R. N., and D. I. Basiulis. 1989. Adhesion and electrical insulation of thin polymeric coatings under saline exposure. *In:* J. S. Hanker, and B. L. Giammara (Eds.) Biomedical Materials and Devices. Materials Research Society. Pittsburgh, PA. 10:627–633.

L'Hostis, E., C. Compère, D. Festy, B. Tribollet, and C. Deslouis. 1997. Characterization of biofilms formed on gold in natural seawater by oxygen diffusion analysis. Corrosion 53:4–10.

Li, D., and R. Lu. 1990. Purification and properties of periplasmic hydrogenase from Desulfovibrio vulgaris D-2. Acta Microbiol. Sinica 30:267–272.

Li, D., Y. Ma, W. F. Flanagan, B. D. Lichter, and J. P. Wikswo Jr. 1997. Detection of hidden corrosion of aircraft aluminum alloy by magnetometry using a superconducting quantum interference device. Corrosion 53:93–98.

Liken, G. E. 1981. Some perspectives of the major biogeochemical cycles. Scope 17. John Wiley. New York, NY. 93–112.

Lin, C., and B. H. Olson. 1995. Occurrence of cop-like copper resistance genes among bacteria isolated from a water distribution system. Can. J. Microbiol. 41:642–646.

Little, B., P. Wagner, S. M. Gerchakov, M. Walch, and R. Mitchell. 1986a. The involvement of a thermophilic bacterirum in corrosion processes. Corrosion 42:533–536.

Little, B. J., P. Wagner, J. S. Maki, and R. Mitchell. 1986b. Factors influencing the adhesion of microorganisms to surfaces. J. Adhesion 20:187–210.

Little, B. J., and J. R. Depalma. 1988. Marine biofouling. Treatise on Material Science and Technology 28:89–119.

Little, B., P. Wagner, W. G. Characklis, and W. Lee. 1990. Microbial corrosion. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley. New York, NY. 635–670.

Little, B. J., P. Wagner, and J. Jones-Meehan. 1994. Sulfur isotopic fractionation in sulfitde corrosion products as an indicator for microbiologically influenced corrosion (MIC). *In:* J. R. Kearns and B. J. Little (Eds.) Microbiologically Influenced Corrosion Testing. American Soci-

ety for Testing and Materials. Philadelphia, PA. ASTM STP 1232:180–187.

Liu, X., F. Roe, A. Jesaitis, and Z. Lewandoski. 1998. Resistance of biofilms to the catalase inhibitor 3-amino-1,2,4-triazole. Biotechnol. Bioeng. 59:156–162.

Lovley, D. R., J. F. Stolz, G. L. Nord, and E. J. Phillips. 1987. Anaerobic production of magnetite by a dissimilatory iron-reducing microorganism. Nature (Lond.) 330:252–254.

Lovley, D. R. 1991. Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol. Rev. 55:259–287.

Lovley, D. R., and E. J. P. Phillips. 1994. Reduction of chromate by Desulfovibrio vugaris and its c_3 cytochrome. Appl. Environ. Microbiol. 60:726–728.

Lü, R., Q. Liu, C. Xiao, S. Bai, H. Chen, and F. Wang. 1984. Study on microbiocidal efficiency of chlorine dioxide for fouling harmful microbes in desalting water systems. Acta Microbiol. Sinica 24:243–249.

Lü, R., Q. Liu, Y. Zhang, and C. Xiao. 1989. Studies on harmful microbes in recirculating cooling water system of oil refinery. Acta Microbiol. Sinica 29:204–215.

Lusty, C. J., and M. Doudoroff. 1966. Poly-β-hydroxybutyrate depolymerases of Pseudomonas lemoignei. Proc. Natl. Acad. Sci. USA 56:960–965.

Lüthi, E., N. B. Jasmat, and P. L. Bergquist. 1990a. Overproduction of an acetylxylan esterase from the extreme thermophile "Caldocellum saccharolyticum" in Escherichia coli. Appl. Microbiol. Biotechnol. 34:214–219.

Lüthi, E., D. R. Love, J. McAnulty, C. Wallace, P. A. Caughey, D. Saul, and P. L. Bergquist. 1990b. Cloning, sequence analysis, and expression of genes encoding xylan-degrading emzymes from the thermophile "Caldocellum saccharolyticum." Appl. Environ. Microbiol. 56:1017–1024.

Luu, W. C., H. S. Kuo, and J. K. Wu. 1997. Hydrogen permeation through nickel-plated steels. Corrosion Sci. 39:1051–1059.

Lynch, J. L., and R. G. J. Edyvean. 1988. Biofouling in oilfield water systems—a review. Biofouling 1:147–162.

MacDonald, M. J., D. L. Hartley, and M. K. Speedie. 1985. Location of cellulolytic enzyme activity in the marine fungus Trichocladium achrasporum. Can. J. Microbiol. 31:145–148.

MacDonald, R. M. 1986. Nitrification in soil: An introductory history. *In:* J. I. Prosser (Ed.) Nitrification. IRL Press. Oxford, UK. 1–16.

MacKenzie, C. R., D. Bilous, H. Schneider, and K. G. Johnsom. 1987. Indution of cellulolytic and xylanolytic enzyme systems in Streptomyces spp. Appl. Environ. Microbiol. 53:2853–2839.

Madigan, M. T., J. M. Martinko, and J. Parker. 2000. Brock Biology of Micoorganisms (9th ed.). Prentice-Hall. Upper Saddle River, NJ.

Maki, J. S., D. Rittschof, A. R. Schmidt, A. S. Snyder, and R. Mitchell. 1989. Factors controlling attachment of bryozoan larvae: A comparison of bacterial films and unfilmed surfaces. Biol. Bull. 177:295–302.

Maki, J. S., B. J. Little, P. Wagner, and R. Mitchell. 1990a. Biofilm formation on metal surfaces in Antarctic waters. Biofouling 2:27–38.

Maki, J. S., D. Rittschof, M.-O. Samuelson, U. Szewzyk, A. B. Yule, S. Kjelleberg, J. D. Costlow, and R. Mitchell. 1990b. Effect of marine bacteria and their exopolymers on attachment of barnacle cypris larvae. Bull. Marine Sci. 46:499–511.

Mansfeld, F., H. Shih, A. Postyn, J. Devinny, R. Islander, and C. L. Chen. 1990. Corrosion monitoring and control in concrete sewer pipes. National Association of Corrosion Engineers. Houston, TX. Corrosion.

Mansfeld, F. 1994. Effectiveness of ion vapor-deposited aluminum as a primer for epoxy and urethane topcoats. Corrosion 50:609–612.

Mansfeld, F. 1995. Use of electrochemical impedance spectroscopy for the study of corrosion by polymer coatings. J. Appl. Electrochem. 25:187–202.

Margulis, L. 1981. The endosymbiotic theory. Symbiosis in Cell Evolution. W. H. Freeman. San Francisco, CA. 1–14.

Marshall, K. C., R. Stout, and R. Mitchell. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. 68:337–348.

Marshall, K. C. 1976. Solid-liquid and solid-gas interfaces. Interfaces in Microbial Ecology. Harvard University Press. Cambridge, MA. 27–52.

Marshall, K. C. 1980. Adsorption of microorganisms to soils and sediments. *In:* G. Bitton and K. C. Marshall (Eds.) Adsorption of Microorganisms to Surfaces. John Wiley. New York, NY. 317–329.

Marshall, K. C. 1992. Biofilms: An overview of bacterial adhesion, activity, and control at surfaces. ASM News 58:202–207.

Martrhamuthu, S., G. Rajagopal, S. Sathianarayannan, M. Eashwar, and K. Balakrishnan. 1995. A photoelectrochemical approach to the ennoblement process: Proposal of an adsorbed inhibitor theory. Biofouling 8:223–232.

Mas-Castellà, J., J. Urmeneta, R. Lafuente, A. Navarrete, and R. Guerrero. 1995. Biodegradation of poly-β-hydroxyalkanoates in anaerobic sediments. Int. Biodeter. Biodegr. 35:155–174.

Matamala, G., W. Smeltzer, and G. Droguett. 1994. Use of tannin anticorrosive reaction primer to improve traditional coating systems. Corrosion 4:270–275.

Matis, K. A., A. I. Zouboulis, A. A. Grioriadou, N. K. Lazaridis, and L. V. Ekateriniadou. 1996. Metal biosorption-flotation, application to cadmium removal. Appl. Microbiol. Biotechnol. 45:569–573.

Mattila, K., L. Carpen, T. Hakkarainen, and M. S. Salkinoja-Salonen. 1997. Biofilm development during ennoblement of stainless steel in Baltic Sea water: A microscopic study. Int. Biodeter. Biodegr. 40:1–10.

May, E., F. J. Lewis, S. Pereira, S. Taylor, and M. R. D. Seaward. 1993. Microbial deterioration of building stone—a review. Biodeter. Abstr. 7:109–123.

Mayr, E. 1998. Two empires or three? Proc. Natl. Acad. Sci. USA 95:9720–9723.

McCain, J. W., C. J. Mirocha. 1995. Screening computer diskettes and other magnetic media for susceptibility to fungal colonization. Int. Biodeter. Biodegr. 33:255–268.

McFeters, G. A. 1991. Disinfection susceptibility of waterborne Pseudomonads and Legionellae under simulated space vehicle conditions. Intersociety Conference on Environmental Systems San Francisco, CA. SAE Technical Paper911404, ICES.

McFeters, G. A., F. P. Yu, B. H. Pyle, and P. S. Stewart. 1995. Physiological methods to study biofilm disinfection. J. Ind. Microbiol. 15:333–338.

McLean, R. J. C., J. C. Nickel, and M. E. Olson. 1995. Biofilm associated urinary tract infections. *In:* H. M. Lappin-Scott and J. W. Costerton (Eds.) Microbial Biofilms. Cambridge University Press. Cambridge, UK. 261–273.

McLean, R. J. C., M. Whiteley, D. J. Stickler, and W. C. Fuqua. 1997. Evidence of autoinducer activity in naturally occurring biofilms. FEMS Microbiol. Lett. 154:259–263.

Mergaert, J., A. Webb, C., anderson, A. Wouters, and J. Swings. 1993. Microbial-degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. Appl. Environ. Microbiol. 59:3233–3238.

Mergeay, M. 1991. Towards an understanding of the genetics of bacterial metal resistance. Trends Biotechnol. 9:17–24.

Meshkov, D. 1994. The influence of spaceflight conditions on sensitization of men to bacterial and chemical allergens. 45th Congress of the International Astronautical Confederation Congress. Jerusalem, Israel. IAF/IAA-94-G.1.125.

Meyer, L. 1864. Chemische untersuchungen der thermen zu landeck in der grafschaft glatz. J. Prakt. Chem. 91:1–15.

Milde, K., W. Sand, W. Wolff, and E. Bock. 1983. Thiobacilli of the concrete walls of the Hamburg sewer system. J. Gen. Microbiol. 129:1327–1333.

Millard, S. G., K. R. Gowers, and J. H. Bungey. 1995. Galvanostatic Pulse Techniques: A Rapid Method of Assessing Corrosion Rates of Steel in Concrete Structure. NACE International. Houston, TX. Corrosion.

Miller, J. D. A. 1970. Microbial Aspects of Metallurgy. Elsevier Publishing. New York, NY. 61–105.

Mills, A. L., and D. K. Powelson. 1996. Bacterial interactions with surfaces in soils. *In:* M. Fletcher (Ed.) Bacterial Adhesion: Molecular and Ecological Diversity. Wiley-Liss. New York, NY. 25–57.

Milstein, O., R. Gersonde, A. Huttermann, M.-J. Chen, and J. J. Meister. 1992. Fungal biodegradation of lignopolystyrene graft copolymers. Appl. Environ. Microbiol. 58:3225–3232.

Mitchell, R., and J. S. Maki. 1989. Microbial surface films and their influence on larval settlement and metamorphosis in the marine environment. *In:* M.-F. Thompson, R. Sarojini, and R. Nagabhushanam (Eds.) Marine Biodeterioration: Advanced Techniques Applicable to the Indian Ocean. Oxford & IBH Publishing, PVT Ltd. New Delhi, India. 489–497.

Mitchell, R., J.-D. Gu, M. Roman, and S. Soulkup. 1996. Hazards to space missions from microbial biofilms. *In:* W. Sand (Ed.) Biodeterioration and Biodegradation. VCH Verlagsgesellschaft. Frankfurt, Germany. DECHEMA Monographs, Vol. 133:3–16.

Mitchell, R., and J.-D. Gu. 2000. Changes in the biofilm microflora of limestone caused by atmospheric pollutants. Int. Biodeter. Biodegr. 46:299–303.

Mittelman, M. W., and G. Geesey. 1985. Copper-binding characteristics of exopolymers from a freshwater-sediment bacterium. Appl. Environ. Microbiol. 49:846–851.

Mittelman, M. W. 1995a. Biofilm development in purified water systems. *In:* H. M. Lappin-Scott, and J. W. Costerton (Eds.) Microbial Biofilms. Cambridge University Press. Cambridge, UK. 133–147.

Mittelman, M. W., and J. C. Danko. 1995b. Corrosion of a concrete dam structure: Evidence of microbially influenced corrosion activity. *In:* P. Angell et al. (Eds.) 1995 International Conference on Microbial Influenced Corrosion. National Association of Corrosion Engineers. Houston, TX. 15/1–15/7.

Mittelman, M. W. 1996. Adhesion to biomaterials. *In:* M. Fletcher (Eds.) Bacterial Adhesion: Molecular and Ecological Diversity. Wiley-Liss. New York, NY. 89–127.

Mitton, B., T. E. Ford, E. LaPointe, and R. Mitchell. 1993. Biodegradation of Complex Polymeric Materials. National Association of Corrosion Engineers. Houston, TX. Corrosion.

Mitton, D. B., R. M. Latanison, and F. Bellucci. 1996. The effects of post-cure annealing on the protective properties of polyimides on chromium substrates. J. Electrochem. Soc. 143:3307–3316.

Mitton, D. B., S. Toshima, R. M. Latanison, F. Bellucci, T. E. Ford, J.-D. Gu, and R. Mitchell. 1998. Biodegradation of polymer-coated metallic substrates. *In:* G. P. Bierwagen (Ed.) Organic Coatings for Corrosion Control. ACS. Washington DC, ACS Symp. Ser. 689:211–222.

Moat, A. G., and J. W. Foster. 1988. Growth and its regulation. Microbial Physiology (2nd ed.). John Wiley. New York, NY. 523–578.

Moore, L., and M. Postle. 1994. Risk-benefit analysis and case study on tributyl tin. Int. Biodeter. Biodegr. 34:401–412.

Moosavi, A. N., J. L. Dawson, and R. A. King. 1986. The effect of sulfate-reducing bacteria on the corrosion of reinforced concrete. *In:* S. C. Dexter (Ed.) Biologically Induced Corrosion: Proceedings of the International Conference on Biologically Induced Corrosion. National Association of Corrosion Engineers. Houston, TX. 291–308.

Morton, R. L., W. A. Yanko, D. W. Graham, and R. G. Arnold. 1991. Relationships between metal concentrations and crown corrosion in Los Angeles County sewers. Res. J. WPCF 63:779–798.

Murray, F. E. S., R. Mitchell, and T. Ford. 1993. *In:* Experimental Methods for the Study of Microbial Mediated Corrosion. NACE International. Houston, TX. Corrosion.

Myers, T. 1988. Failing the test: Germicides or use dilution methodology? ASM News 54:19–21.

Nakagawa, H., S. Akihawa, T. Suenaga, Y. Taniguchi, and K. Yoda. 1993. Fiber reinforced concrete: Developments and application to buildings. Adv. Composite Mat. 3:123–131.

Nakajima-Kambe, T., F. Onuma, N. Kimpara, and T. Nakahara. 1995. Isolation and characterization of a bacterium which utilizes polyester polyurethane as a sole carbon and energy source. FEMS Microbiol. Lett. 129:39–42.

Nakanishi, K., M. Marui, and T. Yasui. 1992. Comparison of xylan and methyl β-xyloside-induced xylanases from Streptomyces sp. J. Ferment. Bioeng. 74:392–394.

Nakayama, K., T. Saito, Y. Fukui, Y. Shirakura, and K. Tomita. 1985. Purification and properties of extracellular poly(3-hydroxybutyrate) depolymerases from Pseudomonas lemoignei. Biochim. Biophys. Acta 827:63–72.

Narayan, R. 1993. Biodegradation of polymeric materials (anthropogenic macromolecules) during composting. *In:* H. A. J. Hoitink and H. M. Keener (Eds.) Science and Engineering of Composting: Design, Environmental, Microbiological and Utilization Aspects. Renaissance. Worthington, OH. 339–362.

Nefedov, Y., N. D. Novikova, and I. N. Surovezhin. 1988. Products of biodegradation of polymers as a factor in the possible pollution of the air of hermetically sealed environments with toxic substances [in Russian]. Kosmicheskaya Biologiya i Aviakosmicheskaya Medistina 22:67–71.

Neu, T. 1996. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. Microbiol. Rev. 60:151–166.

Nieboer, E., B. L. Gibson, A. D. Oxman, and J. R. Kramer. 1995. Health effects of aluminum: A critical review with emphasis on aluminum in drinking water. Environ. Rev. 3:29–81.

Nielsen, P. H., W. Lee, Z. Lewandowshi, M. Morison, and W. G. Characklis. 1993. Corrosion of mild steel in an alternationg oxic and anoxic biofilm system. Biofouling 7:267–284.

Novikova, N. D., and S. N. Zalogoyev. 1985. Formation of volatile substances during polymer destruction by Pseudomonas aeruginosa [in Russian]. Kosmicheskaya Biologiya i Aviakosmicheskaya Medistina 19:74–76.

Novikova, N. D., M. I. Orlova, and M. B. Dyachenko. 1986. Reproductive capacity of microflora on polymers used in sealed environments [in Russian]. Kosmicheskaya Biologiya i Aviakosmicheskaya Medistina 20:71–73.

Nozhevnikova, A. N., O. R. Kotsyurbenko, and M. V. Simankova. 1994. Acetogenesis. *In:* H. L. Drake (Ed.) Acetogenesis at low temperature. Chapman and Hall. New York, NY. 416–431.

NRC (National Research Council). 1987. Agenda for Advancing Electrochemical Corrosion Science and Technology. National Academy Press. Washington DC, Publication NMAB438-2:87–96.

Odian, G. 1991. Reactions of polymers. Principles of Polymerization (3rd ed.). John Wiley. New York, NY. 691–745.

Odom, J. M. 1993a. Industrial and environmental activities of sulfate-reducing bacteria. *In:* J. M. Odom and R. Singleton Jr. (Eds.) The Sulfate-Reducing Bacteria: Contemporary Perspectives. Springer-Verlag. New York, NY. 189–210.

Odom, J. M., and R. Singleton Jr. 1993b. The Sulfate-reducing Bacteria: Contemporary Perspectives. Springer-Verlag. New York, NY. 289.

Olesen, B. H., R. Avci, and Z. Lewandowski. 1998. Ennoblement of Stainless Steel Studied by X-ray Photoelectron Spectroscopy. NACE International. Houston, TX. Corrosion.

Olmstead, W. M., and H. Hamlin. 1900. Converting portions of the Los Angles outfall sewer into a septic tank. Engin. News 44:317–318.

Ortega-Calvo, J. J., M. Hernandez-Marine, and C. Saiz-Jimenez. 1991. Biodeterioration of building materials by cyanobacteria and algae. Int. Biodeter. 28:165–185.

Ortega-Calvo, J. J., P. M. Sanchez-Castillo, M. Hernandez-Marine, and C. Saiz-Jimenez. 1993. Isolation and characterization of epilithic chlorophytes and cynobacteria from two Spanish cathedrals (Salamanca and Toledo). Nova Hedwigia 57:239–253.

Ortega-Calvo, J. J., X. Arino, M. Hernandez-Marine, and C. Saiz-Jimenez. 1995. Factors affecting the weathering and colonization of monuments by phototrophic microorganisms. Sci. Total Environ. 167:329–341.

Ortega-Morales, O., G. Hernandez-Duque, L. Borges-Gomez, and J. Guezennec. 1999. Characterization of epilithic microbial communities associated with Mayan stone monuments in Yucatan, Mexico. Geomicrobiol. J. 16:221–232.

Osswald, P., R. Courtes, P. Bauda, J. C. Block, J. D. Bryers, and E. Sunde. 1995. Xenobiotic biodegradation test using attached bacteria in synthetic seawater. Ecotoxicol. Environ. Safety 31:211–217.

O'Toole, G., H. B. Kaplan, and R. Kolter. 2000. Biofilm formation as microbial development. Ann. Rev. Microbiol. 54:49–79.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. Science 276:734–740.

Padival, N. A., J. S. Weiss, and R. G. Arnold. 1995. Control of Thiobacillus by means of microbial competition: Implications for corrosion of concrete sewers. Water Environ. Res. 67:201–205.

Paradies, H. H., I. Haenßel, W. Fisher, and D. Wagner. 1990. Microbial Induced Corrosion of Copper Pipes. New York, NY. INCRA Report No. 444:16.

Paradies, H. H. 1995. Chemical and physical aspects of metal biofilms. *In:* C. C. Gaylarde and H. A. Videla (Eds.) Bioextraction and Biodeterioration. Cambridge University Press. Cambridge, UK. 197–269.

Parikh, M., R. A. Gross, and S. P. McCarthy. 1993. The effect of crystalline morphology on enzymatic degradation kinetics. *In:* D. Kaplan, E. Thomas, and C. Ching (Eds.) Fundamentals of Biodegradable Materials and Packaging. Technomic Publishing. Lancaster, PA. 159–170.

Parker, C. D. 1945a. The corrosion of concrete. 1: The isolation of a species of bacterium associated with the corrosion of concrete exposed to atmospheres containing hydrogen sulphide. Austral. J. Exp. Biol. Med. Sci. 23:81–90.

Parker, C. D. 1945b. The corrosion of concrete. 2: The function of Thiobacillus concretivorus (nov. spec.) in the corrosion of concrete exposed to atmospheres containing hydrogen sulphide. Austral. J. Exp. Biol. Med. Sci. 23:91–98.

Parker, C. D. 1947. Species of sulfur bacteria associated with the corrosion of concrete. Nature (Lond.) 159:439.

Parker, C. D., and A. Prisk. 1953. The oxidation of inorganic compounds of sulphur by various sulphur bacteria. J. Gen. Microbiol. 8:344–364.

Parker, C. D., and D. Jackson. 1965. The microbial flora of concrete surfaces. Hydrogen Sulphide Corrosion of Concrete Sewers. Melbourne and Metropolitan Board of Works. Melbourne, Australia. Technical Paper No. A8, Part 6:1–29.

Pedersen, K. 1996. Investigations of subterranean bacteria in deep crystalline bedrock and their importance for the disposal of nuclear waste. Can. J. Microbiol. 42:382–391.

Pendyala, J., R. Avci, G. G. Geesey, P. Stoodley, M. Hamilton, and G. Harkin. 1996. Chemical effect of biofilm colonization on 304 stainless steel. J. Vac. Sci. Technol. A14:1955–1760.

Peng, C.-G., and J. K. Park. 1994. Principal factors affecting microbiologically influenced corrosion of carbon steel. Corrosion 50:669–675.

Pierson, D. L., and S. K. Mishra. 1992. Microbiological challenges of space habitation. 43rd Congress of the International Astronautical Confederation Congress, Washington DC.

Pierson, B. K., and M. N. Parenteau. 2000. Phototrophs in high iron microbial mats: Microstructure of mats in iron-depositing hot springs. FEMS Microbiol. Ecol. 32:181–196.

Piervittori, R., O. Salvadori, and A. Laccisaglia. 1994. Literature on lichens and biodeterioration of stonework. I. Lichenologist 26:171–192.

Pitt, C. G. 1992. Non-microbial degradation of polyesters: Mechanisms and modifications. *In:* M. Vert, J. Feijen, A. Albertsson, G. Scott, and E. Chiellini (Eds.) Biodegradable Polymers and Plastics. Royal Society for Chemistry/ Redwood Press. Melksham, UK. 7–17.

Pometto III, A. L., B. Lee, and K. E. Johnson. 1992. Production of an extracellular polyethylene-degrading

enzyme(s) by Streptomyces species. Appl. Environ. Microbiol. 58:731–733.

Pometto III, A. L., K. E. Johnson, and M. Kim. 1993. Pure-culture and enzymatic assay for starch-polyethylene degradable plastic biodegradation with Streptomyces species. J. Environ. Polym. Degr. 1:213–221.

Pope, D. H., D. Duquette, P. C. Wayner Jr., and A. H. Johannes. 1989. *In:* Microbiologically Influenced Corrosion: A State-of-the-art Review (2nd ed.). National Association of Corrosion Engineers. Houston, TX. MTI Publication No. 13:4:1–3.

Postgate, J. R. 1984. The Sulfate-Reducing Bacteria. (2nd ed.). Cambridge University Press. New York, NY. 132–152.

Potekhina, J. S., N. G. Sherisheva, L. P. Povetkina, A. P. Pospelov, T. A. Rakitina, F. Warnecke, and G. Gottschalk. 1999. Role of microorganisms in corrosion inhibition of metals in aquatic habitats. Appl. Microbiol. Biotechnol. 52:639–646.

Power, K., and K. C. Marshall. 1988. Cellular growth and reproduction of marine bateria on surface-bound substrate. Biofouling 1:163–174.

Prieto, B., T. Rivas, B. Silva, R. Carballal, and M. E. Lopez de Silanes. 1995. Colonization by lichens of granite dolments in Galicia (NW Spain). Int. Biodeter. Biodegr. 34:47–60.

Pyle, B. H., S. C. Broadaway, and G. A. McFeters. 1992. Efficacy of copper and silver ions with iodine in the inactivation of Pseudomonas cepacia. J. Appl. Bacteriol. 72:71–79.

Raychaudhuri, S., P. D. Sutphin, J. T. Chang, and R. B. Altman. 2001. Basic microarray analysis: Grouping and feature reduction. Trends Biotechnol. 19:189–193.

Reese, E. T. 1957. Biological degradation of cellulose derivatives. Ind. Engin. Chem. 49:89–93.

Reinsel, M. A., J. T. Sears, P. S. Stewart, and M. J. Mclnerney. 1996. Control of microbial souring by nitrate, nitrite or glutaraldehyde injection in a sandstone column. J. Ind. Microbiol. 17:128–136.

Rethke, D. 1994. Testing of Russian ECLSS-Sabatier and potable water processor. Intersociety Conference on Environmental Systems. Friedrichschafen, Germany. SAE Technical Paper 941252, ICES.

Reynolds, T. B., and G. R. Fink. 2001. Baker's yeat, a model for fungal biofilm formation. Science 291:878–881.

Rijnaarts, H. H. M., W. Norde, E. J. Bouwer, J. Lyklema, and A. J. B. Zehnder. 1993. Bacterial adhesion under static and dynamic conditions. Appl. Environ. Microbiol. 59:3255–3265.

Rittman, B. E. 1993. The significance of biofilms in porous media. Water Res. Rev. 29:2195–2202.

Rittschof, D., J. Maki, R. Mitchell, and J. D. Costlow. 1986. Ion and neuropharmacological studies of barnacle settlement. Netherlands J. Sea Res. 20:269–275.

Roe, F. L., Z. Lewandowshi, and T. Funk. 1996. Simulation microbiologically influenced corrosion by depositing extracellular biopolymers on mild steel surfaces. Corrosion 52:744–752.

Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil. 1994. Influence of pluming materials on biofilm formation and growth of Legionella pneumophila in potable water systems. Appl. Environ. Microbiol. 60:1842–1851.

Rölleke, S., A. Witte, G. Wanner, and W. Lubitz. 1998. Medieval wall paintings—a habitat for archaea: Identification of archaea by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified gene fragments coding for 16S rRNA in a medieval wall painting. Int. Biodeter. Biodegr. 41:85–92.

Ross, J. 1994. An aquatic invader is running amok in U.S. waterways. Smithsonian 24:40–51.

Rossmoore, H. W., and L. A. Rossmoore. 1993. MIC in metalworking processes and hydraulic systems. *In:* G. Kobrin (Eds.) A Practical Manual on Microbiologically Influenced Corrosion. NACE International. Houston, TX. 31–40.

Saito, T., K. Suzuki, Yamamoto, T. Fukui, K. Miwa, K. Tomita, S. Nakanishi, S. Odani, J.-I. Suzuki, and K. Ishikawa. 1989. Cloning, nucleotide sequence, and expression in Escherichia coli of the gene for poly-3-hydroxybutyrate depolymerase from Alcaligenes faecalis. J. Bacteriol. 171:184–189.

Saito, H., Y. Miyata, H. Takazawa, K. Takai, and G. Yamauchi. 1995. Corrosion Rate Measurements of Steel in Carbonated Concrete. NACE International. Houston, TX. Corrosion.

Saiz-Jimenez, C. 1995. Deposition of anthropogenic compounds on monuments and their effect on airborne microorganisms. Aerobiologia 11:161–175.

Saiz-Jimenez, C. 1997. Biodeterioration vs biodegradation: The role of microorganisms in the removal of pollutants deposited on historic buildings. Int. Biodeter. Biodegr. 40:225–232.

Sakaguchi, T., J. G. Burgess, and T. Matsunaga. 1993. Magnetite formation by a sulphate-reducing bacterium. Nature (Lond.) 253:47–49.

Salama, N., K. Guillemin, T. K. McDaniel, G. Sherlock, L. Tompkins, and S. Falkow. 2000. A whole-geneome microarray reveals genetic diversity among Helicobacter pylori strains. Proc. Natl. Acad. Sci. USA 97:14668–14673.

Sánchez, A., A. Ballester, M. L. Blázquez, F. González, J. Muñoz, and A. Hammaini. 1999. Biosorption of copper and zinc by Cymodocea nodosa. FEMS Microbiol. Rev. 23:527–536.

Sand, W., K. Milde, and E. Bock. 1983. Simulation of concrete corrosion in a strictly controlled H_2S breeding chamber. *In:*G. Rossi, and A. E. Torma (Eds.) Recent Progress in Biohydrometallurgy. Associazione Mineraria. Sarda, Italy. 667–677.

Sand, W., and E. Bock. 1984. Concrete corrosion in the Hamburg sewer system. Environ. Technol. Lett. 5:517–528.

Sand, W. 1987a. Importance of hydrogen sulfide, thiosulfate and methylmercaptan for growth of thiobacilli during smulation of concrete corrosion. Appl. Environ. Microbiol. 53:1645–1648.

Sand, W., E. Bock, and D. C. White. 1987b. Biotest system for rapid evaluation of concrete resistance to sulfur-oxidizing bacteria. Mat. Perf. 26:14–17.

Sand, W., B. Ahlers, and E. Bock. 1991. The impact of microorganisms—especially nitric acid producing bacteria—on the deterioration of natural stones. *In:* N. S. Baer, C. Sabbioni, and A. I. Sors (Eds.) Science, Technology and European Cultural Heritage. Butterworth-Heinemann. Oxford, UK. 481–484.

Sand, W. 1997. Microbial mechanisms of deterioration of inorganic substrates—a general mechanistic overview. Int. Biodeter. Biodegr. 40:183–190.

Sandaa, R.-A., O. Enger, and V. Torsvik. 1999. Abundance and diversity of Archaea in heavy-metal-contaminated soils. Appl. Environ. Microbiol. 65:3293–3297.

Sanders, P. F., and W. A. Hamilton. 1986. Biological and corrosion activities of sulphate-reducing bacteria in industraial process plant. *In:* S. C. Dexter (Ed.) Biologically Induced Corrosion: Proceedings of the International Conference on Biologically Induced Corrosion. National Association of Corrosion Engineers. Houston, TX. 47–68.

Santini, J. M., L. I. Sly, R. D. Schnagi, and J. M. Macy. 2000. A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: Phylogenetic, physiological, and preliminary biochemical studies. Appl. Environ. Microbiol. 66:92–97.

Scamans, G. M., J. A. Hunter, and N. J. H. Holroyd. 1989. A surface-engineering approach to the corrosion aluminum. Treatise on Materials Science and Technology 31:485–500.

Schembri, M. A., K. Kjærgaard, and P. Klemm. 1999. Bioaccumulation of heavy metals by fimbrial designer adhesins. FEMS Microbiol. Lett. 170:363–371.

Schink, B., and M. Stieb. 1983. Fermentative degradation of polyethlene glycol by a strictly anaerobic, Gram-negative, nonsporeforming bacterium, Pelobacter venetianus sp. nov. Appl. Environ. Microbiol. 45:1905–1913.

Schmidt, E. L. 1982. Nitrification in soil. *In:* F. J. Stevenson (Ed.) Nitrogen in Agricultural Soils. Soil Science Society of America. Madison, WI. Agronomy Monograph 22:253–288.

Schmidt, R. 1997. Monte Carlo simulation of bioadhesion. Int. Biodeter. Biodegr. 40:29–36.

Schmidtt, C. R. 1986. Anomalous microbiological tuberculation and aluminum pitting corrosion—case histories. *In:* S. C. Dexter (Ed.) Biologically Induced Corrosion: Proceedings of the International Conference on Biologically Induced Corrosion. National Association of Corrosion Engineers. Houston, TX. 69–75.

Schüler, D., and R. B. Frankel. 1999. Bacterial magnetosomes: Microbiology, biomineralization and biotechnological applications. Appl. Microbiol. Biotechnol. 52:464–473.

Schultzen-Lam, S., G. Harauz, and T. J. Beveridge. 1992. Participation of a cyanobacterial S layer in fine-grain mineral formation. J. Bacteriol. 174:7971–7981.

Selwitz, C. M. 1992. The use of epoxy resins in field projects for stone stabilization. Mat. Res. Soc. Symp. Proc. 267:925–934.

Sequeira, C. A. C., and A. K. Tiller. 1988. Microbial Corrosion. Elsevier Science. London New York, 1:34–36.

Severini, F., R. Gallo, and S. Ipsale. 1988. Environmental degradation of polypropylene. Polym. Degr. Stab. 22:185–194.

Sharp, R. R., J. B. Bryers, W. G. Jones, and M. S. Shields. 1998. Activity and stability of a recombinant plasmid-borne TCE degradative pathway in suspended cultures. Biotechnol. Bioeng. 57:287–296.

Siedlarek, H., D. Wagner, W. R. Fischer, and H. H. Paradies. 1994. Microbiologically influenced corrosion of copper: The ionic transport properties of biopolymers. Corrosion Sci. 36:1751–1763.

Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt. 1986. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD.

Sneider, R. P., B. R. Chadwick, R. Pembrey, J. Jankowski, and I. Acworth. 1994. Retention of the Gram-negative bacterium SW8 on surfaces under conditions relevant to the subsurface environment: Effects of conditioning films and substratum nature. FEMS Microbiol. Ecol. 14:243–254.

Solomin, G. I. 1985. Problem of combined toxocological and higenic evaluation of polymer construction materials [in Russian]. Kosmicheskaya Biologiya i Aviakosmicheskaya Medistina 19:4–11.

Somlev V., and S. Tishkov. 1994. Anaerobic corrosion and bacterial sulfate reduction: Application for the purification of industrial wastewater. Geomicrobiol. 12:53–60.

Sonne-Hansen, J., I. M. Mathrani, B. K. Ahring. 1993. Xylanolytic anaerobic thermophiles from Icelandic hotsprings. Appl. Microbiol. Biotechnol. 38:537–541.

Srinivasan, R., P. S. Stewart, T. Griebe, and C.-I., Chen. 1995. Biofilm parameters influencing biocide efficacy. Biotechnol. Bioeng. 46:553–560.

Staley, J. T., M. P. Bryant, N. Pfenning, and J. G. Holt. 1989. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD.

Starkey, R. L. 1986. Anaerobic corrosion—perspectives about causes. *In:* S. C. Dexter (Eds.) Biologically Induced Corrosion: Proceedings of the International Conference on Biologically Induced Corrosion. National Association of Corrosion Engineers. Houston, TX. 3–7.

Stenbüchel, A. 1991. Polyhydroxyalkanoic acids. *In:* D. Byrom (Ed.) Biomaterials: Novel Materials From Biological Sources. Macmillan. New York, NY. 127–213.

Stern, R. V., and G. T. Howard. 2000. The polyester polyurethanase gene (pue A) from Pseudomonas chlororaphis encodes a lipase. FEMS Microbiol. Lett. 185:163–168.

Sternberg, D., P. Vigayakumar, and E. T. Reese. 1977. β-glucosidase: Microbial production and effect on enzymatic hydrolysis of cellulose. Can. J. Microbiol. 23:139–147.

Stewart, P. S. 1996a. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antmicrob. Agents Chemother. 40:2517–2522.

Stewart, P. S. M. A. Hamilton, B. R. Goldstein, and B. T. Schneider. 1996b. Modeling biocide action against biofilms. Biotechnol. Bioeng. 49:445–455.

Stolz, J. F., and R. S. Oremland. 1999. Bacterial respiration of arsenic and selenium. FEMS Microbiol. Rev. 23:615–627.

Stoodley, P., D. DeBeer, and H. M. Lappin-Scott. 1997. Influence of electric fields and pH on biofilm structure as related to the bioelectric effect. Antmicrob. Agents Chemother. 41:1876–1879.

Stranger-Joannesen, M. R. Sorheim, D. Zanotti, and A. Bichi. 1993. The ESA-LPTO simulation campaigns: Microbial contamination of the closed manned habitats. 44th Congress of the International Astronautical Confederation Congress, IAF/IAA-93-G.4.162. Graz, Austria.

Stroes-Gascoyne, S., K. Pedersen, S. Daumas, C. J. Hamon, S. A. Haveman, T. L. Delaney, S. Ekendahl, N. Jahromi, J. Arlinger, L. Hallbeck, and K. Dekeyser. 1996. Microbial Analysis of the Buffer/container Experiment at AECL's Underground Research Laboratory. Whiteshell Laboratories, Atomic Energy of Canada Ltd. Pinawa, Manitoba, Canada. AECL-11436:1–219.

Stuart, E. S., R. W. Lenz, and R. C. Fuller. 1995. The ordered macromolecular surface of polyester inclusion bodies in Pseudomonas oleovorans. Can. J. Microbiol. 41 (Suppl.):84–93.

Stumm, W., and J. J. Morgan. 1996. Oxidation and reduction, equilibria and microbial mediation. Aquatic Chemistry:

Chemical Equilibria and Rates in Natural Waters (3rd ed.). John Wiley. New York, NY. 425–515.

Suci, P. A., J. D. Vrany, and M. W. Mittelamn. 1998. Investigation of interactions between antimicrobial agents and bacterial biofilms using attenuated total reflection Fourier transform infrared spectroscopy. Biomaterials 19:327–339.

Sugio, T., Y. Tsujita, T. Katagiri, K. Inagaki, and T. Tano. 1988. Reduction of Mo6+ with elemental sulfur by Thiobacillus ferrooxidans. J. Bacteriol. 170:5956–5959.

Sugio, T., K. Hirayama, K. Inagaki, H. Tanaka, and T. Tano. 1992. Molybdenum oxidation by Thiobacillus derrooxidans. Appl. Environ. Microbiol. 58:1768–1771.

Sullivan, B. K., C. A. Oviatt, G. Klein-MacPhee. 1993. Fate and effects of a starch-based biodegradable plastic substitute in the marine environment. *In:* D. Kaplan, E. Thomas, and C. Ching (Eds.) Fundamentals of Biodegradable Materials and Packaging. Technomic Publishing. Lancaster, PA. 281–296.

Sunesson, A., W. H. J. Vaes, C. Nilsson, G. Blomouist, B., andersson, and R. Carlson. 1995. Identification of volatile metabolites from five fungal species cultivated on two media. Appl. Environ. Microbiol. 61:2911–2918.

Swift, M. J., O. W. Heal, and J. M. Anderson. 1979. Decomposer organisms. Decomposition in Terrestrial Ecosystems: Studies in Ecology. Blackwell Scientific Publications. London, UK. 5:66–117.

Szycher, M. 1989. Medical-grade polyurethanes: Their crucial role in artificial hearts. *In:* J. S. Hanker and B. L. Giammara. Biomedical Materials and Devices. Materials Research Society. Pittsburgh, PA. 110:41–50.

Tall, B. D., H. N. Williams, K. S. George, R. T. Gray, and M. Walch. 1995. Bacterial succession within a biofilm in water supply lines of dental air-water syringes. Can. J. Microbiol. 41:647–654.

Tanio, T., T. Fukui, T. Saito, K. Tomita, T. Kaiho, and S. Masamune. 1982. An extracellular poly(β-hydroxybutyrate) depolymerase from Alcaligenes faecalis. Eur. J. Biochem. 124:71–77.

Tatnall, R. E. 1986. Experimental methods in biocorrosion. *In:* S. C. Dexter (Ed.) Biologically Induced Corrosion: Proceedings of the International Conference on Biologically Induced Corrosion. National Association of Corrosion Engineers. Houston, TX. 246–253.

Tayler, S., and E. May. 1991. The seasonality of heterotrophic bacteria on sandstones of ancient monuments. Int. Biodeter. 28:49–64.

Tayler, S., and E. May. 1994. Detection of specific bacteria on stone using an enzyme-linked immunosorbent assay. Int. Biodeter. Biodegr. 33:155–167.

Tebo, B. M., and A. Y. Obraztsova. 1998. Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV) and Fe(III) as electron acceptors. FEMS Microbiol. Lett. 162:193–198.

Thorp, K. E. G., A. S. Crasto, J.-D. Gu, and R. Mitchell. 1994. Biodegradation of composite materials. *In:* T. Naguy (Ed.) Proceedings of the Tri-Service Conference on Corrosion. US Government Printing House. Washington DC, 303–314.

Thorp, K. E. G., A. S. Crasto, J.-D. Gu, and R. Mitchell. 1997. Contribution of Microorganisms to Corrosion. National Association of Corrosion Engineers. Houston, TX. Corrosion.

Tiano, P. 1993. Biodeterioration of stone monuments: A critical review. *In:* K. L. Garg, N. Garg, and K. G. Mukerji (Eds.) Recent Advances in Biodeterioration and Biodegradation. Naya Prokash. Calcutta, India. 301–321.

Tiano, P., P. Accolla, and L. Tomaselli. 1995. Phototrophic biodeteriogens on lithoid surfaces: An ecological study. Microb. Ecol. 29:299–309.

Tilstra, L., and D. Johnsonbaugh. 1993. A test method to determine rapidly if polymers are biodegradable. J. Environ. Polym. Degr. 1:247–255.

Tomaselli, L., G. Lamenti, M. Bosco, and P. Tiano. 2000. Biodiversity of photosynthetic micro-organisms dwelling on stone monuments. Int. Biodeter. Biodegr. 46:251–258.

Tomei, F. A., J. S. Maki, and R. Mitchell. 1985. Interactions in syntrophic associations of endospore-forming, butyrate-degrading bacteria and H_2-consuming bacteria. Appl. Environ. Microbiol. 50:1244–1250.

Torre, M. A. D., G. Gómez-Alarcón, P. Melgarejo, and J. Lorenzo. 1991. Fungal colonization of the Salamanca catheral sandstones: Some patterns of degradation. *In:* N. S. Baer, C. Sabbioni, and A. I. Sors (Eds.) Science, Technology and European Cultural Heritage. Butterworth-Heinemann. Oxford, UK. 511–514.

Torre, M. A. D., G. Gómez-Alarcón, and J. M. Palacios. 1993a. "In vitro" biofilm formation by Penecillium frequentans strains on sandstone, granite, and limestone. Appl. Microbiol. Biotechnol. 40:408–415.

Torre, M. A. D. L., G. Gómez-Alarcón, G. Vizcaino, and M. T. Garcia. 1993b. Biochemical mechanisms of stone alteration carried out by filamentous fungi living in monuments. Biogeochem. 19:129–147.

Torres-Sanchez, R., Magana-Vazquez, J. M. Sanchez-Yanez, and L. M. Gomez. 1997. High temperature microbial corrosion in the condenser of a geothermal electric power unit. Mat. Perf. 36:43–46.

Törrönen, A., C. P. Kubicek, and B. Henrissat. 1993. Amino acid sequence similarities between low molecular weight endo-1,4-β-xylanases and family H cellulases revealed by clustering analysis. FEBS Lett. 321:135–139.

Tsao, R., T. A., anderson, and J. R. Coats. 1993. The influence of soil macroinvertebrates on primary biodegradation of starch-containing polyethylene films. J. Environ. Polym. Degr. 1:301–306.

Uhlig, H. H. 1971. Corrosion and Corrosion Control. John Wiley. New York, NY.

Uhlig, H. H., and R. W. Revie. 1985. Corrosion and Corrosion Control: An Introduction to Corrosion Science and Engineering (3rd ed.). John Wiley. New York, NY.

Urzì, C., and M. Realini. 1998. Colour changes of Noto's calcareous sandstone as related to its colonization by microorganisms. Int. Biodeter. Biodegr. 42:45–54.

Vaidya, R. U., and D. P. Butt, L. E. Hersman, and A. K. Zurek. 1997. Effect of microbiological influenced corrosion on the tensile stress-stain response of aluminum and aluminum-partical reinforced aluminum composite. Corrosion 53:136–141.

Vandevivere, P., and D. L. Kirchman. 1993. Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl. Environ. Microbiol. 59:3280–3286.

Vandevivere, P. 1995. Bacterial clogging of porous media: A new modelling approach. Bifouling 8:281–291.

van Loosdrecht, M. C. M., J. Lyklema, W. Norde, G. Schraa, and A. J. B. Zehnder. 1987. The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. 53:1893–1897.

van Loosdrecht, M. C. M., J. Lyklema, W. Norde, G. Schraa, and A. J. B. Zehnder. 1990. Influence of interfaces on microbial activity. Microbiol. Rev. 54:75–87.

van Westing, E. P. M., G. M. Ferrari, and J. H. W. De Witt. 1994. The determination of coating performance with impedance measurements. II: Water uptake of coatings. Corrosion Sci. 36:957–977.

Verbicky, J. W. 1988. Polyimides. Encyclopedia of Polymer Science and Engineering. John Wiley. New York, NY. 12:364–383.

Verbiest, T., D. M. Burland, M. C. Jurich, V. Y. Lee, R. D. Miller, and W. Volksen. 1995. Exceptionally thermally stable polyimides for second-order nonlinear optical applications. Science 268:1604–1606.

Videla, H. A. 1996. Corrosion inhibition by bacteria. Manual of Biocorrosion. CRC Press. Boca Raton, FL. 121–136.

Viktorov, A. N., and N. D. Novikova. 1985. Distinctions in formation of microflora on construction materials used in habitable pressurized compartments [in Russian]. Kosmicheskaya Biologiya i Aviakosmicheskaya Medistina 19:66–69.

Viktorov, A. N., and V. K. IIyin. 1992. The actual problems of microbiological control in regenerative life support systems exploration. 43rd Congress of the International Astronautical Confederation Congress, IAF/IAA-92-0277. Washington DC.

Viktorov, A. N., V. K. Ilyin, and J. Syniak. 1993. The problems of microbial safety in regenerative life support systems exploration. 44th Congress of the International Astronautical Confederation Congress, IAF/IAA-93-G.4.161. Graza, Austria.

Viktorov, A. N. 1994. The characteristics of interaction between normal and conventionally pathogenic human microflora in different types of closed objects. 45th Congress of the International Astronautical Confederation Congress, IAF/IAA-94-5.165. Jerusalem, Israel.

von Wolzogen Kuhr, C. A. H., and I. S. van der Vlugt. 1934. The graphitization of cast iron as an electrochemical process in anaerobic soils. Water (The Hague) 18:147–165.

Wächtershäuser, G. 1988. Before enzymes and templates: Theory of surface metabolism. Microbiol. Rev. 52:452–484.

Wagner, P. A., and R. I. Ray. 1994. Surface analytical techniques for microbiologically influenced corrosion—a review. *In:* J. R. Kearns and B. J. Little (Eds.) Microbiologically Influenced Corrosion Testing. American Society for Testing and Materials. Philadelphia, PA. ASTM STP 1232:153–169.

Wagner, P. 1995. Microbial Degradation of Stressed Fiber Reinforced Polymeric Composites. NACE International. Houston, TX. Corrosion.

Wagner, P. R. Ray, K. Hart, and B. Little. 1996. Microbiological degradation of stressed fiber-reinforced polymeric composites. Mat. Perf. 35:79–82.

Wakefield, R. D. 1997. Masonry Biocides: Assessments of Efficacy and Effects on Stone. Scottish Soc. Conserv. Restor. 5–11.

Walch, M., and R. Mitchell. 1986. Microbial influence on hydrogen uptake by metals. *In:* S. C. Dexter (Eds.) Biologically Induced Corrosion: Proceedings of the International Conference on Biologically Induced Corrosion. National Association of Corrosion Engineers. Houston, TX. 201–209.

Walch, M., T. E. Ford, and R. Mitchell. 1989. Influence of hydrogen-producing bacteria on hydrogen uptake by steel. Corrosion 45:705–709.

Walch, M. 1992. Corrosion, microbial. *In:* J. Lederberg Encyclopedia of Microbiology. Academic Press. San Diego, CA. 1:585–591.

Wang, P.-C., T. Mori, K. Komori, M. Sasatsu, K. Toda, and H. Ohtake. 1989. Isolation and characterization of an Enterobacter cloacae starain that reduces hexavalent chromium under anaerobic conditions. Appl. Environ. Microbiol. 55:1665–1669.

Wang, C.-C., C.-Z. Fan, S.-J., and Wang, and M.-G. Zhang. 1991. Research on powdery corrosion of the Serials Bells from Cai Hou Tomb. Sci. China (Ser. B.) 34:522–529.

Wang, C.-C., Y.-S. Wu, C.-Z. Fan, S.-J., and Wang, and Y.-M. Hua. 1993. Formation mechanism of particulates in the surface layer of Bronze Mirror. Chinese Sci. Bull. 38:429–432.

Wang, J.-S. 1996. Hydrogen induced embrittlement and the effect of the mobility of hydrogen atoms. *In:* A. W. Thompson and N. R. Moody (Eds.) Hydrogen Effects in Materials. The Minerals, Metals and Materials Society. Warrendale, PA. 61–75.

Warcheid, T., M. Oelting, and W. E. Krumbein. 1991. Physicochemical aspects of biodeterioration processes on rocks with special regard to organic pollutants. Int. Biodeter. 28:37–48.

Weimer, P. L., M. J. van Kavelaar. C. B. Michel, and T. K. Ng. 1988. Effect of phosphate on the corrosion of carbon steel and on the composition of corrosion products in two-stage continuous cultures of Desulfovibrio desulfuricans. Appl. Environ. Microbiol. 54:386–396.

Westlake, D. W. S., K. M. Semple, and C. O. Obuekwe. 1986. Corrosion by ferric iron-reducing bacteria isolated from iol production systems. *In:* S. C. Dexter (Ed.) Biologically Induced Corrosion: Proceedings of the International Conference on Biologically Induced Corrosion. National Association of Corrosion Engineers. Houston, TX. 193–200.

White, D. C., D. E. Nivens, G. G. Geesey, and C. K. Clarke. 1986. Role of aerobic bacteria and their extracellular polymers in the facilitation of corrosion: Use of Fourier tranforming infrared spectroscopy and "signature" phospholipid fatty acid analysis. *In:* S. C. Dexter (Ed.) Biologically Induced Corrosion. Proceedings of the International Conference on Biologically Induced Corrosion. National Association of Corrosion Engineers. Houston, TX. 233–243.

Whitfield, C. 1988. Bacterial extracellular polysaccharides. Can. J. Microbiol. 34:415–420.

Widdel, F. 1988. Microbiology and ecology of sulfate- and sulfite-reducing bacteria. *In:* A. J. B. Zehnder (Ed.) Biology of Anaerobic Microorganisms. John Wiley. New York, NY. 469–585.

Widdel, F. 1992. Microbial corrosion. *In:* R. F. Finn, P. Prave, M. Schlingmann, W. Crueger, K. Esser, R. Thauer, and F. Wagner (Eds.) Biotechnology Focus. Hanser Publishers. Munich, Germany. 3:261–300.

Wiencek, K. M., and M. Fletcher. 1995. Bacterial adhesion to hydroxyl- and methyl-terminated alkanethiol self-assembled monolayers. J. Bacteriol. 177:1959–1966.

Wilkinson, J. F., and G. H. Stark. 1956. The synthesis of polysaccharide by washed suspensions of Klebsiella aerogenes. Proc. R. Phys. Soc. Edinburgh 25:35–39.

Williams, S. T., M. E. Sharpe, and Holt, J. G. 1989. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD.

Williams, V., and M. Fletcher. 1996. Pseudomonas fluorescens adhesion and transport through porous media are affected by lipopolysaccharide composition. Appl. Environ. Microbiol. 62:100–104.

Williamson, P. R. 1994. The screening of sponge extracts for antifouling activity using a bioassay with labotatory-reared cyprid larvae of the barnacle Balanus amphitrite. Int. Biodeter. Biodegr. 34:361–373.

Williamson, P. R., K. Wakamatsu, and S. Ito. 1998. Melanin biosynthesis in Crypotococcus neoformans. J. Bacteriol. 180:1570–1572.

Wimpenny, J. W. T., and R. Colasanti. 1997. A unifying hypothesis for the structure of microbial biofilm based on cellular automaton models. FEMS Microbiol. Ecol. 22:1–6.

Wirsen, C. O., and H. W. Jannasch. 1993. Microbial degradation of a starch-based biopolymer in the marine environment. *In:* D. Kaplan, E. Thomas, and C. Ching (Eds.) Fundamentals of Biodegradable Materials and Packaging. Technomic Publishing. Lancaster, PA. 297–310.

Woese, C. R., and G. J. Olsen. 1986. Archaebacterial phylogeny: Perspectives on the Urkingdoms. System. Appl. Microbiol. 7:161–177.

Woese, C. R. 1987. Microbial evolution. Microbiol. Rev. 51:221–171.

Woese, C. R. 1990. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria and Eukatya. Proc. Natl. Acad. Sci. USA 87:4576–4579.

Woese, C. R. 1998. Default taxonomy: Ernest Mayr's view of the microbial world. Proc. Natl. Acad. Sci. USA 95:11043–11046.

Wolfaardt, G. M., J. R. Lawrence, R. D. Robarts, S. J. Caldwell, and D. E. Caldwell. 1994. Multicellular organization in a degradative biofilm community. Appl. Environ. Microbiol. 60:434–446.

Wong, K. K. Y., L. U. L. Tan, and J. N. Saddler. 1988. Multiplicity of β-1,4-xylanase in microorganisms: Function and applications. Microbiol. Rev. 52:305–317.

Wu, Y.-S., C.-S. Wang, C.-Z. Fan, S.-J. Wang, and Z.-C. Li. 1992. A study on corrosion-resistance mechanism of the ancient mirror "Hei Qi Gu". Acta Phys. Sinica 41:170–176.

Xu, X., P. S. Stewart, and X. Chen. 1996. Transport limitation of chlorine disinfection of Pseudomonas aeruginosa entrapped in alginate beads. Biotechnol. Bioeng. 49:93–100.

Yao, Y., and Y. Li. 1995. Research and development of concrete admixtures. Chinese J. Mat. Res. 9 (Suppl.):381–390.

Yoshizako, F., A. Nishimura, and M. Chubachi. 1992. Microbial reduction of cyclohexanone by Chlorella pyrenoidosa Chick. J. Ferment. Bioeng. 74:395–397.

Young, G. H. 1948. Anti-fouling measures. *In:* H. H. Uhlig (Ed.) The Corrosion Handbook. John Wiley. New York, NY. 441–446.

Young, L. L., and C. E. Cerniglia. 1995. Microbial Transformation and Degradation of Toxic Organic Chemicals. Wiley-Liss. New York, NY.

Yu, F. P., and G. A. McFeters. 1994. Physiological responses of bacteria in biofilms to disinfection. Appl. Environ. Microbiol. 60:2462–2466.

Yue, S., R. M. Pilliar, G. C. Weatherly. 1984. The fatigue strength of porous-coated Ti-6%Al-4%V implant alloy. J. Biomed. Mat. Res. 18:1043–1058.

Zachary, A., M. E. Taylor, F. E. Scott, and R. R. Colwell. 1980. Marine microbial colonization on material surfaces. *In:* T. A. Oxley, G. Becker, and D. Allsopp (Eds.) Biodeterioration: Proceedings of the 4th International Biodeterioration Symposium. Pitman Publishing. London, 171–177.

Zaloguyev, S. N. 1985. Results of microbiological studies conducted during operation of Salyut-6 orbital space station [in Russian]. Kosmicheskaya Biologiya i Aviakosmicheskaya Medistina 19:64–66.

Zavarzin, G. A., T. N. Zhilina, and M. A. Pusheva. 1994. Halophilic acetogenic bacteria. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 432–444.

Zehnder, A. J. B., and W. Stumm. 1988. Geochemistry and biochemistry of anaerobic habitats. *In:* A. J. B. Zehnder (Ed.) Biology of Anaerobic Microorganisms. John Wiley. New York, NY. 1–38.

Zhou, Z., and N. Brown. 1995. Slow crack growth in polyethylene gas pipes and resins. Chinese J. Mat. Res. 9 (Suppl.):463–472.

Zinder, S. H. 1993. Physiological ecology of methanogens. *In:* J. G. Ferry (Ed.) Methanogenesis: Ecology, Physiology, Biochemistry and Genetics. Chapman and Hall. New York, NY. 128–206.

Zuo, J., C. Xu, X. Liu, and C. Wang. 1994. Study of the Raman spectrum of nanometer SnO_2. J. Appl. Phys. 75:1835–1836.

Zyska, B. 1996. Performance of paper in Polish books of the period 1900–1994. Restaurator 17:214–228.

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CHAPTER 3.10

Microbial Biofilms

DIRK DE BEER AND PAUL STOODLEY

Introduction

Biofilms are usually thought of as the slimy layer of microorganisms that covers solid surfaces. However, there are a number of features that distinguish biofilm populations from their planktonic (suspended or free floating) counterparts, namely: the association with a surface, high population densities (on the order of 10^{10} cells per ml of hydrated biofilm), an extracellular polymer (EPS) slime matrix, and a wide range of physical, metabolic and chemical heterogeneities. However, some biofilms may not have all features. Indeed a concise universal definition of biofilms has yet to emerge; in part this is because of the wide diversity of biofilm populations. Although much of contemporary microbiology is based on the study of planktonic "cells," it is now thought that biofilms are the primary habitat for many microorganisms. Microbial mats associated with sediment and suspended microbial flocs or aggregates, although different in appearance from conventional biofilms, have many important features in common and thus are included in the definition of "biofilm." Often biofilm cells are embedded within a highly hydrated EPS matrix, and in the absence of corrosion products or scale, biofilms are estimated to be primarily water. The physical properties of the biofilm are largely determined by the EPS, while the physiological properties are determined by the bacterial cells.

Characklis (1990a) identified up to eight processes in the development of biofilms. These can be condensed to three main processes: the attachment of cells to a surface (colonization), growth of the attached cells into a mature biofilm, and the detachment of single cells (erosion) or large pieces (sloughing).

Since free convection is hindered within biofilms, the chemical environment to which the cells are exposed differs from the surrounding water phase. Also, mass transfer to the cells often limits conversion rates. All natural biofilms, mats, aggregates, and flocs can consist of complex microbial communities, and their function is characterized by interactions between different populations within these communities. Many experimental methods to study aggregated biomass are similar, i.e., microscopic and staining techniques. We will refer therefore in this review to all types of aggregated microorganisms. Table 1 gives some examples of biofilm types.

Biofilms, flocs, and microbial mats are responsible for most microbial conversions in natural environments. Natural biofilms can develop on solid surfaces under all conditions facilitating microbial growth thus biofilms are ubiquitous in nature, covering rocks and plants in seawater and freshwater, sediment grains, and sediment surfaces. Microbial mats are formed on most sediments, especially under extreme conditions (temperature, salinity) that inhibit the activity of grazers (Karsten and Kühl, 1996). Flocs are highly fragile structures suspended in fresh and seawater (called river- and marine snow) and typically occur during bloom periods after an increased input of nutrients. Consequently, biofilms exist almost everywhere, and microbial aggregates, are responsible for the majority of the microbial conversions in many aquatic ecosystems. Biofilms have been associated with a wide range of problems both in industry and in medicine (Table 2) and have been utilized for various processes (Table 3).

Microbial cells, living in biofilms are much more difficult to eradicate or control than suspended cells. Yet the susceptibility of biofilm cells to antibiotics and industrial antimicrobial agents is rarely assessed. In part this is due to convention and in part because standard testing protocols against suspended cultures are much easier to develop. Cell density and growth phase can be easily controlled in both batch and chemostat cultures, whereas biofilms are highly variable.

Currently, the most important practical use of biofilms is for biological wastewater treatment, while many emerging technologies are utilizing biofilms for biodegradation and bioremediation in bioreactors. Municipal wastewater is treated in activated sludge plants that are based on the activity of flocs. Their relevance in natural element cycles as well as their economical and med-

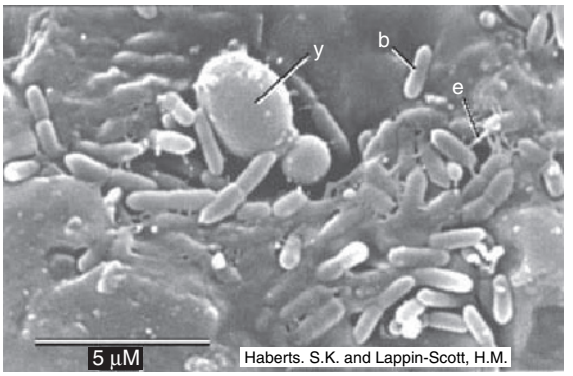

Fig. 1a. Scanning electron photomicrograph showing biofilm formation after 24-h growth on stainless steel AISI 321. The biofilm was established under laminar pipe flow (Re = 2.72) using a modified Robbins device (MRD). The inocula were all environmental isolates and consisted of four Gram-negative rod-shaped bacteria, *Stenotrophomonas maltophilia*, *Pseudomonas alcaligenes*, *Alcaligenes denitrificans*, and *Flavobacterium indologenes*; one nonfermentative yeast, *Rhodotorula glutinis*; and two filamentous fungi, *Fusarium solani* and *Fusarium oxysporum* (Elvers, 1998). The image shows a budding yeast cell "y" and bacterial rods "b" attached to the surface. The bacteria are embedded in an EPS matrix. During preparation for SEM the dehydrated EPS has formed characteristic strands "e." Scale bar = 5 µm. Image supplied by Sara K. Roberts, Biological Sciences, Exeter University.

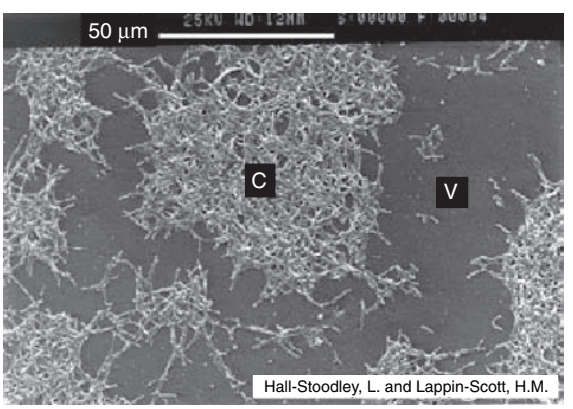

Fig. 1b. Scanning electron photomicrograph showing biofilm formation of *Mycobacterium fortuitum* after 24-h growth on silicone rubber (Hall-Stoodley, 1998). The biofilm was grown under laminar flow in a MRD. The biofilm was composed of cell clusters "c" surrounded by voids "v." Scale bar = 50 µm. Image supplied by Luanne Hall-Stoodley, Biological Sciences, Exeter University.

ical impact has been recognized, and the study of immobilized cell systems has gained considerable momentum in the last decade. The knowledge has been advanced because of new techniques to determine the functioning, structure and microbial populations in biofilms.

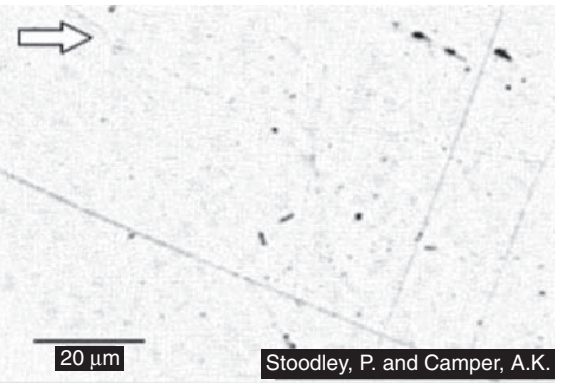

Fig. 2a. Time-lapse movie showing the initial events of biofilm formation. *Pseudomonas aeruginosa* cells are attaching to a 316L stainless steel coupon over 6.5 h. The *Ps. aeruginosa* cells were initially grown in a chemostat with a residence time of 5 h. The stainless steel coupon was mounted in a flat plate flow cell and the inoculum was delivered with an average flow velocity of 2.8 cm $\cdot s^{-1}$ (Re = 6). Note how some of the cells divide, detach and move around on the surface. Arrow indicates flow direction. Scale bar = 20 µm. Images were enhanced for clarity using NIH-Image 1.59 (available at http://zippy.nimh.nih.gov). For the Fig. 2a, see the online version of *The Prokaryotes*.

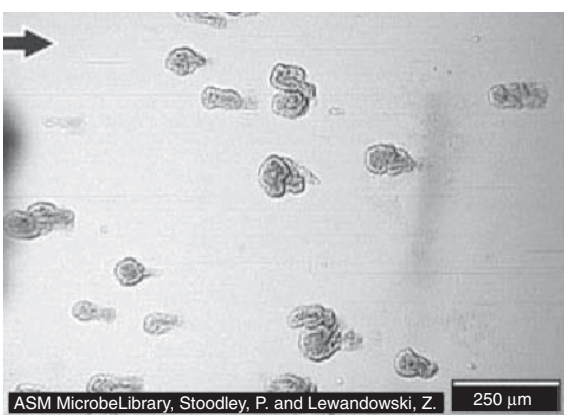

Fig. 2b. Time-lapse movie showing the accumulation of a bacterial biofilm on a glass surface over 14 days. The biofilm was composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae* and was grown in a glass flow cell (average flow velocity = 1.8 m $\cdot s^{-1}$) to simulate conditions in an industrial pipeline. Note the complex structure of biofilm patches (dark) and water channels between them. After 12 days much of the biofilm detached from the surface in a "sloughing event." There was a corresponding decrease in pressure drop across the flow cell, which had been building up as the biofilm accumulated (Lewandowski and Stoodley, 1995). The arrow shows the direction of fluid flow. Scale bar = 250 µm. This sequence of images has been accepted for use in the ASM Biofilms Collection (http://www.asmusa.org/edusrc). For the Fig. 2b, see the online version of *The Prokaryotes*.

Table 1. Examples of different types of biofilm.

Environment	Biofilm type	Thickness (m)	Community	References
Natural	Photosynthetic microbial mats, hot springs and hypersaline lakes	10^{-3}–1	Mixed algal and bacterial community	Stal, 1994
	Stromatolites	1	Bacterial	Stal, 1994
	Benthic/river sediments	10^{-6}–10^{-3}	Mixed bacterial, algal and protozoan communities	Baty, 1996 Costerton, 1994
Medical	Dental plaque	10^{-6}–10^{-4}	Mixed bacterial community	Kinnement, 1996
	Infectious	10^{-6}–10^{-3}	Often bacterial or fungal mono-cultures	Morck, 1994 Buret, 1991
Industrial	Heat exchangers	10^{-6}–10^{-3}	Mixed bacterial and fungal communities	Characklis, 1990
	Drinking water pipes	10^{-6}–10^{-2}	Mixed bacterial and fungal communities	Camper, 1994 Van Der Kooij, 1994
	Wastewater treatment	10^{-4}–10^{-3}	Mixed bacterial and fungal communities, biofilms, aggregates and flocs	Lemmers, Griebe, 1995
	Filtration units	10^{-5}–10^{-4}	Mixed bacterial and fungal biofilms	Flemming, 1996
	Ship hulls	10^{-4}–10^{-2}	Mixed bacterial and algal and marine macroorganisms	Cooksey, 1995

Table 2. Problems associated with biofilms.

Problems	Consequences
Fouling of heat exchangers	Loss of heat exchange efficiency and reduction of flow capacity
Fouling of ships	Energy losses
Oil reservoirs	H_2S souring by sulfate–reducing bacteria
Industrial and drinking water pipelines	Energy losses, pitting and general corrosion, product contamination, pathogen reservoirs
Dental plaque	Dental caries
Medical infections	Colonization of indwelling devices (catheters, artificial joints, contact lenses) —endocarditis

Table 3. Processes which utilize biofilms.

Processes	Uses
Wastewater treatment	Bioremoval of pollutants
Biobarriers[a]	Immobilization of ground water contaminants; microbially enhanced oilfield recovery (MEOR)
Metals leaching	Enhanced recovery of metals

[a]See MacLeod, 1988.

Biofilms and microbial mats are thus important microbial communities in most aquatic ecosystems today. Interestingly, the first known fossils of single microbes and microbial communities share almost identical structural characteristics to those found in recent biofilms and microbial mats (Schopf and Klein, 1992).

Because of their ubiquity in natural and industrial environments, the study of biofilms lends itself to a multidisciplinary approach involving microbiology and engineering. Originally, an engineering approach was used to study biofilm performance on the macro-scale (i.e., for optimization of wastewater treatment plants). Subsequently, engineering concepts were applied to further our understanding of biofilm processes on the micro-scale. An important task for microbiologists studying biofilms is to determine the types of organisms present and to determine their in situ activities. This chapter will focus on recent findings on biofilm structure, mass-transfer phenomena, microbial activities and community structure. Brief descriptions of biofilm cultivation methods and new techniques to determine biofilm structure in situ, community structure and population distributions and in situ microbial activity distributions will be given.

Biofilm Structure

Biofilm structure is the spatial arrangement of bacteria, cell clusters, EPS and particulates. Since the structure can influence transport resistance, it is a significant determinant in the activity of the biofilm. Various conceptual and mathematical models have been proposed to describe the structure and function of biofilms (Characklis, 1990a; Rittmann and Manem, 1992; Wanner and Gujer, 1986). Mathematical models describing transport, conversion, cell growth and biofilm development are based on conceptual models. Biofilms were initially considered as planar structures, impermeable and with homogeneous cell distribution. Mass transfer through the mass boundary layer and within the biofilm was assumed to be diffusional and perpendicular to the surface to which it was attached (the substratum).

Biofilms and mats are matrices of cells and extracellular polymers (EPS). The EPS is produced by the cells and consists of polysaccharides, polyuronic acids, proteins, nucleic acids and lipids (Schmidt and Ahring, 1994; Decho, 1990; Decho and Lopez, 1993). EPS holds the cells together and to the substratum. Owing to the dimensions of microbial mats and biofilms, their structural analysis is strongly dependent on microscopic methods that are briefly discussed and listed in Table 4.

Heterogeneity

Recent microscopic observations indicated that biofilms are not flat and the distribution of microorganisms is not uniform. Instead, multispecies biofilms were observed with complex structures containing "voids," channels, cavities, pores, and filaments and with cells arranged in clusters or layers. Such complex structures were found in a wide variety of biofilms such as methanogenic films from fixed-bed reactors (Robinson et al., 1984), aerobic films from wastewater plants (Eighmy et al., 1983; Mack et al., 1975), nitrifying biofilms (Kugaprasatham et al., 1992), and pure culture biofilms of *Vibrio paraheamolyticus* (Lawrence et al., 1991) and *Pseudomonas aeruginosa* (Stewart et al., 1993).

Depending on growth conditions and age, the thickness of biofilms can range from a few micrometers (a monolayer) up to a centimeter. Owing to the microscopic dimensions of microbial mats and biofilms, their structural analysis strongly depends on the microscopic methods used. Most microscopic techniques involve preparation of the sample, such as dehydration and

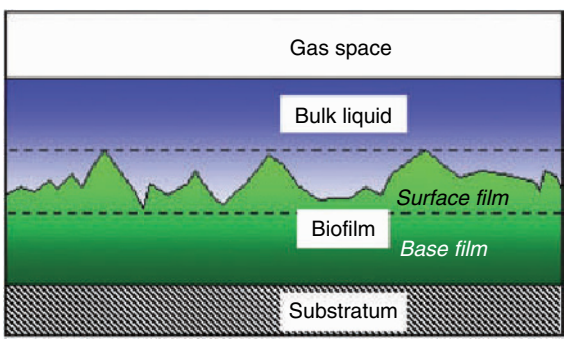

Fig. 3. Conceptual model for biofilm structure proposed at the 1988 Dahlem conference (Wilderer, 1989). The model divides the biofilm system into specific compartments: the substratum, the biofilm, the bulk liquid and a possible head space. The biofilm compartment was further subdivided into a base film and a surface film. Although the model recognized a certain degree of biofilm roughness, it was essentially a planar layered model.

Table 4. List of microscopic techniques for studying biofilms and mats.

Microscopy technique	Spatial resolution	Application	Sample treatment	References
LM	1 µm	EPS and cells	Dehydration, freezing, sectioning, staining	Chayen, 1973
FM	1 µm	EPS and cells	Dehydration, freezing, sectioning, staining	Stewart, 1995; Griebe, 1995; De Beer, 1996
SEM	1 nm	Cell and EPS surfaces	Dehydration, sputter coating	Beeftink, 1986; Paterson, 1995
ESEM	10 nm	Cell and EPS surfaces	None	Little, 1991
TEM	1 nm	Cells and EPS	Dehydration, sectioning, staining	Beeftink, 1986; Bakke, 1984; Sanford, 1995
CSLM	1 µm	EPS, cells, voids	Staining	Lawrence, 1991; De Beer, 1994
AFM	0.1 µm	Cell and EPS surfaces	None	Bremer, 1992; Gunning, 1996

Abbreviations: LM, light microscopy; EPS, extracellular polymer slime; FM, fluorescence microscopy; SEM, scanning electron microscopy; ESEM, environmental scanning electron microscopy; TEM, transmission electron microscopy; CSLM, confocal scanning laser microscopy; AFM, atomic force microscopy.

embedding,which causes the soft biofilm structure to collapse and often to be observed as flattened (Stewart et al., 1995). Because this structure most conveniently agreed with the basic assumption for one-dimensional (1-D) modeling, it was accepted as the general structure of biofilms. However, a study by Siebel and Characklis (1990) using interference contrast (Nomarsky) microscopy challenged this assumption. They reported that binary population biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* could form uneven biofilms consisting of patchy clusters of cells surrounded by a smooth monolayer. After the introduction of confocal scanning laser microscopy (CSLM), however, the perception changed drastically. The findings with new microscopic techniques indicate that the assumptions for 1-D geometry need to be carefully analyzed. CSLM images of undisturbed biofilms show that biofilms can consist of biomass clusters separated by interstitial voids (De Beer et al., 1994). [Voids were made visible with a negative staining by fluorescein that is strongly quenched by biomass. Cells (stained with a DNA stain) and EPS (stained with calcofluor and Alcian blue) were observed in the clusters, while no cells or EPS could be detected in the voids.] Fluorescent beads (0.3 μm) added to the medium immediately penetrated the voids, but not the cell clusters. It was concluded that voids were water channels in open connection with the bulk water phase. Fluid flow in the biofilm was later directly demonstrated, and quantified, by using the beads as particle tracers to visualize flow through the water channels (Stoodley et al., 1994). The flow velocity of individual beads at various depths in the biofilm channels were calculated by measuring the bead track length, using confocal microscopy. The resulting flow profiles were consequently used to determine the fluid shear stress acting on the channel wall and the surface of the biofilm cell clusters (deBeer et al., 1994; Stoodley et al., 1994). These observations were made on biofilms grown in the lab, either as undefined culture or as mixed pure culture. Similar observations were reported from both pure culture biofilms and biofilms with undefined microbial communities from various sources (Massol-Deya et al., 1995; Gjaltema et al., 1994; Zhang, 1994; Neu and Lawrence, 1997; Okabe et al., 1996; Okabe et al., 1997). The presence of voids has considerable consequences for mass transfer inside the biofilms (advection) and exchange of substrates and products with the water phase (effective exchange surface), as will be discussed in the relevant section. These new findings have led to a concept that incorporates two key features: structural heterogeneity and the water flow within the biofilm.

Extracellular Polymeric Substances (EPS)

The proportion of EPS can vary between 50–80% of the organic matter and is the main structural component of biofilms. The physical properties of the biofilm are largely determined by the EPS, while the physiological properties are determined by the bacterial cells. A common perception was that EPS consists mainly of polysaccharides, and many detection techniques focus on this group of compounds (Christensen and Characklis, 1990; Neu and Lawrence, 1997; Beeftink and Staugaard, 1986; Williams and Wimpenny, 1978; De Beer, 1996). Also, research relating EPS to biofilm functioning, cell-cell and cell-surface interactions was concentrated on the polysaccharide fraction of EPS. However, recent analyses showed that biofilms contain EPS consisting of a mixture of protein, polysaccharides, lipids and nucleic acids (Nielsen et al., 1997; Schmidt and Ahring, 1994; Jahn, 1995; Frolund et al., 1996). Protein appeared the most abundant EPS component (50% or more) in activated sludge (Frolund et al., 1996); biofilms (Jahn and Nielsen, 1995), and anaerobic aggregates (Ahring et al., 1993), while polysaccharides were much less abundant (5–20%). Detailed knowledge is available on the polysaccharide content of both laboratory grown and natural biofilm EPS (Sutherland, 1994; Sutherland, 1996); however, data on the actual composition of nonpurified biofilm EPS as it occurs in situ are lacking. Thus we face the situation that the actual composition of EPS, including the protein fraction, is largely unknown, as are its chemical and physical properties. Since EPS is the second important fraction of biofilms, beside cells, research on the chemistry and properties of EPS has a high priority.

More research on the composition and function of EPS is needed, since EPS has been linked with many processes and properties integral to biofilm behavior, i.e., attachment, detachment, mechanical strength, antibiotic resistance, and exo-enzymatic degradation activity. The mechanical stability of a biofilm is important for stable process maintenance (sloughing of biofilms, floc stability). To remove unwanted biofilms, surfactants are used to weaken the strength of the matrix. Furthermore, there is evidence that biofilms maintain their structural heterogeneity by releasing EPS-degrading enzymes (Davies et al., 1998). This interesting process is thought to involve cell-cell communication, i.e., quorum-sensing, through the generation of homoserine lactones (Greenberg, 1997).

Binding of water is important for dehydrating activated sludge (Nielsen et al., 1996). Pollutants may bind considerably to the EPS of biofilms; 60% of biofilm-bound BTX, but less than 20%

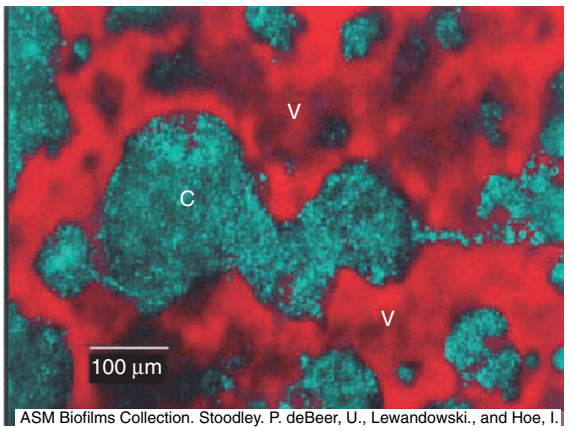

Fig. 4a. Biofilm composed of *P. aeruginosa*, *P. fluorescens*, and *K. pneumoniae* grown in a glass flow cell for 5 days (de Beer et al., 1994a). The image was taken using CSLM which allows high resolution 3-D imaging of fully hydrated samples. Differential staining with propidium iodide (a nucleic acid stain) and fluorescein (red) showed that the biofilm consisted of cell clusters "c" separated by interstitial voids "v" or water channels. Scale bar = 100 µm. Image available from the ASM Biofilms Collection (http://www.asmusa.org/edusrc/edu34.htm).

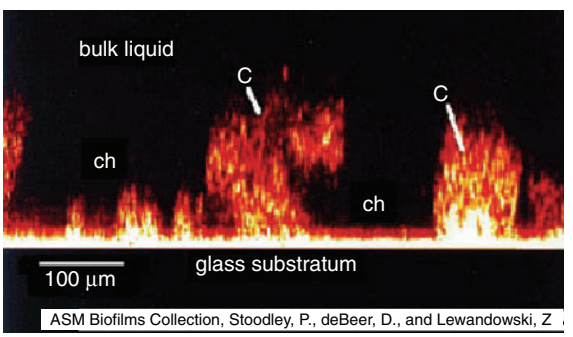

Fig. 4b. Side view of the same biofilm in Fig. 4a. Cells appear red and orange and are grouped in distinct cell clusters "c" separated by water channels "ch." Some of the cell clusters formed "mushroom" shapes, which greatly increase the available surface area for nutrient and waste product exchange with the bulk liquid. The horizontal white line is the glass surface. Scale bar = 100 µm. Image available from the ASM Biofilms Collection (http://www.asmusa.org/edusrc/edu34.htm).

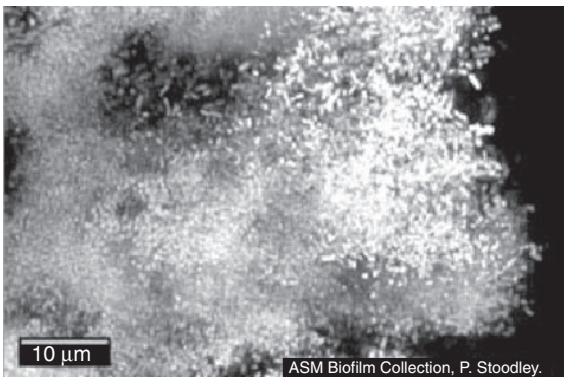

Fig. 4c. Movie sequence showing 3-D structure of a mixed-species biofilm taken by CSLM. The biofilm was composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae* and was grown in a flow cell with an average liquid flow velocity of 6.6 cm · s^{-1}. The biofilm was heterogeneous and was made up of microbial cell clusters (individual cells are stained with propidium iodide and appear as bright dots) held in an EPS matrix (not stained in this image). The biofilm was approximately 150-µm thick and protrudes out towards the viewer. The image is composed of 27 overlaid optical sections taken at 6-µm depth intervals. The motion is an artifact used to give the 3-D effect. Scale bar = 10 µm. For the Fig. 4c, see the online version of *The Prokaryotes*.

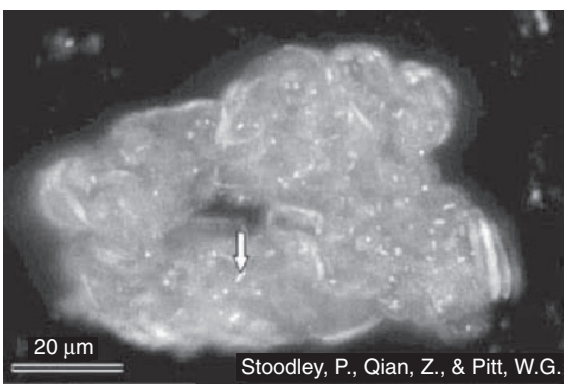

Fig. 4d. Movie sequence showing 3-D structure of a pure culture *Pseudomonas aeruginosa* biofilm taken by CSLM (Qian et al., 1996). The biofilm was heterogeneous and consisted of cell clusters and surrounding water channels. This image shows a donut-shaped cell cluster that protrudes out towards the viewer. The bright dots are stained bacterial cells (representative cell indicated by arrow) and the lighter, hazy material is probably EPS slime. The biofilm was grown in a polycarbonate flow cell on a glass slide. Scale bar = 20 µm. For the Fig. 4d, see the online version of *The Prokaryotes*.

of the biofilm-bound heavy metals (Späth et al., 1998), was located in the EPS.

EPS can mask the original surface properties of the cells and render hydrophobic surfaces hydrophilic. The phenomenon of flotation of anaerobic aggregates occurs by attachment of gas bubbles to the hydrophobic aggregate surface. Aggregates with low amounts of EPS showed a strong tendency to float, leading to severe biomass losses from the reactors. The presence of carbohydrates in the feed increases the amount of EPS, especially on the aggregate surface, inhibiting attachment of gas bubbles and preventing flotation (De Beer, 1996; Neu and Lawrence, 1997).

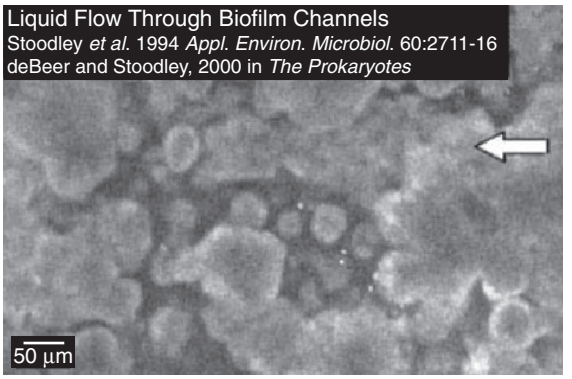

Fig. 5. Time lapse CSLM movie sequence showing fluorescent latex beads moving through biofilm water channels (Stoodley et al., 1994). The bacterial biofilm, composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae*, was grown in a flow cell on a glass cove slip. The sequence of images was taken over 44 s. The beads were moving with a velocity of approximately 15 µm · s^{-1}, the average liquid flow velocity of the bulk liquid was 6.6 cm · s^{-1}. The arrow indicates flow direction of the bulk liquid. Note that in some cases the flow around the cell clusters is counter that of the channel current. The biofilm clusters were autofluorescent and appear lighter than the surrounding water channels. The optical section was taken at a depth of 70 µm in the 175-µm thick biofilm. Scale bar = 50 µm. For the Fig. 5, see the online version of *The Prokaryotes*.

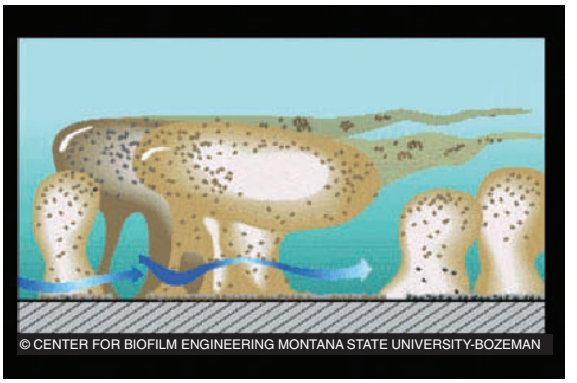

Fig. 6. Recent "Center for Biofilm Engineering" conceptual biofilm model incorporating structural complexity and liquid flow through biofilm channels. The model also incorporates biofilm streamers, which form as a function of fluid shear. The schematic was composed by Peg Dirckx of the Center for Biofilm Engineering (with permission from the Center for Biofilm Engineering, Bozeman, Montana, USA).

The diffusion coefficient of solutes in biofilms is influenced by the microstructure of EPS (Neu and Lawrence, 1997; De Beer et al., 1997). Biofilms have been considered to be highly porous polymer gels (Christensen and Characklis, 1990) and diffusion studies demonstrate their gel-like characteristics (De Beer et al., 1997). Also, recent in situ rheological testing of *P. aeruginosa* biofilms, grown in the absence of divalent cations, showed that the biofilm behaved like a non-cross-linked polymer gel (Stoodley et al., 1999c). In this case, the EPS matrix can be considered to be a two-phase system with a solid network of polymers and free interstitial water as depicted by Stewart (Stewart, 1998). Only then does the structure of the network effect diffusivity (Westrin, 1991), particularly when the pore size of the network is of the same order of magnitude as the molecular diameter of the solute. Based on this assumption, it is possible to infer some properties of the microstructure of EPS from the diffusional behavior of large molecules. It was found that the diffusion of small molecules is not strongly inhibited by the biofilm matrix, whereas diffusion of large molecules is impeded (Bryers and Drummond, 1996). Similar effects of the molecular size were found using microinjection of fluorescent dyes (fluorescein, MW 332, Ø > 1 nm, diffusivity not affected, and phycoerithrin, MW 240,000, Ø = 11 nm, 40% reduction of diffusivity; De Beer et al., 1997). The pore size of the biofilm matrix (ca. 80 nm) was calculated from these data.

However, it has been reported that the forces keeping the polymers together are not strong covalent bonds, but weak hydrophobic and electrostatic interactions and hydrogen bonds (Flemming, 1998). These forces are weakened by surface active agents, complexing agents, pH and ionic strength. Flemming concluded that a significant portion of the EPS may not be in bound form; if so, the polymers would increase the viscosity and reduce the diffusion coefficient, which could explain in part contradictory findings on D_{eff}. Boyd and Chakrabarty (1994) hypothesized that altering the length of polymer chains through the activity of alginate lyase may control the viscosity of *P. aeruginosa* biofilms. It is clear that further research is needed on both the physical and chemical properties of EPS, which appears to play a critical role in the structure and function of biofilms.

Morphogenetic Factors

In conclusion, the architectural features of biofilms can be viewed in terms of a hierarchical arrangement, the basic components of the biofilm being the cells and the EPS. These can combine to form secondary structures such as discrete cell clusters (which may take on various forms and dimensions) and a base film. Finally, the arrangement of base film, cell clusters, and the void areas between the clusters gives the overall biofilm architecture. The relative impor-

tance of each of these features in determining the biofilm heterogeneity can be highly variable.

HYDRODYNAMICS Hydrodynamic conditions control two interdependent parameters (mass transfer and shear stress) and will, therefore, significantly influence many of the processes involved in biofilm development. Two types of flows are relevant to most natural and industrial processes: laminar flow and turbulent flow. Generally, when the flow rate of a liquid is low, flow will be laminar, and when the rate is high, it will be turbulent. Transition between these two types of flow will be dependent on channel geometry and fluid properties and, in many cases, can be predicted by the Reynolds number (*Re*), a dimensionless parameter commonly used by engineers (Vogel, 1994). In closed pipes, flow is generally turbulent at a *Re* above 1200. The *Re* is also useful as a comparative indicator of flow conditions in a diverse range of systems. Briefly, in laminar flow shear stresses are low and mixing is poor, whereas in turbulent flow shear stresses are high and mixing is good. Under these conditions, shear and mixing have opposing influences on biofilm accumulation and on the resulting biofilm structure (van Loosdrecht et al., 1995). Increased shear tends to increase the detachment rate by the physical removal of individual cells (erosion) or larger pieces of biofilm (sloughing), while increased mixing tends to increase the growth rate by reducing transport limitations and increasing the nutrient supply. The rate of transport of dissolved and particulate species (nutrients, biocides, etc.) into the biofilm and the removal of waste products from the biofilm also will have a profound influence on the chemistry (pH and eH, etc.) of the local microenvironment. It has been hypothesized that there may be an optimal flow for biofilm formation below which accumulation would be limited by mass transfer and above which accumulation would be limited by detachment (Lewandowski, 1991).

To date most of the detailed investigation on biofilm structure has been conducted on biofilms grown in the laboratory under laminar flows. These biofilms tend to be cell clusters which are roughly circular or amorphous and in which there is no obvious axial alignment. However, in turbulent flows, the influence of drag becomes apparent, and biofilms are form-filamentous "streamers" which can oscillate rapidly in the flow (Bryers and Characklis, 1981; McCoy et al., 1981; Siegrist and Gujer, 1985; Stoodley et al., 1998; Stoodley et al., 1999a; Stoodley et al., 1999b). The increased energy losses in pipelines have been attributed to the possible formation of streamers (Picologlou et al., 1980). More recently, it has been shown that mixed biofilms growing in turbulent flow can form ripple structures that steadily migrate downstream (Stoodley et al., 1999d). The ripple morphology and migration velocity varied with bulk liquid flow velocity, with a response time on the order of minutes. The ripples had a maximum migration velocity of approximately 1 $mm \cdot h^{-1}$. Dalton et al. (1996) have observed cyclical colonization by marine *Vibrio* and *Pseudomonas* species growing in laminar flow, in which microcolonies repeatedly formed and dispersed over periods between 1 and 2 days. However, in this case, it appears that the structural changes were caused by gliding motility of the individual cells, possibly in response to nutrient conditions in the biofilm. It is generally assumed that microcolonies (also termed cell clusters) are formed mainly through cell division during the early stages of biofilm formation. The observation by Dalton et al. (1996) reveals that clusters also can form by the grouping together of attached cells. Both of these observations (Dalton et al., 1996; Stoodley et al., 1999d) demonstrate that the structural arrangement of biofilms is not only spatially but also temporally complex.

Influence of hydrodynamics on the structure of a *Pseudomonas aeruginosa* PAO1 biofilms grown in parallel glass flow cells under laminar and turbulent pipe flow (Stoodley et al., 1999b). The biofilms were grown on a minimal salts medium with glucose ($400 mg \cdot liter^{-1}$) as the carbon source. Images were taken 7 days after inoculation. Black arrow indicates direction of bulk liquid flow. Scale bar = 50 μm.

It may be possible to predict biofilm morphotypes from theoretical consideration of the relative influences of mass transfer and shear (Stoodley et al., 1999a). At high shear flows, where the influence of drag is high but mass transfer limitations are low, drag-reducing planar structures may be expected. In low shear flows, where the mass transfer limitations are high but drag is low, highly porous structures with high surface exchange areas might be expected. Intermediate forms may exist between these extreme conditions.

BIOFILM VISCOELASTICITY In addition to the long-term influence of hydrodynamics on the structure of biofilms grown under steady shear, biofilm structure also can be influenced by short-term changes in fluid shear. Structural changes to mixed and pure culture biofilms caused by variations in fluid shear demonstrate that biofilms can be visco-elastic and have a very low elastic modulus (ca. 30 Pa, i.e., biofilms are highly compliant; Stoodley et al., 1999c). The biofilms exhibited liquid flow when the fluid

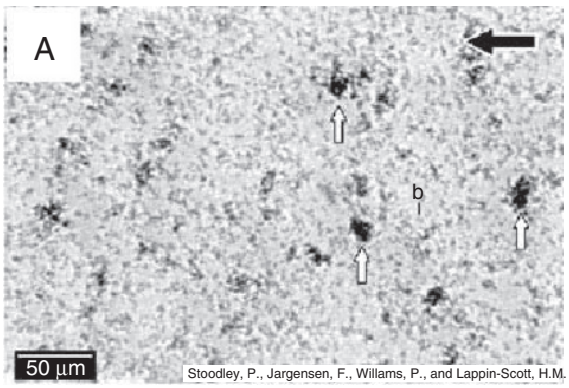

Fig. 7a. The biofilm grown under laminar flow (flow velocity = 0.033 m · s^{-1}, *Re* = 120) was composed of small cell clusters (white arrows) with single cells "b" in the void spaces.

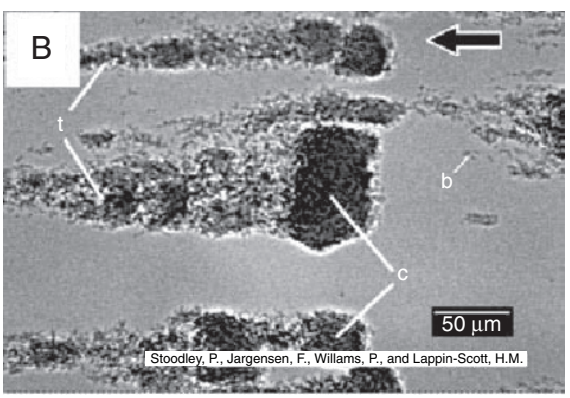

Fig. 7b. The biofilm grown under turbulent flow (flow velocity = 1.0 m · s^{-1}, *Re* = 3600) was composed of larger clusters which had become elongated in the downstream direction to form tapered streamers. Each streamer consisted of an upstream "head" cell cluster "c," which was attached to the glass surface, and a downstream tail "t." Some of the tails were free to oscillate in the flow, while others were more firmly attached to the substratum. The void spaces between the streamers were almost devoid of single cells "b."

shear stress exceeded the yield point. The yield point occurred between 1.2 and 2.0 times the shear at which the biofilm was grown. It is possible that liquid-like behavior may explain the formation of flowing ripples in similar biofilms (see Fig. 8).

Also, the thickness of cell clusters was reduced by up to 30% when the flow velocity was increased from 0 to 1.5 m · s^{-1}. It is thought that the flexibility of certain seaweeds, anemones and other benthic macroorganisms may allow the organisms to withstand the large variations in drag to which they are subjected by wave action (Koehl, 1984). In these types of organisms, drag reduction can be achieved when the organisms "collapse" into a more streamlined shape. It is possible that the flexibility of some biofilms is an adaptive characteristic, which allows these biofilms to remain attached when exposed to varying shears (as would be expected in turbulent flow and many natural flowing water systems).

Biofilm rheology also may help explain the large energy losses that biofilms can cause in water pipelines. High-pressure drops (δP) have been linked to the observed formation of filamentous streamers, and it has been calculated that the measured δP was significantly greater than that expected from an equivalent rigid structure (Picologlou et al., 1980). It is known that rigid structures that are anchored in flowing fluids can dissipate the kinetic energy of the fluid through skin friction and pressure drag (Vogel, 1994). Skin friction is dependent on surface area and is more significant in laminar flows. Pressure drag is shape-dependent and is more significant in turbulent flows. Biofilms that are behaving visco-elastically also can dissipate kinetic energy through both elastic and viscous action (Stoodley et al., 1999c). Rapid elastic deformations, which may occur when biofilm streamers oscillate, would result in the generation of heat in the biofilm matrix as bonds repeatedly stretch and contract. It is expected that, because of the thin nature of the biofilm, this heat would be quickly transferred to the bulk liquid. Viscous behavior also can generate heat through friction as individual polymer strands move past each other when biofilm flows. As yet, it is not clear what the relative contribution of each of these mechanisms is to the formation of pressure drops in pipe flow.

In addition, biofilm structure may be influenced electrochemically. It has been found that electrical fields can cause biofilms to rapidly contract and expand (Stoodley et al., 1997). It was found that the thickness of a mixed species biofilm cell cluster was reduced to 74% of the original thickness when the platinum wire which it had been grown on was cathodic. This change was similar to that caused physically by hydrodynamic shear (see above). This effect is common in gels and may lead to increased exchange between water phase and biofilm and partly explain the bioelectric effect (Wellman, 1996).

Growth and Detachment Detailed studies on growth and detachment have been performed by van Loosdrecht and his group. Tijhuis et al. (1996) suggested that the degree of heterogeneity is determined by the balance between the growth rate and abrasion. Indeed, slow growing organisms (e.g., nitrifiers and methanogens)

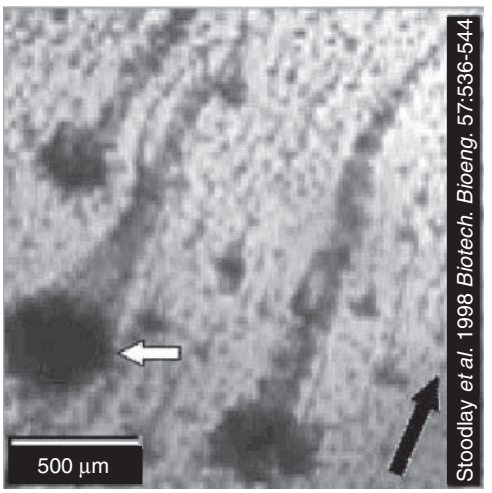

Fig. 7c. Time lapse movie showing biofilm streamers oscillating in turbulent flow. The bacterial biofilm, composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae*, was grown on a glass observation window in a polycarbonate flow cell. The oscillation frequency of the streamers was directly proportional to the velocity of bulk liquid, suggesting that the oscillations were caused by vortex shedding of the upstream "head" (white arrow) of the streamer (Stoodley et al., 1998). Black arrow indicates flow direction; scale bar = 500 µm. For the Fig. 7c, see the online version of *The Prokaryotes*.

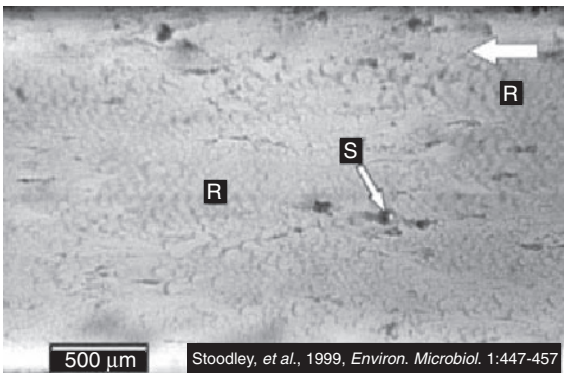

Fig. 8. Time lapse movie showing the migration of biofilm ripple-like structures across a glass surface. The mixed species biofilm was composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Stenatrophomonas maltophilia*, and *Klebsiella pneumoniae* (Stoodley et al., 1999d). Ripple patches are labelled "R." Elongated streamers "S" also formed in the biofilm. The streamers did not migrate across the surface, but some of them were observed to detach during the observation period (see labelled streamer "S"). The biofilm was 15 days old and grown in turbulent pipe flow at a bulk liquid flow velocity of 1 $m \cdot s^{-1}$ (Re = 3600). The flow direction is indicated by the arrow. Frames were captured at 1-h intervals over 15-h. Scale bar = 500 µm. For the Fig. 8, see the online version of *The Prokaryotes*.

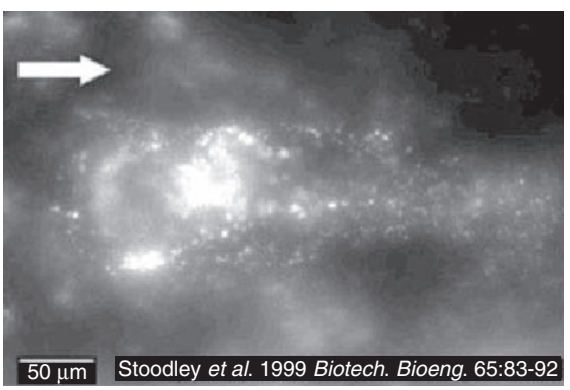

Fig. 9. Time-lapse movie showing the influence of fluid shear on biofilm structure (Stoodley et al., 1999c). The biofilm streamer was stained with fluorescent beads, which appear as bright dots and were used as fiducial points to monitor structural changes. The sequence of images shows the changes to structure as the fluid shear was increased stepwise from 0 to 10.11 Pa and then reduced stepwise back to 0. When the load was removed, the biofilm "sprang" back, clearly demonstrating an elastic response. Arrow indicates flow direction. Scale bar = 50 µm. For the Fig. 9, see the online version of *The Prokaryotes*.

form relatively flat biofilms or spherical aggregates; faster (heterotrophic) growth results in formation of more heterogeneous biofilms with cell clusters and streamers. Characklis (1990a) has demonstrated the relative contributions of nutrient loading and flow velocity on biofilm thickness. At low loading rates (0.1 g carbon $\cdot m^{-2} \cdot h^{-1}$) biofilm thickness (ca. 50 µm) was nutrient-limited and relatively independent of liquid flow velocity. However, at higher loading rates (2.4 g carbon $\cdot m^{-2} \cdot h^{-1}$), the biofilm thickness was approximately 1000 µm at a liquid flow velocity of 1.5 $m \cdot s^{-1}$ but was reduced to 200 µm when the biofilm was grown at a liquid flow velocity of 3 $m \cdot s^{-1}$. In this case, the biofilm thickness was presumably limited by shear-induced detachment as the flow rate was increased. It is well established that generally thinner biofilms form (and less biofilm biomass accumulates) under low nutrient conditions, and it has been proposed that biofilm accumulation may be limited by removing nutrients under controlled conditions in an upstream biofilter (Griebe and Flemming, 1998). However, there is less information on the influence nutrients have on the structure of biofilms. Møller et al. (1997) reported that the structure of a mixed microbial community grown under laminar flow changed in response to a switch in substrate (while maintaining a constant labile carbon loading rate) (Møller et al., 1997). When grown on 2,4,6-trichlorobenzoic acid, the biofilm consisted of mounds of cells separated by void areas. However, when the substrate was switched to trypticase soy broth, the biofilm became thicker, and growth

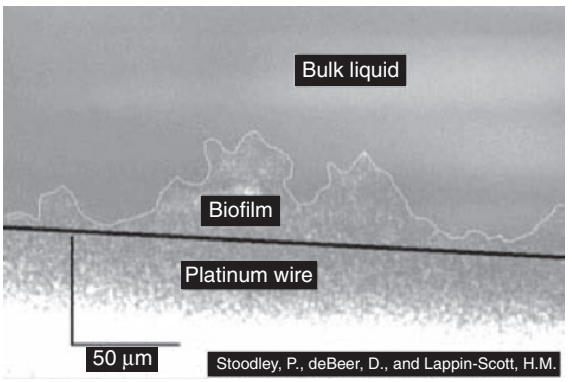

Fig. 10. Time lapse movie sequence showing the expansion and contraction of a mixed-species biofilm growing on a platinum wire (Stoodley et al., 1997). The biofilm expanded when the wire was cathodic but contracted when it was anodic. The biofilm could fully expand and contract at a maximum frequency of approximately 5 Hz. At higher frequencies the biofilm appeared to fibrillate. Similar contractions and expansions could be induced by pH alone. The edge of the wire is indicated by the black line. The edge of the biofilm has been outlined in white for clarity. Scale bar = 50 µm. For the Fig. 10, see the online version of *The Prokaryotes*.

in the void areas resulted in a less heterogeneous structure. Also, a change in nutrient concentration can cause a change in the structure of an established biofilm as well as changes in thickness and surface coverage. Stoodley et al. (1999a) reported that the structure of a 21-day mixed-species biofilm growing in turbulent flow initially consisted of ripples and streamers (see Fig. 8) but changed to large cell clusters (ca. 500 µm in length) when the carbon and nitrogen concentration was increased by a factor of 10. The biofilm also significantly increased in thickness and surface coverage. When C and N concentrations were reduced to their original levels, there was a loss of biomass and the ripples and streamers reappeared. We speculate that cell surface properties, in particular hydrophobicity or hydrophilicity, also can determine the biofilm structure. Cell surface hydrophobicity results in minimization of the contact surface between liquid and biofilm and thus in planar biofilms or spherical aggregates. Hydrophilic cells will more easily form protrusions like streamers and cell clusters. Typically, dividing cells (Allison et al., 1990) and many (facultative) aerobic heterotrophs (Daffonchio et al., 1995) are hydrophilic. Heterotrophic conditions thus result in heterogeneous biofilms. Examples of hydrophobic microorganisms are benthic cyanobacteria (Fattom and Shilo, 1984), methanogens, syntrophic bacteria, and to a lesser extent, sulfate reducers (Daffonchio et al., 1995). Indeed cyanobacterial mats and methanogenic biofilms are usually relatively flat; however, detailed observations on mat structure and heterogeneity have not been conducted to the same extent as on bacterial biofilms.

Cell-Cell Signaling Finally, cell-cell communication must be considered as a morphogenetic mechanism. By sensing cell-produced compounds, e.g., *N*-acyl-homoserine lactones where the acyl group determines action or strain specificity, cells recognize the local cell density (therefore called "quorum sensing") and react by switching on or off certain sets of functional genes. Quorum sensing regulates the expression of the LUX genes in the bioluminescent bacterium *Vibrio fisheri* and the release of virulence genes in pathogens like *Pseudomonas aeruginosa*, and it plays a role in the symbiotic host association of *Rhizobium leguminosarum* in root nodules. Genes for quorum sensing have been found in ca. 25 different bacterial species, and this communication mechanism is believed to be common among Gram-negative bacteria (Greenberg, 1997). Quorum sensing also determines the structure of *P. aeruginosa* biofilms (Davies, personal communication): the presence of *N*-3-oxododecanoyl-L-homoserine lactone enhances the production of polyuronic acids, which are important components of bacterial EPS. The lactone concentration is increased, due to restricted out-diffusion, at higher cell densities or after adhesion to a surface, thereby enhancing biofilm formation. At higher concentrations (as can occur in dense and thick biofilms), the same compound induces production of *N*-butyryl-L-homoserine lactone, which then induces the production of alginate lyase that can dissolve EPS and lead to rapid cell mobilization and formation of voids in the biofilm matrix. Mutants of *P. aeruginosa*, defective in quorum–sensing, form flat and homogeneous biofilms, while the wild-type organism forms heterogeneous biofilms (Davies et al., 1998). If these mutants are grown with *N*-3-oxododecanoyl-L-homoserine lactone added to the medium, a patchy biofilm resembling the wild-type biofilm is formed. Two counteracting lactones, one stimulating cell aggregation (biofilm formation) and one stimulating biofilm dissolution, can thus regulate biofilm structure. Halogenated furanones, produced by marine algae, interfere with the cell-cell signaling mechanism, resulting in strongly decreased biofilm accumulation (Maximilien et al., 1998). It can be expected that the newly found mechanism of cell-cell signaling will be very important in future biofilm studies. This will lead to an explanation of many

biofilm characteristics and to more ways to manipulate biofilms.

Mass Transfer and Microbial Activity

Substrates for biofilm growth usually are supplied by the water phase, and metabolic products are eventually released into the water phase. The rates of exchange between the biofilm and the water phase are determined by the mass transfer processes of diffusion and advection. Microbial conversions in biofilms are, therefore, dependent and often limited by mass transfer. The process rates should be determined in situ. Activity determinations on biofilm samples cannot give reliable data as the microenvironment cannot be accurately replicated in vitro. Conversions are related mostly to cell growth and division, and the development of reliable methods to determine growth rates of single cells in situ has proved difficult. However, the combination of two photon confocal microscopy with fluorescent gene activity reporters should remove much of the ambiguity associated with current techniques.

Mass Transfer in Biofilms

A common property of microbial mats, biofilms, flocs, and aggregates is the occurrence of mass transfer resistance. This is due to the limited water flow inside the matrix and the presence of a hydrodynamic boundary layer between the matrix and the surrounding water phase (Jørgensen and Revsbech, 1985; Jørgensen, 1994; Lewandowski et al., 1993; De Beer et al., 1993; De Beer et al., 1994; De Beer and Stoodley, 1995; Ploug and Jørgensen, 1998). Transport of solutes is thought to be primarily by diffusion inside the biofilm matrix and in the boundary layer adjacent to the solid surface. Consequently, when the internal chemical composition (substrates and products) differs from bulk water conditions, steep gradients develop. This has strong effects on the type and rates of microbial conversions. Mass transfer resistance often limits conversion rates. However, many processes can occur only inside biofilms because of special prevailing conditions. For example, anaerobic conversions like denitrification, sulfate reduction, and methanogenesis primarily take place in anoxic environments found in the deeper zones of biofilms and mats. However, recent studies showed that anaerobic processes also can occur in the oxic part of sediments and mats, indicating that anaerobic bacteria have special physiological adaptations or that anaerobic micro-niches may exist in the oxic zone (Canfield and Des Marais, 1991; Frund and Cohen, 1992; Krekeler et al., 1997). A characterization of the microenvironments and their interaction with mass transfer processes is needed to understand conversions inside biofilms.

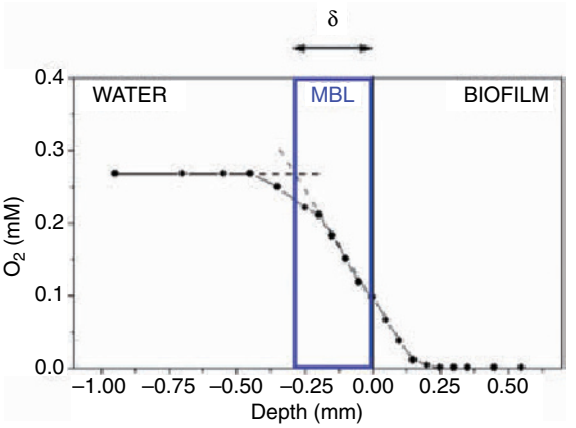

Fig. 11. Oxygen profile measured in a moderately active biofilm. The crosspoint of the dashed lines indicates the upper boundary of the hypothetical mass boundary layer (MBL), having a thickness δ. The image shows that a significant part of the mass transfer resistance can be situated outside the biofilm.

The simplest biofilm concept is a planar geometry, with microbial activity distributed homogeneously and all transport parameters constant regardless of depth. Adjacent to the biofilm is a mass boundary layer (MBL) in which the transport gradually changes from diffusional to advective in the mixed bulk liquid. This is illustrated in Fig. 11, showing an O_2 microprofile in and above a respiring biofilm. The strength of this concept is its simplicity, which facilitates mathematical modeling of transport, conversion and growth (Wanner and Gujer, 1986; Rittmann and Manem, 1992; Wanner and Reichert, 1996).

The mass-transfer resistances can be separated into external, in the MBL, and internal, in the matrix itself. The resistance in the MBL is proportional to its thickness, which depends mainly on the flow velocity of the liquid (Jørgensen and Des Marais, 1990). The mass transport coefficient, k_s, and the thickness of the MBL, δ, can be calculated from the liquid flow velocity ($_u\exists$). For example (Shaw and Hanratty, 1977):

$$k_s = 0.0889 u_\infty Sc^{-0.704} \quad (1)$$

with Sc as the Schmidt mumber,

$$Sc = \frac{\eta\rho}{D_{eff}} \quad (2)$$

with η as the dynamic viscosity, ρ as the density of the water phase, and

$$\delta = \frac{D_{eff}}{k_s} \quad (3)$$

Ultimately, the effective diffusion coefficient (D_{eff}) and the penetration depth (ρ; diffusion distance) of the limiting substrate determine the mass transfer resistance (η) in the matrix.

The relative importance of the MBL and intra-matrix resistance for conversion rates has been described for flat geometry with first- and zero-order kinetics (De Beer, 1998). Qualitatively expressed, the greater is the microbial activity of the matrix, the smaller is the penetration depth of the limiting substrate. Consequently, the relative contribution of the mass transfer resistance inside the matrix decreases. Therefore, the higher the microbial activity, the more important the MBL is for the regulation of microbial activity. The penetration depth of O_2, often the limiting substrate, is typically 100 μm in active biofilms, while the thickness of the MBL is in the range of 50–300 μm. Consequently, external mass transfer, and thus flow velocity (see Eq.), often determines the activity of biofilms.

Internal mass transfer is usually considered to be diffusional and, consequently, frequently described using a single effective diffusion coefficient (D_{eff}). The flux of a compound, therefore, depends both on its diffusion coefficient and the slope of the concentration profile:

$$J = D_{eff} = \frac{dc}{dx} \quad (4)$$

where J is the flux ($mol \cdot m^{-2} \cdot s^{-1}$), D_{eff} is the effective diffusion coefficient in the biofilm, and dc/dx is the concentration gradient.

Both diffusion and advection of solutes are important in biofilms. The biofilm matrix hinders both phenomena; obviously the matrix is an effective barrier not only for water movement (advection) but also for the random movement of solutes (diffusion). The effective diffusion coefficient (D_{eff}) is proportional to the biofilm porosity (θ) and inversely proportional to the square of the diffusional distance, the average path length (φ):

$$D_{eff} = \frac{D_w \theta}{\varphi^2} \quad (5)$$

Diffusion is the only transport mechanism when there is no flow inside the biofilm, while advection usually becomes the dominant mechanism when the matrix is sufficiently permeable to allow liquid flow. Contrary to advectional transport, diffusion becomes rapidly less effective with increasing distance. A simple calculation example demonstrates this; the root-mean-square displacement (x) due to diffusion is described by Berg, 1983:

$$\langle x^2 \rangle^{1/2} = \sqrt{2Dt} \quad (6)$$

where t = time.

From this equation, it is evident that displacement due to diffusion is time-dependent: for a diffusion coefficient with a representative value of $1 \times 10^{-9}\ m^2 \cdot s^{-1}$, the average displacement due

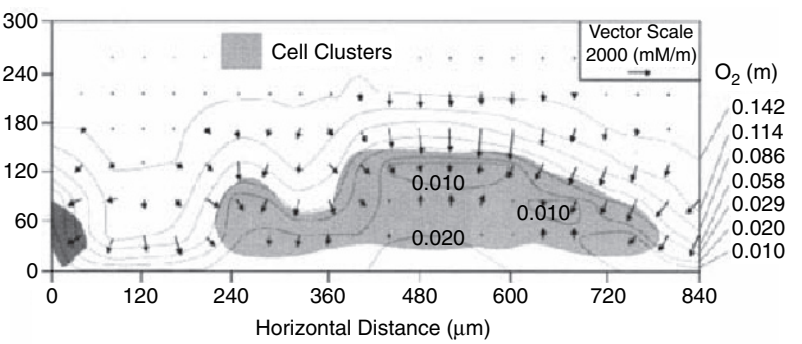

Fig. 12. Cross section through a heterogeneous biofilm showing dissolved oxygen (DO) contours associated with the cell clusters (shaded) and surrounding water channels. Twenty two adjacent DO profiles were measured by microelectrode to give a 2D array of DO measurements. The microelectrode was guided by micromanipulator and confocal microscopy was used to locate the positions of the clusters and channels. In this particular biofilm the cell clusters were held away from the flow cell wall (located toward the bottom of the figure) by "stalk-like" structures. Since this cross section did not intersect any of these stalks the biofilm cluster appears to be "floating" above the wall of the flow cell. The arrows are the calculated DO fluxes and the length of the arrow is proportional to the magnitude of the flux. Note the penetration of DO into the biofilm channel which is centered at 120 micron on the x-axis. Also note that in the center of the large cell cluster the flux arrows are pointing upwards, indicating that DO was being supplied from underneath the cell cluster. In this case the anaerobic region occurred in the center of the cell cluster, approximately 100 microns away from the substratum, and not in a layer adjacent to the wall as would be predicted for a flat biofilm. The vertical distance in microns is shown on the left Y-axis.

to diffusion is 100 μm in 5 s, 1 mm in 10 min, 1 cm in 14 hours, and 1 dm in 2 months. Diffusion is a very effective transport process for short distances (the size of bacterial cells), but it is much slower over longer distances (the thickness of biofilms). Solute transport due to advection equals the velocity of the liquid, and even if it is in the order of 1 $\mu m \cdot s^{-1}$, advection will be as important as diffusion for biofilms, which typically range from a few hundred μm to several mm thick. Microscopic observations have showed that biofilms can be highly porous, thus the common assumption that diffusion is the sole transport mechanism must be treated with care. Indeed it was shown that water can flow between the cell clusters as described in the heterogeneity section. Fluorescent beads quickly penetrated the voids, and their movement, followed by confocal microscopy, showed that water can flow through the channel-like structures (De Beer, 1994; Stoodley, 1994; 1997). With oxygen microsensors it was further demonstrated that the oxygen concentration inside the voids is much higher than that in the adjacent cell-clusters, thus diffusion will occur in and a horizontal direction or even from voids at the base of the biofilms in an upwards direction into the cell clusters (Fig. 12; De Beer, 1994). It was calculated that in such heterogeneous biofilms the interfacial oxygen flux is 2–3 times higher than that for a flat biofilm. At low flow velocity this effect disappears and a 1-D model can be applied (De Beer, 1995). Upon injecting a fluorescent dye in the voids of a biofilm an elongated plume developed, while injection inside the pores resulted in a spherical plume (De Beer, 1995). Therefore it was concluded that in voids flow occurs and induces advective transport at higher flow velocities, but in cell clusters diffusion is the only transport mechanism.

Numerous studies reporting measurements of the D_{eff} of various compounds in biofilms and microbial aggregates have been reviewed (Christensen and Characklis, 1990; Libicki et al., 1988; Siegrist and Gujer, 1985). Literature values show a wide range of variation, D_{eff} being 1–900% of the diffusion coefficient in water (D_w), reflecting the variety of biofilms studied as well as the different measurement methods. The D_{eff}/D_w ratios for substrates with small molecular weights, such as oxygen, glucose, ammonium, and nitrate, in spontaneously growing biofilms and microbial aggregates are assumed to be around 0.9 (Christensen and Characklis, 1990). Diffusion of macromolecules such as DNA, dextrans, and proteins may be more strongly impeded by biofilm matrices, resulting in decreased D_e/D_w ratios. Diffusion experiments with such molecules have been reported only for gel matrices; however, biofilms have been considered to be highly hydrated gels (Christensen and Characklis, 1990). An extensive review on diffusion phenomena in gels is given by Westrin (1991). It has been shown that the D_e/D_w of proteins diffusing through agarose gels is inversely correlated with their molecular weight (Boyer and Hsu, 1992; Arnold et al., 1985). This is due to gel-matrix polymers obstructing diffusion (Rodbard and Chranbach, 1970) as well as to hydrodynamic drag at the matrix polymer-solvent interface (Brenner and Gaydos, 1977). The impeded diffusion of large molecules in gels is strongly influenced by the microstructure of the gel matrices; consequently, information about the microstructure of the biofilm matrix may be derived from diffusion data.

Diffusivities in biofilms have been estimated by measuring transient or steady state fluxes through biofilms in diffusion chambers or in uptake experiments (Libicki et al., 1988). If the experiments are performed with a nonreacting compound or with killed biofilms, D_{eff} can be calculated by fitting the measured fluxes with a diffusion model. In the case of a reacting compound, a reaction-diffusion model is necessary.

The determination of diffusivities by these methods assumes a homogeneous and flat biofilm. It was, however, shown that biofilms are not always flat but may contain streamers, cell clusters, voids, pores and channels that may affect strongly the transport properties of the biofilm. Thus the diffusivity may be overestimated from advective transport through the pores. This may explain why D_e higher than D_w values have been reported (Libicki et al., 1988; Siegrist and Gujer, 1985).

To describe transport inside biofilms, transport in the voids (advection and diffusion) and in the base film and cell clusters (diffusion only) must be distinguished. For this, measuring techniques with high spatial resolution are needed. A powerful technique for determinating local diffusional properties of biofilms is the Fluorescence Recovery After Photobleaching (FRAP) method (Axelrod et al., 1976). Application of optical techniques is limited to transparent structures. First, a biofilm is soaked with fluorescently labelled compounds. The FRAP method measures the diffusion of fluorescent molecules into a small area in which all molecules are bleached by a high intensity laser beam. After that volume is depleted of fluorescent molecules by the laser flash, other molecules diffuse in from the surrounding area. By quantitatively monitoring this diffusion using CSLM and by fitting these data using a mathematical model, the diffusion coefficient of the compound can be calculated. The spatial resolution of the method is ca. 30 μm. It was found that the diffusion of small molecules (MW 300) was not significantly impeded by

biomass. Large molecules, such as dextrans, proteins, and DNA, were impeded (ca. 30% of that in water for large proteins, MW 200,000, and 20% for DNA, MW 3×10^6; Bryers and Drummond, 1996).

A conceptually related technique is based on microinjection of a fluorescent tracer and on monitoring the expansion of the fluorescent plume by CSLM (De Beer et al., 1997). Instead of diffusion into a depleted area (FRAP), the out-diffusion is followed into the surrounding area. The distribution pattern of the dye is fitted with an implicit equation to obtain a local value for D, the diffusion coefficient. A refinement of this technique is to detect the fluorescence with an optical fiber connected to the microinjection capillary (De Beer et al., 1997; Kühl and Revsbech, 1998). This microsensor can be used in thick nontransparent biofilms. With this technique, similar observations were obtained as with FRAP: the diffusion coefficient of small molecules (MW 300) is close to that in water, while the mobility of large molecules (MW 240,000) is decreased to ca. 30%.

Both FRAP and microinjection are non steady state methods of measuring the diffusivity at a particular location in the biofilm. The value obtained is the molecular diffusivity, corrected for the tortuosity of the matrix (D_w) but not the porosity (Libicki et al., 1988). For determination of D_{eff} (D_w/j), flux measurements during steady-state transport are needed.

Recently, two microsensor approaches were developed to determine local diffusivity (D_{eff}) with high spatial resolution. A microsensor method to determine local diffusivities or local mass transfer properties (Yang and Lewandowski, 1995; Rasmussen and Lewandowski, 1998) is based on measuring the limiting current during the reduction of Fe^{3+}. A strong inhibition of Fe^{3+} transport was measured in biofilms, especially in deeper parts of the cell clusters, with values lower than 10% of that in water. These findings contradict others that concluded small molecules are not impeded in their motility (Bryers and Drummond, 1996; De Beer et al., 1997). Possibly, because of the measuring conditions employed (0.5 M KCl and 25 mM $K_3Fe(CN)_6$), the viscosity in the biofilm was increased by cell lysis or weakening of the EPS bonds, thus decreasing the diffusivity. Recently, a diffusion microsensor was developed, based on out-diffusing of a tracer gas from a needle-type capillary, with a tip size of 140 µm. The concentration of the tracer (H_2 or C_2H_2) in the tip of the capillary is measured using a normal microelectrode incorporated into the capillary. The signal is governed by the diffusional resistance in a sphere around the tip of the sensor, and thus is proportional to the local diffusion coefficient (Revsbech et al., 1998). With this sensor the D_{eff} in methanogenic aggregates was estimated at 50% of that in water (Santegoeds, 1999a).

In a recent study using NMR imaging, D_{eff} was determined in microbial mats, which were stratified; their diffusivity values ranged from 30–60% of that in water (Wieland, 1999). These measurements were confirmed with the gas-diffusion sensor (Revsbech et al., 1998).

In conclusion, data on the diffusion coefficient in biofilms are highly variable. This may be partially due not only to differences in the techniques used but also to the possibility of large variations within individual biofilms and between different types of biofilms.

Stratification (e-donor) and Internal Cycling

In sediments, a stratification into zones with different microbial conversions is well documented (Berner, 1981). Sediments are subjected to a continuous or intermittent influx of organic matter, which is mineralized in several steps. Therefore, a wide diversity of conversions takes place, and as a consequence several profiles develop. The deeper regions are more anoxic and reduced than the top, which is usually aerobic. The organics are degraded and oxidized by bacteria using electron acceptors in the characteristic sequence O_2, NO_3^-, MnO_2, Fe^{3+}, SO_4^{2-}, and CO_2 (Reeburgh, 1983). This sequence coincides with the standard free energy changes of the reactions involved, and it is assumed that the larger the energy yield of a conversion, the greater the likelihood it will dominate other competing reactions. The mineralization processes involved are aerobic mineralization, denitrification, iron reduction, sulfate reduction, and methanogenesis. Since electron acceptors are usually supplied from the water phase, the different processes will occur adjacent to each other, going from surface to deeper zones, in the same characteristic sequence mentioned. The stratification is not necessarily strict, and processes do not necessarily exclude each other, e.g., there is no thermodynamic argument why methanogenesis does not occur under aerobic conditions. Inhibition and regulation on the cell level are strong determinants for the stratification. Denitrification is inhibited usually by oxygen, although an exception was reported (Robertson, 1983). Consequently, denitrification is located directly adjacent to the aerobic zone, with some possible overlap in the microaerobic zone (Lorenzen, 1998; De Beer and Sweerts, 1989; Robertson, 1995). Sulfate reducers were thought to be very sensitive to oxygen, and thus restricted to anoxic zones. However, recently sulfate reducers were found in oxic zones of biofilms, sediments, and microbial mats (Dilling, 1990; Krekeler et al.,

1997). Furthermore, there is evidence that sulfate reducers can be sulfidogenically active under aerobic conditions (Canfield and Des Marais, 1991). Even more confusing was the finding of sulfate reducers that oxidize sulfide aerobically (Fuseler, 1996). Sediments are not fundamentally different from biofilms, and both can be considered as matrices with localized (evidence suggests that cells are not immobilized but can swim around inside the cell clusters) microorganisms. All these processes may occur in biofilms as well, although in the relatively thin and heterogeneous biofilms, a stratification of processes and organisms may not be as pronounced and may be more difficult to study.

Owing to the short distances in biofilms, the different conversion processes can be tightly coupled and internal cycling occurs. Organic matter is subjected to hydrolysis and fermentation, producing volatile fatty acids and hydrogen. These products and the original organic matter serve as e-donors for anaerobic- and aerobic respiration and methanogenesis. In the oxic zone, products from anaerobic redox processes like sulfide, methane, and Fe^{2+} are oxidized. Thus internal cycles are possible, which can not be quantified from interfacial fluxes. Such sequences of processes can only be detected by invasive techniques with high spatial resolution, i.e., by microsensors.

An important internal cycle in biofilms is sulfate reduction coupled to sulfide oxidation. It is well known that sulfate reduction contributes considerably to the mineralization process in marine sediments (Jørgensen, 1977, 1985; Jørgensen and Des Marais, 1990; Thamdrup, 1996). Because of re-oxidation by aerobic and anaerobic processes, sulfide does not reach the top of the sediment. The importance of sulfate reduction in marine environments is usually explained by the high sulfate concentrations in seawater. However, also in freshwater systems, the sulfur cycle can be important. In aerobic wastewater biofilms, a significant part of the mineralization occurs by sulfate reducers. It was demonstrated with microsensors for S^{2-} that 50% of the oxygen was used for sulfide oxidation, implying that 50% of the mineralization of organic matter is degraded by sulfate reduction (Kühl and Jørgensen, 1992). In similar biofilms, a combination of molecular techniques and microsensor techniques was used to relate microbial activity with microbial population distributions (Ramsing et al., 1993). A good correlation was found with the distribution of sulfate reducers and sulfide production in these biofilms, which were both confined to the anoxic zones. However, owing to the metabolic versatility of sulfate reducers and their resistance to oxygen, such a good correlation between microbial populations and microbial conversions is not obvious. Recently, the development of sulfate reduction in an aerobic biofilm was studied with microsensor and molecular analyses (Santegoeds et al., 1998). It was found that sulfate reduction coupled to sulfide oxidation began only after 6 weeks, although anoxic zones were present by the first week. Once started, this process became of major importance, resulting in up to 70% of the mineralization in the biofilm. Thus sulfate reduction can be an important process even in thin biofilms that are exposed to oxygen.

In the absence of nitrate, sulfide is oxidized by oxygen, and thus the oxygen and sulfide profiles overlap. Upon addition of nitrate, a separation between the sulfide and oxygen profile occurs, because nitrate penetrates beyond the oxic layer and becomes the e-acceptor for sulfide oxidation (Kühl and Jørgensen, 1992). Furthermore, iron and manganese form important shuttles for redox equivalents in marine sediments (Canfield, 1991). Their role in biofilms is not known.

When illuminated, photosynthesizing biofilms are found. In such communities, a most complex internal cycling exists as these biofilms are in principle self-supporting. In the photic zone, CO_2 is bound in the biomass and O_2 produced. In the aerobic and anaerobic zones, the biomass is degraded to CO_2. In such systems, a large array of microbial processes can be found. Especially in microbial mats from extreme environments, such as hot springs or hypersaline lakes, undisturbed stratification of the different processes occurs. Microbial mats are actually complete ecosystems, where primary production is balanced by aerobic and anaerobic respiratory processes. Such communities are therefore highly interesting model systems. For further information on these systems, we refer to reviews (Pearl, 1996; Stal, 1994).

The main processes in the nitrogen cycle are ammonification due to degradation of organic matter, nitrification, and denitrification. Since the product of denitrification is N_2 gas, the nitrogen cycle in biofilms is not closed but depends on input of nitrogen compounds. Since nitrification is an aerobic process and denitrification proceeds primarily in the absence of oxygen, a clear stratification can be expected. Indeed, using microsensors, it could be confirmed that nitrate is formed in the oxic and consumed in the anoxic zone (Schramm and Amann, 1999; Schramm et al., 1996; De Beer, 1998; De Beer et al., 1997b). Nitrification is usually limited by oxygen penetration and confined to an outer layer of ca. 100-μm thick (De Beer et al., 1993; Schramm, 1998a). The intermediate nitrite can be found in a narrow zone near the oxic-anoxic interface, where it is formed by either incomplete nitrification or denitrification (De Beer et al., 1997b). In

biofilms, denitrification is regulated by oxygen in several ways: firstly by inhibition, secondly by nitrification in the oxic zone, and finally by transport. Denitrification takes place adjacent to the oxic zone. At higher oxygen levels, the thicker oxic zone forms a transport resistance for nitrate from the water phase.

The competition between sulfate reduction and methanogenesis for e-donors has been investigated intensively. Sulfate reduction has more favorable thermodynamics than methanogenesis (Widdel, 1988). Thus methanogenesis is typically a more important process in low-sulfate environments (freshwater), and sulfate reduction dominates in marine sediments. Anaerobic reactors are usually designed for methane production, while sulfide production is an unwanted process because of odor and corrosion problems. In practice both processes are active, resulting in biogas that is polluted with sulfide. Based on modelling, it was concluded that, under sulfate-limiting conditions, the outer layers of anaerobic biofilms or aggregates would be sulfidogenic, leaving a microniche for methanogens in the center (Overmeire et al., 1994). Such a division was indeed found: methane and sulfide microprofiles showed that sulfate reduction is confined to the outer 100 μm, while methanogenesis occurs in the center (Santegoeds, 1999a). This was also observed in aggregates preincubated for months in excess sulfate and e-donor, i.e., where sulfate reducers were expected to be present in the center as well. It was hypothesized that methanogens are needed for initial aggregate formation, while sulfate reduction develops subsequently. However, it is strange that sulfate-reducing bacteria (SRB) do not eventually colonize the aggregate center.

From the previously reviewed literature, it can be concluded that the sequence of e-acceptor use found in sediments is also present in biofilms. This was almost comprehensively demonstrated with microsensors in wastewater biofilms (De Beer, 1999). First O_2 is used by heterotrophic and autotrophic processes (nitrification and sulfide oxidation). Then NO_2^- and NO_3^-, formed by nitrification or originating from the water phase, are consumed in the zone directly adjacent to the oxic layer. Denitrification can be coupled to sulfide oxidation. Sulfate reduction is found below the denitrifying zone. Methanogenesis is also spatially separated from sulfate reduction and occurs in the deepest zones of the biofilms.

Special Physiology of Biofilm Cells?

Since biofilms function differently in many aspects from planktonic cells, it has often been speculated that a special biofilm physiology exists (Cochran et al., 2000). Biofilms usually have a lower specific conversion rate, high resistance towards biocides and antibiotics, and less sensitivity to temperature changes. It has been argued that all these phenomena can be explained by mass transfer resistance (van Loosdrecht et al., 1990). Mass transfer resistance reduces the transport of substrates and biocides to the cells, even if they form only a monolayer. Due to mass-transfer resistance, only a partial penetration of substrate occurs; but under conditions of reduced cellular activity (e.g., by cooling) a larger part of the biofilm gets penetrated with substrate, which counteracts the reduced specific conversion rates in each cell. Two other explanations for the reduced efficacy of biocides and antibiotics are: the relatively low growth rates of biofilm cells, which make them less susceptible (Brown and Gilbert, 1993), and the reduced penetration either by binding of compounds to the biofilm matrix or, in case of reactive biocides, by deactivation in the outer layers of the biofilm (Stewart et al., 1996). It appears that rather than a special biofilm physiology, the growth and activity of the cells within the biofilm may be governed by the physiochemical conditions that prevail in the biofilm microenvironment.

However, more and more evidence indicates that cells recognize and respond to the presence of other cells and surfaces in their environment. It has been shown that after attachment certain genes required for EPS synthesis are activated (Davies and Geesey, 1995) and that the production of pheromones (homoserine lactones) induces biofilm formation (Davies et al., 1998). Thus the development of a mature biofilm involves the same compounds involved in quorum sensing (Greenberg, 1997). It was carefully hypothesized that cell-cell signalling might be involved in the resistance of biofilms to biocides (Costerton et al., 1999). See further in the paragraph on "Morphogenetic factors."

Microbial Populations

Previously, microbial population analysis was based on enrichment and cultivation techniques. It has become clear that plate counts very often do not represent the true microbial community, as many strains are resistant to cultivation. Microbial analysis has become much more reliable (and easier) due to the development of noncultivation techniques. Owing to the relative ease of molecular techniques, many data are collected from a wide variety of microbial communities; however, the role of the detected populations is often not known. This can be attributed to the difficulty of functional analysis of complex communities: the conversions of a

community can be measured, but it is difficult and often impossible to assign the conversion to certain populations. However, there are some exceptions. Some microbial populations can be analyzed with a combination of microsensor and molecular techniques. Then, it is possible to determine the location of certain microbial processes (with microsensors) and to determine the location of certain microbial populations (with fluorescent in situ hybridization, FISH). Comparing these data can lead to estimations of activities and kinetics of populations in situ.

The combination of molecular and microsensor techniques was first used in a biofilm from a trickling filter (Ramsing et al., 1993). Sulfide profiles were measured with AgS microsensors from which the distribution of sulfate reduction and sulfide oxidation was determined. With oligonucleotide probes (SRB385) the distribution of SRB was determined, although it is now known that the probe is not targetting SRB exclusively. Once a reasonable agreement between the distribution of SRB and sulfate-reducing zones was found, the in situ activity could be estimated. However, SRB were also found in the oxic zones. Although 50% of the mineralization was done by sulfate reduction, only 10^8–10^9 SRB per ml were found, which was probably less than 1% of the total number of cells.

In a more detailed study, the development of the number and distribution of SRB as well as that of sulfate reduction was followed in a biofilm developing in a wastewater treatment plant. Although anoxic conditions were present from the first week on, no sulfate reduction could be detected until the sixth week. More surprisingly, SRB were present also in the initial biofilm as shown with various molecular techniques (DGGE, denaturing gradient gel electrophoresis; FISH; Santegoeds et al., 1998). A better correlation between molecular and functional analysis was found when comparing the presence of the functional gene for bisulfite reductase (DSR) and sulfate reduction (Santegoeds, 1999a), as the start of the sulfate reduction coincided with a strong increase of the signal for DSR. Thus, the presence of a functional gene in a complex microbial community seems more predictive for its behavior than a population analysis. However, in activated sludge DSR could be clearly demonstrated, but sulfate reduction was absent, even upon exposure to anoxia in the presence of a suitable cocktail of e-donor (Schramm and Amann, 1999). This points again to the fact that observed populations and biodiversity do not necessarily reflect the behavior of the community at the time of sampling. Many ecosystems and microbial communities are open and thus exchange of strains is occurring frequently. For example, wastewater treatment systems, which are the subject of many diversity studies, have an enormous input of microorganisms that are collected from a wide area or are grown in the sewer system. It is likely that the observed sulfate reducers in activated sludge originate from the input and the SRB populations need time to adapt to the conditions in the wastewater treatment plant.

In methanogenic aggregates a good correlation was found between the distribution of SRB and sulfate reduction, as well as between the distribution of methanogenic bacteria (MB) and methanogenesis. SRB were mostly found on the outside, while MB form the core of the aggregates. In between was a layer of syntrophic bacteria, which were found to supply both the SRB and MB with H_2 and acetate. In aggregates from a reactor optimized for sulfate reduction, few MB and little methanogenesis were found. In aggregates from a reactor optimized for methanogenesis, no SRB or sulfate reduction was detected (Santegoeds, 1999a). The difference between anaerobic aggregates and the previously studied biofilms and activated sludge is age: while the biofilms and flocs had a life-cycle of months or weeks, anaerobic aggregates develop over years. Probably, the SRB populations found in flocs and biofilms originated from outside the biofilms and flocs, and since they did not reduce sulfate, they were not adapted to the environment inside the studied communities. Thus, population analysis based on nucleic acid analysis must be regarded with care. It can be concluded that although certain physiological groups may be present, they may not necessarily be active. The presence of a population will only reflect the functioning of a microbial community if the community is mature and its populations well adapted to its environment.

A good correlation between activity and presence of populations is often found in nitrifying biofilms. In a high-loaded biofilm reactor from a fish hatchery, nitrate microprofiles showed nitrification in the aerobic surface layer and denitrification in the deeper anoxic zone. With FISH, populations of ammonium- and nitrite-oxidizing *Nitrosomonas* and *Nitrobacter* strains were found predominantly in this outer zone (Schramm et al., 1996). Nitrification is generally thought to be mediated mainly by *Nitrosomonas* and *Nitrobacter* strains, because these are the main species that can be isolated from environmental samples. However, with molecular techniques these strains are seldomly detectable in environmental samples. In aggregates from a rather low-loaded, fluidized bed reactor, intense nitrification was measured, but no nitrifying populations were found with the probes for *Nitrosomonas* and *Nitrobacter*. DNA was extracted, 16S-RNA coding fragments amplified

with PCR and sequenced, and after comparison with the databases, new probes were designed. The nitrifiers that were found were new *Nitrosospira* and *Nitrospira* strains, which could not be grown in culture. With FISH and by using these probes, quantification of the different populations was possible, and from the microprofiles, local activities were obtained. Thus, nitrifying activities could be estimated under different well-chosen conditions, even allowing in situ determination of Monod parameters (Schramm et al., 1998b) of up-to-now uncultured strains. The newly found strains had much lower specific activity and K_s than the known Nitrosomonas and Nitrobacter, implying a different survival strategy. In biofilms, they were found mainly in areas with low oxygen concentrations (Schramm, 1998a). *Nitrosospira* and *Nitrospira* strains are adapted to low nutrient and oxygen concentrations (K-strategy), while *Nitrosomonas* and *Nitrobacter* can compete with their much higher conversion (and probably growth) rates at high nutrient and oxygen concentrations (G-strategy).

Previously described in in situ studies, activity of populations was determined using a combination of FISH and microautoradiography, but activity determinations of single cells are also possible (Nielsen et al., 1998). Populations of filamentous bacteria from activated sludge were identified with probes for type 021N (strain identification according to Eikelboom key) and for *Thiotrix*. Uptake of six different organic substrates (^{14}C- or ^{3}H-labelled) under aerobic conditions revealed that no filaments took up all substrates and that strains, indistinguishable by morphology and molecular probes, showed differences in uptake patterns. This means again that great care must be taken when interpretating population structure of a complex community from the way its components function (Okabe, 1997).

Biofilm Control

Biofouling is the detrimental development of biofilms in engineered systems, such as industrial process equipment, drinking water distribution systems, and ship hulls. Biofilms can decrease heat transfer in heat exchangers, increase the pressure drop in pipelines, enhance corrosion, and may be a source of bacterial contamination of drinking water (McCoy, 1987; Camper, 1994; Characklis, 1990b). Biofilms are a nuisance in these systems and control of their development may be necessary to maintain process efficiency and safety. Biofilm control is often performed with biocides, of which the most commonly used is chlorine, a strong oxidizing agent and disinfectant. Biocides are much less effective against biofilms than suspended cells (Chen et al., 1993; Nichols, 1988; LeChevallier et al., 1988). Cells in biofilms are protected from biocide action and are killed only at biocide concentrations orders of magnitude higher than those necessary to kill suspended cells. It has been speculated that the lower sensitivity of biofilm cells to biocides is the result of physiological differences associated with lower growth rate (Brown and Gilbert, 1993). Alternatively, biocide may not reach the cells due to diffusional resistance of the biofilm matrix or to neutralization of biocide inside the matrix (Stewart et al., 1996). There is evidence for both theories.

Using a microsensor for chlorine it was shown that chlorine penetrates very slowly in biofilms (De Beer et al., 1994). The shape of the chlorine profiles, the long equilibration times, and the dependence on the bulk chlorine concentration showed that the penetration was a function of simultaneous reaction and diffusion of chlorine in the biofilm matrix. Frozen cross-sections of biofilms, stained with metabolic stain 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; a redox dye), showed that the chlorine penetration overlapped with non-respiring zones near the biofilm-bulk fluid interface. Thus, chlorine was reduced effectively at the surface of the biofilm, which prevented its penetration to the cells in the deeper layers and thereby protected them. See further information in the section on "cell-cell signaling."

Methods for Studying Biofilms

Cultivation

Laboratory-Scale Cultivation All reactors used for cultivation of biofilms must be designed so that a selective advantage exists for cells to grow in biofilms and not in suspension. It must be kept in mind that cells in biofilms have a transport limitation not shared by suspended cells, which gives suspended cells in a reactor a selective advantage. In general, growth of suspended cells will interfere with biofilm behavior and thus complicate the interpretation of the experiment. Suspended cells can more efficiently compete for the limiting substrates, leaving little available for biofilms attached to the reactor surface. Consequently, in biofilm experiments suspended growth must be suppressed. Alternatively, certain selective pressures may favor biofilm formation. For example, biofilm formation may protect cells within the biofilm from the action of biocides or grazing predators (Costerton et al., 1994; Stewart et al., 1996). When biofilm growth is to be studied under controlled conditions, e.g., pure or defined mixed

cultures and constant conditions, ideally reactor conditions should be chosen so that the residence time of the liquid is shorter than the generation time of the cells. Then the suspended cells will be washed out and the substrate is available for biofilm growth. This was nicely demonstrated by experiments that stepwise decreased the residence time of a culture (Beeftink, 1987a). When the residence time became shorter than the generation time of the cells, wash-out occurred accompanied by a temporary increase of the substrate concentration. Subsequently, attached cell mass proliferated and the substrate concentration decreased again.

The particular design of a reactor system depends on the type of experiment, i.e., the type of process, conditions desired, and monitoring techniques applied. Besides the residence time of the medium, also the hydrodynamic regime and the microenvironment in the reactor, i.e., axial gradients, should be considered. The presence of stagnant zones must be avoided. The hydrodynamics should be predictable, and sudden expansions or contractions or sharp bends should be avoided where possible. For the medium supply, one can choose a once through system, in which fresh, sterile medium is pumped through the biofilm compartment. This ensures good control over the substrate concentrations. However, to obtain a sufficiently high flow rate to avoid axial gradients and stagnant zones, large amounts of medium are needed. Most reactor systems are operated with a continuous supply of medium, sufficient to ensure that the residence time of the medium is shorter than the generation time of the cultures, and a recycle allowing reconditioning of the medium (e.g., aeration and pH control) and control over the hydrodynamics of the biofilm. The design is often a compromise dictated by common sense. If experiments are to be done on pure cultures, a simple system that can be sterilized is preferred. Certain accomodations must be made for specific measurements, e.g., observation windows for in situ microscopy, removable slides for biofilm sampling, openings for the introduction of microsensors, and so forth.

Flow Cells One of the most widely used reactors for biofilm study is the flow cell. The system can be very simple, made of two microscopic slides and a spacer (Caldwell and Lawrence, 1988; Wolfaardt et al., 1995; Lawrence et al., 1991), which is ideal for microscopic monitoring of biofilms. The volume is small, therefore, a one-way medium supply can be used. It can easily be sterilized and so be used for pure cultures. A more complicated flow cell is needed when the biofilm is monitored with microsensors. Either a closed flow cell with openings for introduction of the sensors (De Beer et al., 1994) or an open flow cell is used (Horn and Hempel, 1997). Since these flow cells are larger, a recycle is needed to obtain good mixing of the water phase. A special type of flow cell is the Robbins device (Whiteley et al., 1997; Sly et al., 1990), essentially a pipe with sample holders, with the surface flush with the lining of the pipe. The sample holders are removable, allowing multiple biofilm sampling. Also, flow cells with observation windows for microscopy have been equipped with pressure- and flowmeters to study the interaction of hydrodynamics and biofilm accumulation (Stoodley et al., 1998).

A special flow cell was developed for immobilization of marine snow flocs (Ploug and Jørgensen, 1998). The device (called net-jet) consists of a cylinder with a fine stocking separating the top and bottom part. The hydrodynamics are not well understood, but this simple device allows fixation of flocs in an upward water stream. The flocs can be penetrated by microsensors under settling conditions, resembling the normal hydraulic regime. In this flow cell, also activated sludge flocs with a diameter of 0.2–1.5 mm were investigated (De Beer, 1998). If flow cells are well designed, the hydrodynamics can be well characterized (in contrast with many other types of bioreactor), allowing the influence of fluid shear and mass transfer on biofilm processes such as adhesion, detachment and biotransformation rates.

Annular Biofilm Reactor The annular reactor is essentially a chemostat consisting of a cylinder rotating in an outer cylinder with the reactor content between the outer and inner cylinder (Characklis, 1988). The outer cylinder can be equipped with removable sample plates that are flush with the surface. The inner cylinder rotates at variable speed, thus allowing adjustment of the hydraulic regime over a wide range, independent of the residence time of the medium. With this device, many studies have been done on initial biofilm formation (Escher and Characklis, 1990), the effect of biofilms on shear stress (Characklis, 1990), and the effect of hydraulics on biofilm formation (Gjaltema et al., 1994). It is difficult to maintain a pure culture in these reactors. The hydraulics are not well-described and not uniform (Gjaltema et al., 1994). Therefore, it now is recognized that this device cannot easily be used for quantitative studies.

Fowler Cell Hydrodynamics are important for the attachment of cells and development of biofilm. The Fowler cell (Fowler, 1988) is a radial flow cell, consisting of two plates mounted parallel to each other. The inlet is mounted in the

center so that flow occurs radially, from the center to the periphery. The flow velocity is the highest in the center and decreases with increasing radial distance. The shear forces can be calculated assuming a flat geometry.

The Modified Robbins Device (MRD) The MRD consists of a square or rectangular channel in a polycarbonate block in which 25 sampling-port studs are inserted along the length of the MRD (Hall-Stoodley et al., 1999). The studs can be fitted with different materials to investigate biofilm formation on different surfaces. The MRD is usually sterilized with ethylene oxide and the studs can be removed aseptically. The advantage of the system is that several samples can be taken simultaneously at different times to study biofilm development. Quantification, such as viable and total cell counts, total protein, and carbohydrate content, is possible on scraped samples. Although it is not possible to observe the biofilms in situ, microscopic analysis is possible using conventional staining techniques of slide-mounted samples or by electron microscopy of the colonized surfaces. The MRD is also relatively inexpensive. It can be used in both batch recirculating and once-through culture systems or can be connected to a chemostat. The major disadvantages of the MRD are the inability to directly observe the biofilm, the possible formation of significant nutrient gradients along the length of the device, and possible eddy generation around the sampling studs.

Constant-Depth Fermenter Biofilms actually never reach a steady state, in which cell growth is balanced by decay and abrasion. Instead, biofilm development is characterized by colonization, growth and sloughing events, and then by regrowth. A special type of biofilm reactor is designed in which the top of the biofilm is constantly (a few times per minute) scraped off and a constant thickness is maintained (Peters and Wimpenny, 1988). Biofilms are grown in plugged holes in a plate, over which a plastic scraper rotates. In this model system, flat biofilms were obtained, which have several advantages including: ease of microprofile interpretation, facilitated determination of D, multiple sampling (30 biofilm surfaces per plate), mass balances throughout the reactor accurately related to the biofilm surface, successional population changes to a steady-state situation followed, and spatial heterogeneity of populations determined (Wimpenny, 1996). The obvious disadvantage of this approach is that the control of the biofilm thickness is very artificial. While in normal conditions an irregular surface develops, perfectly flat biofilms are formed in this model system.

Reactors for Biofilms on Carriers A step in the direction of applied biofilm reactors is to downscale them to laboratory-size systems. Most applied biofilm reactor systems are based on biofilms on carrier-aggregated biomass, such as gas-lift reactors (GLR), fluid-bed reactors (FBR) and upflow anaerobic sludge blanket (UASB) reactors. Gas-lift reactors are attractive laboratory systems as the behavior is almost scale-independent (Beeftink, 1987b), facilitating good comparison with full-scale systems. Moreover, they are well mixed and the amount of samples that can be taken without disturbing the reactor is large. The GLR consist of two reactor compartments, a riser and a downcomer. Gas is pumped in the riser, resulting in an upflow of the water-gas-aggregate mixture. At the top of the riser the gas is separated and the water-aggregate mixture goes down in the reactor through the downcomer. In aerobic GLR, the gas is used for efficient aeration and mixing (Van Houten et al., 1994; van Loosdrecht et al., 1997; Gjaltema et al., 1997); in an anaerobic GLR oxygen-free gas is recycled for mixing only (Beeftink and Staugaard, 1986; Van Houten et al., 1994). The advantage of mixing with gas over mechanical devices (impellers) is the relatively low power input and thus low shear forces. The reactor is completed with an internal or external settler to separate aggregates from the effluent stream.

The FBR is based on suspending aggregates on an upwardly directed liquid flow. This reactor is less well mixed and axial substrate and product gradients develop. This may be a disadvantage for practial use, but for microbial ecology studies such gradient systems can be very useful (Csikor et al., 1994; Buffiere et al., 1995; Shieh and Hsu, 1996; Schramm, 1998a; De Beer et al., 1993).

The UASBs are used commonly for anaerobic treatment of concentrated wastewater. In these reactors, the hydrodynamic regime is so quiet that the aggregates are constantly settling in a sludge layer at the bottom (Hulshoff Pol, 1989; Lettinga, 1995). Laboratory-scale UASB with a volume of 1–3 liter have been used to study methanogenic and sulfidogenic consortia (Thaveeshi et al., 1995; Harada et al., 1994; Koster, 1989) as well as start-up phenomena of methanogenic biofilm reactors (Hulshoff Pol, 1989).

Microscopic and Staining Methods

Microscopy Scanning electron microscopy (SEM), transmission electron microscopy (TEM), normal light microscopy (LM), fluorescence microscopy (FM) and confocal scanning laser microscopy (CSLM) all have been used to study biofilms (Surman et al., 1996). Most microscopic methods involve some preparation of the

sample, including staining, fixation, freezing, dehydration, embedding, and sectioning. For this reason, it is important to realize that biofilms are soft and mostly consist of water (>95%) (Christensen and Characklis, 1990). Preparations for microscopy may significantly change the matrix structure by shrinking and deformation (Stewart et al., 1995), and the resulting artifacts have influenced the concept of biofilm structure for years. Most relevant is the underestimation of the spatial heterogeneity, as several steps in the preparation may level the soft biofilm structures. Then, EPS appears as strands connecting the cells. EPS morphology changes by dehydration: diffuse polymeric matter is condensed to strands, leading to overestimation of the pore-size. From SEM images the pore-size appears to be in the order of 1 μm. Good TEM preparations show a pore size of ca. 100 nm (Beeftink and Staugaard, 1986).

Images acquired by ESEM (Little et al., 1991) and atomic force microscopy (AFM; Bremer et al., 1992) with sub-μm resolution (no dehydration) do not show these strands, but rather a smooth smear. A possible artifact from ESEM is the filling of recesses by water, "drowning" the roughness elements of the surface. The sensor needle of the AFM might disturb the surrounding water, causing the polymers to move and resulting in a blurred image (Bremer et al., 1992). Samples examined by LM, FM, AFM, ESEM and CSLM can be unfixed. The recent application of CSLM has been especially instrumental in changing our concepts of biofilm structure (Lawrence et al., 1991; Massol-Deya et al., 1995; De Beer et al., 1994). With the CSL microscope, living transparent tissues can be sectioned optically, under growth conditions. In as much as out-of-focus fluorescence is effectively removed by the pinhole, the images are much sharper than standard microscopic images. Lawrence et al. (1991) published an excellent description of confocal microscopy techniques for biofilm research. Scanning electron microscopy, ESEM and AFM can be used for surface scanning, while the other techniques to some extent allow observation below the surface.

Staining Specific staining is an important tool to unravel the spatial distribution of different biofilm components, most importantly in cells, EPS and voids (Table 5). For viewing cells, stains nonspecific for DNA, such as acridine orange, diamidino-phenylindole (DAPI), ethidium bromide, and hexidium iodide are most useful. These dyes can be combined with confocal microscopy, thus giving an image of cell distributions in undisturbed biofilms or mats. Species-specific staining by oligonucleotide probes or antibodies will be treated elsewhere.

Much less attention has been paid to visualization of EPS. Staining of EPS for fluorescent microscopy or CSLM is possible for proteins (fluorescein isothiocyanate), polyuronic acids and polysaccharides (lectin conjugates, calcofluor). Calcofluor stains most polysaccharides (attaching to β-1,4 and β-1,3 polysaccharides; Haigler et al., 1980), while lectins are more specific. Also, EPS dyes will stain cells that become visible as discrete points, whereas EPS appears as a continuous sheet. *The Handbook of Fluorescent Probes and Research Chemicals* (a catalogue of molecular probes) is a highly valuable source of information about dyes and staining techniques (Haugland, 1996). Also, EPS can be stained by ruthenium red for TEM or observed directly by SEM. Voids can be made visible with negative staining using fluorescein that is quenched by the presence of biomass. Using CSLM, voids appear as bright fluorescent areas, while biomass remains dark (see Fig. 4a, De Beer et al., 1994; Lawrence et al., 1991). Also fluorescent microbeads can be used that penetrate the voids but not the cell clusters (Stoodley, 1998).

In conclusion, several new microscopic techniques now make it possible to get a much more detailed view of biofilm structure.

Microbial Activity

As biofilms consist of thin but often dense layers of cells, many trophic interactions between different populations may occur on a small scale. Examples are nitrification and denitrification, sulfate reduction and sulfide oxidation, photosynthesis and respiration. Few methods are suitable to measure in situ these coupled processes on such a small scale. Activity tests on subsamples give at best an impression of the distribution of potential activities, but do not reflect the actual rates (Okabe et al., 1996). Analysis based on in situ detection of mRNA is not developed as yet for complex communities, and its value as a quantitative method is doubtful. With autoradiography, it was possible to detect substrate use on a cell level (Andreasen and Nielsen, 1997); however, this method does not give local rates. Microsensor techniques are probably the most suitable for unraveling processes in thin complex communities. The local net consumption or production rates can be calculated from microprofiles with a spatial resolution of 25–50 micron. The main prerequisite is that the different processes must be spatially separated, with a distance of some tens of microns (with the exception of coupled photosynthesis and respiration).

Microsensors Depending on growth conditions and age, the thickness of biofilms, aggregates and

Table 5. Dyes for structural analysis of biofilms and microbial mats.

Structure	Dye	Microscopy	Staining
Cells	Classical stains (crystal violet, Gram, etc.)	LM	All cells
	Acridine orange DAPI	FM and CSLM	All cells
	Ethidium bromide	FM and CSLM	Dead cells
	Eropidium iodide		
	Hexidium iodide	FM	Living cells
	CTC, formamide	FM and CSLM	Respiratory active cells
Voids and channels	Dextran conjugate	FM	Voids
	Beads	FM and CSLM	Voids
	Fluorescein	CSLM	Voids
EPS	Alcian blue	LM	EPS (carbohydrates)
	Lectins	FM and CSLM	EPS (carbohydrates)
	Calcofluor	FM and CSLM	EPS (carbohydrates)
	FITC	FM and CSLM	EPS (proteins)
	Heavy metals	TEM	Cells, EPS

Abbreviations as stated in Table 4; DAPI, 4′,6-diamidino-2-phenylindole; and CTC, cyanoditolyl tetrazolium chloride.

flocs can reach from μm to cm, and the structural heterogeneity can be pronounced. The active zones are typically on the order of a few mm or less. This requires experimental techniques with a high spatial resolution, and here microsensors have proven highly useful tools to study the biofilm and mat microenvironments and microbial activities in immobilized cell systems (including sediments). Microsensors are needle-shaped devices with a tip size of 1–20 μm and can measure the concentration of a specific compound. Owing to the small sensing tip, highly localized measurements are possible. Although the technique is invasive, the small tips do not influence structures or processes significantly. With microsensors the spatial distribution of substrates and products can be determined, and from this, the distribution of microbial activity can be inferred.

Niels-Peter Revsbech introduced microsensors in microbial ecology (Revsbech, 1983) and also constructed the first reliable O_2 microsensors for profiling sediments and biofilms (Revsbech, 1989). Other microsensors relevant for microbial ecology were developed and used, such as for N_2O (Revsbech et al., 1988), pH (Hinke, 1969; Lee and De Beer, 1995), NH_4^+ (De Beer and van den Heuvel, 1988), NO_3^- (De Beer and Sweerts, 1989; Larsen et al., 1996), S^{2-} (Revsbech, 1983), H_2S (Jeroschewski, 1996), NO_2^- (De Beer et al., 1997), CH_4 (Damgaard and Revsbech, 1997; Damgaard, 1995), CO_2 (De Beer et al., 1997), and HClO (De Beer et al., 1994) determination. Reviews have been published on construction and use of microsensors and on interpretation of results (Thomas, 1978; Revsbech, 1986; Kühl and Revsbech, 1998; De Beer, 1998; De Beer, 1999). Here only a brief summary is given. Microsensors are based on amperometric, potentiometric or optical principles.

Amperometric sensors are based on current measurements induced by the electrochemical reduction or oxidation of the substrate in the tip, with a rate proportional (usually linearly) to its concentration. Useful O_2, N_2O, H_2S and HClO sensors are based on this principle. Many research groups have used O_2 microsensors for study of photosynthesis and respiration. Such studies are done in biofilms (De Beer et al., 1994; Kühl and Jørgensen, 1992; Lewandowski, 1991; Zhang, 1994; Harmer and Bishop, 1992; Zhang, 1996), activated sludge flocs (Schramm, 1998a; Lens et al., 1995), and marine snow (Ploug et al., 1997). The N_2O sensor has been used for denitrification studies in biofilms and microbial mats (Revsbech et al., 1988); the development of nitrate sensors has made this sensor obsolete for this purpose. The new H_2S sensor is used to study sulfate reduction and sulfide oxidation in biofilms (Santegoeds et al., 1998) and activated sludge (Schramm et al., 1998b). The HClO microsensor is used in biofilm disinfection studies (De Beer et al., 1994; Xu et al., 1995).

The variety of measurable substrates has been expanded by applying enzymes or bacteria as catalysts for the formation or consumption of redox-active compounds in the sensor. Glucose oxidase has been used for glucose sensors (Cronenberg et al., 1991), cultures of methane oxidizers in methane sensors (Damgaard and Revsbech, 1997) and pure cultures of incomplete denitrifiers in nitrate and nitrite sensors (Larsen et al., 1997; Larsen et al., 1996).

Potentiometric microsensors measure electrical potential generated at the tip by charge separation. The oldest potentiometric microsensor is the full glass pH sensor (Hinke, 1969). It is versatile (Revsbech, 1986), has a low spatial resolution owing to its 100-μm long tip, and has an ca. 30-second response time. The AgS-membrane S^{2-} electrode has been very useful in studies of

the sulfur cycle in microbial mats and biofilms (Kühl and Jørgensen, 1992; Revsbech, 1983), but it must be used with care and in absence of oxygen. The H_2S sensor has no such problems and can be used for the same research. The H_2S sensor is most suitable for environments with low pH (>8), whereas the S^{2-} sensor may still be necessary in environments with high pH. The liquid membrane ion-exchanging (LIX) microsensor technique was developed by cell physiologists for intracellular measurements (mostly of CO_3^{2-}, Mg^{2+}, Li^+, Na^+ and K^+). These sensors can be very small (with a tip diameter of less that 1 μm, the size of a bacterial cell) and are used to measure NH_4^+ (De Beer and van den Heuvel, 1988), NO_3^- (De Beer and Sweerts, 1989; Jensen, 1993), NO_2^- (De Beer et al., 1997), H^+ (Schulthess et al., 1981), CO_2 (De Beer et al., 1997) or CO_3^- (Müller et al., 1998). The NH_4^+, NO_3^- and NO_2^- sensors are used to study the nitrogen cycle in biofilms and in sediments from freshwater environments (De Beer et al., 1991; Sweerts and De Beer, 1989; Jensen, 1993; De Beer et al., 1997), and the H^+ and CO_2 sensors are used for studies on photosynthesis and respiration in algal mats.

Ion-selective microsensors have some disadvantages. Often their selectivity is not very high. Because of interference by Na^+ or Cl^- ions, measurements cannot be made in marine environments, with the exception of pH, S^{2-} and Ca^{2+}. However, their value for studies in freshwater environments is high, and no alternative exists for NH_4^+, NO_2^-, and NO_3^- microsensors. They last only ca. 1 day; however, they are easy to construct. Finally, these sensors have behaved unpredictably in some circumstances, readings drifting radically, for example, upon penetration of the biofilm. Most likely this is caused by dissolution of hydrophobic biofilm compounds in the LIX membrane. The microsensor can be protected from this phenomenon with a hydrophilic protein layer (De Beer et al., 1997).

Micro-optodes are based on the change of optical properties (fluorescence intensity or fluorescence lifetime) of a layer covering an optical microfiber. Microsensors are developed for O_2, pH and temperature. The presence of the substrate induces quenching of the fluorescence intensity or decrease of the fluorescence lifetime. Klimant et al. (1997) gave a description of the theory and practice of this technique. Advantages of optical sensors are their ease of manufacture, insensitivity to noise, stability of calibration, and mechanical strength. Disadvantages include their size (ca. 20 μm), limited types of sensors available, and cost of the opto-electronics.

Typical experiments measure transient concentration changes at a fixed position and concentration profiles. Transient concentration changes are measured during photosynthesis with the fast light-dark shift (Revsbech, 1983) or for the in situ determination of diffusion coefficients (Cronenberg, 1994a; Cronenberg, 1994b). For these types of experiments we refer to the literature; the interpretation of profiles will be discussed below.

Interpretation of Profiles Profiles give information on microbial activity as well as insight into the microenvironments in biofilms. Microprofiles depend on mass transfer and microbial conversions. Consequently, if the transport processes are known, information on the distribution of microbial activity can be derived from the measured profiles. Because of microbial conversion and mass transfer resistance effects, substrate and product profiles develop inside biofilms. If the biofilm is impermeable, diffusion is the only transport mechanism inside the matrix. The turbulent bulk liquid is usually well mixed by advective transport (transport by liquid flow). Adjacent to the matrix is a viscous boundary layer in which the mixing and flow velocity gradually decrease as the surface is approached. Consequently, the mode of transport changes gradually from advectional in the bulk liquid to diffusional in the laminar boundary layer. Diffusional transport is driven by the concentration differences as expressed in Fick's law:

$$J = D\frac{dc}{dx} \tag{1}$$

where J is the flux ($mol \cdot m^{-2} \cdot s^{-1}$), D is the diffusion coefficient ($m^2 \cdot s^{-1}$), dc is the change in concentration (in mols) over the distance dx (in meters); dc/dx is the concentration gradient. In steady state, local conversion rates are equal to transport rates. Assuming a constant D, the mass balance becomes for a planar geometry:

$$D\frac{d^2c}{dx^2} = r \tag{2}$$

where r is the conversion rate. With Eq. 2 the concentration profiles can be calculated for zero- and first-order kinetics, assuming a homogeneous activity distribution. In reality, the assumptions are often too simple: conversions are of mixed-order kinetics (Monod) and the distribution of activity is varying with depth. Then only with iterative computer modeling, concentration profiles can be calculated. By fitting calculated with measured profiles, a good estimation can be made of the distribution of microbial activity (Revsbech, 1986; Berg, 1998). The executable code of the procedure from Berg et al. (1998) is available by e-mailing the author (pb8n@virginia.edu). A more direct approach is

to derive the local activities from the profiles. Assuming a measured profile consisting of three measured points (a, b, and c), the flux between a and b and b and c can be calculated from Eq. 1:

$$J_{ab} = D_{ab}\frac{(c_a - c_b)}{(x_a - x_b)} \quad \text{and} \quad J_{bc} = D_{bc}\frac{(c_b - c_c)}{(x_b - x_c)}$$

This gives the best estimate for the fluxes through the intermediate points between the measurements, $0.5(x_a + x_b)$ and $0.5(x_b + x_c)$. If the system is in steady state, then the difference in fluxes through these points is equal to the local conversion in point b, which is approximated by:

$$r_b = \frac{J_{ab} - J_{bc}}{0.5(x_a + x_b) - 0.5(x_b + x_c)} \qquad (3)$$

If D is constant with depth and we measure with constant depth intervals, δx, then

$$r_b = D\frac{c_a - 2c_b + c_c}{\Delta x^2} \qquad (4)$$

This approach needs high spatial resolution microprofile measurements. Since noise is magnified, a smoothing procedure is recommended. Alternatively, the profiles can be fitted with a polynome. Then the local fluxes are given by the product of the derivative of that polynomial function and D. The local activities are calculated by the product of the second derivative of the function and D.

When D_{eff} is varying with depth, the local fluxes must be calculated with the local D_{eff}, using Eq. 1, and local activities calculated with Eq. 3. All these calculations can be conveniently done with a spreadsheet.

An example of such an analysis is given in Fig. 12. In a biofilm, profiles of O_2, NO_2^-, NO_3^-, and H_2S were measured. From these profiles the aerobic respiration, nitrate consumption (denitrification), and sulfate reduction rates could be calculated. The analysis shows nicely the stratification of processes, i.e., the sequence of used e-acceptors.

Although activity profiles are valuable, combining these data with bacterial population distribution allows a more complete analysis of the microbial activities in biofilms. By aligning population and activity distributions, it can be determined which microorganisms are responsible for certain conversions (Ramsing et al., 1993; Santegoeds et al., 1998). The use of molecular techniques, techniques for population analysis (e.g., DGGE) and population distribution (using fluorescent in situ hybridization, FISH) has resulted in discovery of new species and their distribution within biofilms. With microsensor analysis and FISH, the ecological niche and kinetic data could be determined from these up-to-now uncultivable species (Schramm, 1998a; Schramm et al., 1998b).

Population Analysis

Cultivation Techniques: Microslicing, Most Probable Number (MPN) Technique, Plate Counting The classical microbial approach for population analysis is based on cultivation techniques, such as plate counting and MPN. However, it has become clear that these techniques select for certain organisms and thus do not give quantitative data (Staley and Konopka, 1985). Subpopulations of environmental samples cannot be cultivated and thus do not appear in MPN or platecounts (Torsvik et al., 1990; Wayne et al., 1987). Even key players of a community may not be identified (Wagner et al., 1993; Wagner et al., 1994; Schramm et al., 1998b). Consequently, cultivation techniques give a strongly biased picture of complex microbial communities in biofilms. Moreover, assessment of microscale distribution of microorganisms is very difficult. Okabe et al. analyzed the distribution of heterotrophic and nitrifying organisms by slicing living biofilms horizontally in 100–250 μm thick sections and enumerating organisms by MPN and plate counting (Okabe et al., 1996a; 1996b). However, since active zones in biofilms are often in the order of 50–100 μm, even this fine-scale technique is too coarse. To avoid these limitations, techniques were developed for identification and counting without cultivation steps.

Molecular Techniques Species-specific detection of strains is possible with antibodies and analysis of nucleic acids. Both techniques have been used on preparations of dispersed communities (fingerprinting) and on intact biofilms or preparations in which the integrity is maintained (in situ detection). Fingerprinting with antibodies has been described for nitrifiers (Sanden et al., 1994; Both et al., 1992) and methanogens (Kobayashi et al., 1988). More often, in situ analyses (with fluorescently labelled antibodies) are used to detect the spatial distribution of certain microorganisms (Zellner et al., 1995; Kobayashi et al., 1988; Buswell et al., 1998; Coughlin et al., 1997; Sonne Hansen and Ahring, 1997; Zellner et al., 1997; Stewart et al., 1997; Morin et al., 1996; Roberts and Keevil, 1992; Hunik et al., 1993). Beside technical difficulties, such as nonspecific binding, the preparation of specific antibodies requires pure cultures. Consequently, the antibody technique is not really culture-independent, as only antibodies can be developed for organisms that can be cultivated.

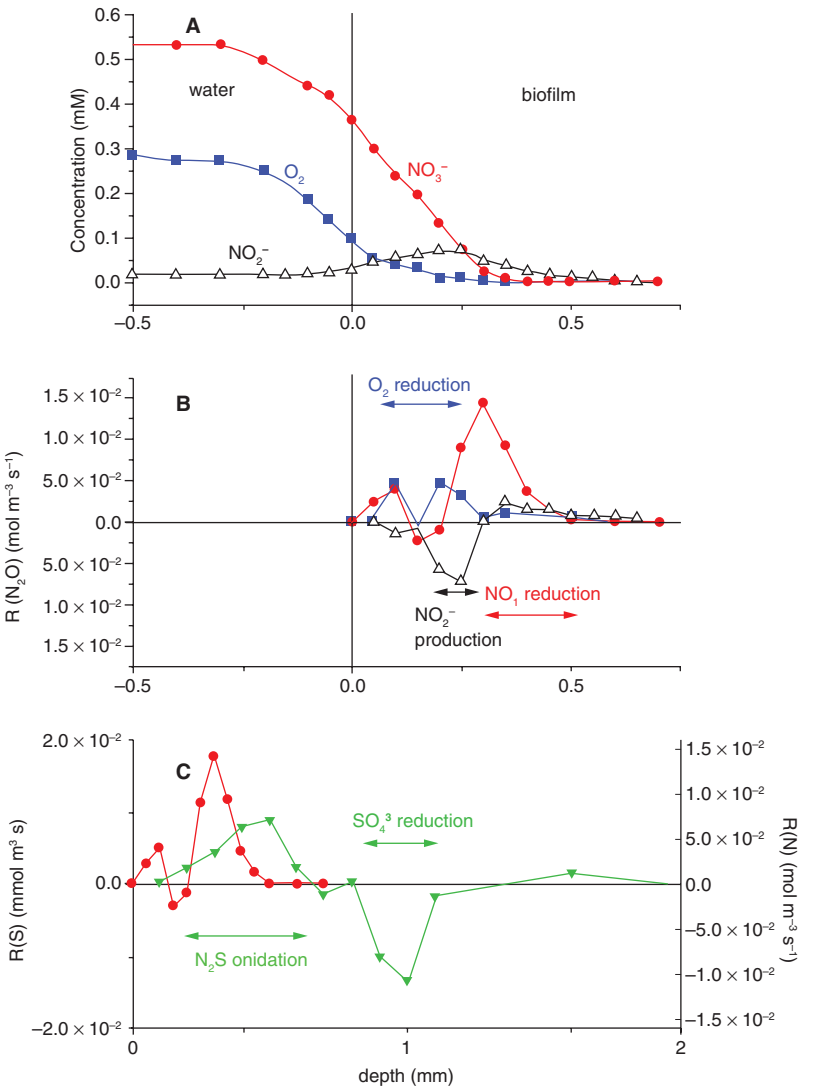

Fig. 13. Oxygen, nitrate, and nitrite profiles in a thick biofilm from a wastewater treatment plant (A) and local conversion rates calculated from these profiles (B). In (C) also the total sulfide conversion rates are plotted (sulfide profiles not shown), demonstrating that sulfide production occurs in the deep biofilm (ca. 1-mm depth) and sulfide oxidation overlaps with the denitrifying zone.

With the nucleic acid approach, population analysis is possible without cultivation. Currently, most efforts are directed to the analysis of ribosomal RNA, recently reviewed by Schramm and Amann (1999). For several reasons, 16S RNA sequence analysis is a powerful tool for the classification of microorganisms (Woese, 1987; Maidak et al., 1994). Ribosomes are present in all organisms, thus this piece of genetic material is universal. Part of the RNA molecules is identical for all microorganisms, while other regions are less well-conserved. Thus sequences can be found that are specific for different taxonomic levels, from species, genera, kingdoms and domains. Public databases contain 16S RNA sequences from many of the described bacterial species. Microbial cells contain 1,000 to 30,000 copies of 16S RNA molecules, allowing sensitive assays and identification of single cells by fluorescent oligonucleotide hybridization.

Fingerprinting techniques for populations based on 16S rDNA analysis are ARDRA (amplified ribosomal DNA restriction analysis; Heyndrickx et al., 1996), DGGE (Muyzer and De Waal, 1994) or TGGE (temperature gradient gel electrophoresis), and T-RFLP (terminal restriction fragment length polymorphism; Liu et al., 1997). These techniques all involve isolation of DNA, amplification of the genes or gene fragments encoding for 16S RNA by PCR (polymerase chain reaction).

In the procedure of ARDRA and T-RLFP, first the complete 16S RNA genes are amplified by PCR; this is followed by a digestion with restriction enzymes and size separation of the fragments by gel electrophoresis. ARDRA is used for fast screening of isolates, particularly giving information on similarities. ARDRA is less suitable for community analysis because of the complexity of resulting band patterns. T-RLFP

results in community fingerprints which can demonstrate the diversity and dynamics of microbial communities. However, different species have often similar fragment lengths, leading to an underestimation of the diversity. The more sensitive alternatives are DGGE and TGGE (Schramm and Amann, 1999).

For DGGE and TGGE only fragments are amplified (200–500 bp) and a GC-rich sequence of 40 bp is added at one end. With an increasing gradient of DNA denaturing agents, denaturation of double-stranded to single-stranded DNA fragments will occur at a position in the gel that depends on the composition of the DNA fragment (G+C content, sequence). Upon denaturation, the migration of that gene fragment stops. Consequently, fragments of the same length but with different sequences can be effectively separated, resulting in a band pattern reflecting the microbial diversity of the community. The sensitivity of DGGE is ca. 1%, meaning that bacterial populations making up 1% or more of the total community can be detected (Muyzer and Smalla, 1998). These methods (DGGE and TGGE) are very useful for detecting population changes of complex microbial communities. Bands in the gel can be further identified by hybridization analysis with specific probes (complementary fluorescently labelled DNA fragments). Also, bands can be retrieved from the gel and sequenced after amplification and cloning. Comparing the sequence with a data base allows identification or affiliation of the band within a phylogenetic tree. Although molecular methods may suffer from biases, they are probably less biased than cultivation methods. DNA has to be extracted, and not all cells may lyse. Furthermore, preferential amplification of DNA during the PCR can occur, and consequently, the band intensity in DGGE gels must not be interpreted as a quantitative measure for species abundance (Schramm et al., 1998b). Quantitative analysis is possible with hybridization, either dot-blot hybridization (Stahl et al., 1988) or in situ hybridization (Amann, 1995) with labelled oligonucleotide sequences targetting rRNA. After sequencing, it is possible to design a probe (with a fluorescent marker) targetting that sequence, and use it for FISH. Then, by a combination of FISH with microscopic analysis and cell counting, quantitative analysis is possible of uncultivated, and even uncultivable, species in environmental samples.

Fluorescent in situ hybridization (FISH) is a recently developed, very powerful tool to quantify populations within a microbial community and to determine the spatial distributions of populations on different taxonomic levels. Instead of extracting nucleic acids, the cells are gently permeabilized so that fluorescently labelled 16S RNA probes, small fragments of up to 20 bp, can enter the cells and hybridize with rRNA. Probes can be labelled with different fluorescent dyes, each with distinct excitation and emission spectra. Thus, two or more probes can be used simultaneously to detect different (up to seven) populations within one sample (Amann et al., 1996). However, the spatial distribution of populations as determined with FISH does not give information on the activity of the cells. The populations detected are there, but can be inactive or dead. Conversion rates can be derived from microprofiles determined with microsensors. At the moment, the combination of in situ techniques, i.e., molecular and microsensor techniques, gives the most accurate characterization of microbial interactions and activities in complex microbial communities.

Literature Cited

Ahring, B. K., J. E. Schmidt, M. Winther-Nielsen, A. J. L. Macario, and E. Conway de Macario. 1993. Effect of medium composition and sludge removal on the production, composition and structure of thermophilic (55°C) acetate-utilizing granules from an upflow anaerobic sludge blanket reactor. Appl. Environ. Microbiol. 59:2538–2545.

Allison, D. G., D. J. Evans, M. R. W. Brown, and P. Gilbert. 1990. Possible involvement of the division cycle in dispersal of Escherichia coli biofilms. J. Bacteriol. 172:1667–1669.

Amann, R. I. 1995. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. Molec. Microbiol. Ecol. Man. 4:543–554.

Amann, R., J. Snaidr, M. Wagner, W. Ludwig, and K. H. Schleifer. 1996. In situ visualisation of high genetic diversity in a natural microbial community. J. Bacteriol. 178:3496–3500.

Andreasen, K., and P. H. Nielsen. 1997. Application of microautoradiography to the study of substrate uptake by filamentous microorganisms in activated sludge. Appl. Environ. Microbiol. 63(9):3662–3668.

Arnold, F. H., H. W. Blanch, and C. R. Wilke. 1985. Analysis of affinity separations II: The characterization of affinity columns by pulse techniques. Chem. Eng. J. 30:25–36.

Axelrod, D., D. E. Koppel, J. Schlesinger, E. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. Biophys. J. 16:1055–1069.

Bakke, R., M. G. Trulear, J. A. Robinson, and W. G. Characklis. 1984. Activity of Pseudomonas aeruginosa in steady state biofilms. Biotechnol. Bioeng. 26:1418–1424.

Beeftink, H. H., and P. Staugaard. 1986. Structure and dynamics of anaerobic bacterial aggregates in a gas-lift reactor. Appl. Environ. Microbiol. 52(5):1139–1146.

Beeftink, H. H. 1987. Anaerobic bacterial aggregates; variety and variation. University of Amsterdam. Amsterdam.

Beeftink, H. H., and J. C. van den Heuvel. 1987. Novel anaerobic gas-lift reactor AGLR with retention of biomass, start-up routine and establishment of hold up. Biotechnol. Bioeng. 30(2):233–238.

Berg, H. C. 1983. Random walks in biology. Princeton University Press. Princeton, NJ.

Berg, P., N. Risgaard-Pedersen, and S. Rysgaard. 1998. Interpretation of measured concentration profiles in sediment pore water. Limnol. Oceanogr. 43(7):1500–1510.

Both, G. J., S. Gerards, and H. J. Laanbroek. 1992. Temporal and spatial variation in the nitrite oxidising bacterial community of a grassland soil. FEMS Microbiol. Ecol. 101(2):99–102.

Boyd, A., and A. M. Chakrabarty. 1994. Role of alginate lyase in cell detachment of Pseudomonas aeruginosa. Appl. Environ. Microbiol. 60:2355–2359.

Boyer, M. B., and J. T. Hsu. 1992. Experimental studies of restricted protein diffusion in an agarose matrix. AIChE J 38:259–272.

Bremer, P. J., G. G. Geesey, and B. Drake. 1992. Atomic force microscopy examination of the topography of a hydrated bacterial biofilm on a copper surface. Curr. Microbiol. 24:223–230.

Brenner, H., and L. J. Gaydos. 1977. The constrained brownian movement of spherical particles in cylindrical pores of compatible radius. J. Coll. Interfac. Sci. 58:312–356.

Brown, M. R. W., and P. Gilbert. 1993. Sensitivity of biofilms to antimicrobial agents. J. Appl. Bacteriol. Symp. (Suppl.) 74:87–97.

Bryers, J. D., and W. G. Characklis. 1981. Early fouling biofilm formation in a turbulent flow system: Overall kinetics. Wat. Res. 15:483–491.

Bryers, J., and F. Drummond. 1996. Local macromolecule diffusion coefficients in structurally non-uniform bacterial biofilms using fluorescence recovery after photobleaching (FRAP). Biotechnol. Bioeng. 60(4):462–473.

Buffiere, P., J. P. Steyer, C. Fonade, and R. Moletta. 1995. Comprehensive modeling of methanogenic biofilms in fluidized bed systems: Mass transfer limitations and multisubstrate aspects. Biotechnol. Bioeng. 48:725–736.

Buret, A., K. H. Ward, M E. Olson, and J. W. Costerton. 1991. An in vivo model to study the pathobiology of infectious biofilms on biomaterial surfaces. J. Biomed. Mat. Res. 25(7):865–874.

Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. M. Mcguiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1998. Extended survival and persistence of Campylobacter spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. Appl. Environ. Microbiol. 64(2):733–741.

Caldwell, D. E., and J. R. Lawrence. 1988. Study of attached cells in continuous-flow slide culture. *In:* J. W. T. Wimpenny (Ed.) Handbook of Laboratory Model Systems for Microbial Ecosystems. CRC Press. Boca Raton, FL. 117–138.

Camper, A. K. 1994. Coliform regrowth and biofilm accumulation in drinking water systems: A review. *In:* G. G. Geesey, Z. Lewandowski, and H.-C. Flemming (Eds.) Biofouling/biocorrosion in Industrial Systems. Lewis. Chelsea, UK. 91–105.

Canfield, D. E., and D. J. Des Marais. 1991. Aerobic sulfate reduction in microbial mats. Science 251:1471–1473.

Characklis, W. G. . Biofouling: effects and control. *In:* International Workshop Industrial Biofilms Biocorrosion. 7–27.

Characklis, W. G. 1988. Model biofilm reactors. *In:* J. W. T. Wimpenny (Ed.) Handbook of Laboratory Model Systems for Microbial Ecosystems. CRC Press. Boca Raton, FL. 155–174.

Characklis, W. G. 1990. Microbial fouling. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley and Sons. New York, NY. 523–584.

Characklis, W. G., and K. Marshall. 1990. Biofilms: A basis for an interdisciplinary approach. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley and Sons. New York, NY. 3–15.

Chayen, J., L. Bitensky, and R. Butcher. 1973. Practical Histochemistry. John Wiley & Sons.

Chen, C.-I., T. Griebe, and W. G. Characklis. 1993. Biocide action of monochloramine on biofilm systems of Pseudomonas aeruginosa. Biofouling 1–17.

Christensen, B. E., and W. G. Characklis. 1990. Physical and chemical properties of biofilms. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley and Sons. New York, NY. 93–130.

Cochran, W. L., G. A. McFeters, and P. S. Stewart. 2000. Reduced susceptibility of thin Pseudomonas aeruginosa biofilms to hydrogen peroxide and monochloramine. J. Appl. Microbiol. 88:22–30.

Costerton, W. J., Z. L. Lewandowski, D. Debeer, D. Caldwell, D. Korber, and G. James. 1994. Biofilms, the customized microniche. J. Bacteriol. 176(8):2137–2142.

Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. Science 284:1318–1322.

Coughlin, M. F., B. K. Kinkle, A. Tepper, and P. L. Bishop. 1997. Characterization of aerobic azo dye-degrading bacteria and their activity in biofilms. Wat. Sci. Technol. 36(1):215–220.

Cronenberg, C. C. H., H. Van Groen, D. De Beer, and J. C. Van den Heuvel. 1991. Oxygen-independent glucose microsensor based on glucose oxidase. Anal. Chim. Acta 242:275–278.

Cronenberg, C. C. H. 1994. Biofilms investigated with needle-type glucose sensors. University of Amsterdam. Amsterdam.

Cronenberg, C. C. H., S. P. P. Ottengraf, J. H. van den Heuvel, F. Pottel, D. Sziele, K. Schugerl, and K. H. Bellgardt. 1994. Influence of age and structure of Penicillium chrysogenum pellets on the internal concentration profiles. Bioprocess Engineering 10:209–216.

Csikor, Z., P. Mihaltz, L. Czako, and J. Hollo. 1994. New interpretation of expansion in biofilm-coated particle fluidization. Appl. Microbiol. Biotechnol. 41:608–614.

Daffonchio, D., J. Thaveeshi, and W. Verstraete. 1995. Contact angle measurement and cell hydrofobicity of granular sludge from UASB reactors. Appl. Environ. Microbiol. 61(10):3676–3680.

Dalton, H. M., A. E. Goodman, and K. C. Marshall. 1996. Diversity in surface colonization behavior in marine bacteria. J. Ind. Microbiol. 17:228–234.

Damgaard, L. R., Larsen, L. H., and Revsbech, N. P. 1995. Microscale biosensors for environmental monitoring. Trends Anal. Chem. 14:300–303.

Damgaard, L. R., and N. P. Revsbech. 1997. A microscale biosensor for methane. Anal. Chem. 69(13):2262–2267.

Davies, D. G., and G. G. Geesey. 1995. Regulation of the alginate biosynthesis gene algC in Pseudomonas aeruginosa during biofilm development in continuous culture. Appl. Environ. Microbiol. 61(3):860–867.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298.

De Beer, D., and J. C. van den Heuvel. 1988. Response of ammonium-selective microelectrodes based on the neutral carrier nonactin. Talanta 35(9):728–730.

De Beer, D., and J. P. R. A. Sweerts. 1989. Measurements of nitrate gradients with an ion-selective microelectrode. Anal. Chim. Acta 219:351–356.

De Beer, D., J. P. R. A. Sweerts, and J. C. van den Heuvel. 1991. Microelectrode measurement of ammonium profiles in freshwater sediments. FEMS Microbiol. Ecol. 86(1):1–6.

De Beer, D., J. C. van den Heuvel, and S. P. P. Ottengraf. 1993. Microelectrode measurements of the activity distribution in nitrifying bacterial aggregates. Appl. Environ. Microbiol. 59(2):573–579.

De Beer, D., R. Srinivasan, and P. S. Stewart. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Appl. Environ. Microbiol. 60(12):4339–4344.

De Beer, D., P. Stoodley, and Z. Lewandowski. 1994. Liquid flow in heterogeneous biofilms. Biotechnol. Bioeng. 44:636–641.

De Beer, D., P. Stoodley, F. Roe, and Z. Lewandowski. 1994. Effect of biofilm structures on oxygen distribution and mass transfer. Biotechnol. Bioeng. 43:1131–1138.

De Beer, D., and P. Stoodley. 1995. Relation between the structure of an aerobic biofilm and transport phenomena. Wat. Sci. Technol. 32(8):11–18.

De Beer, D. 1996. Microenvironments and mass transfer phenomena in biofilms and activated sludge studied with microsensors. *In:* International Symposium on Environmental Biotechnology. Ostend, Belgium. 217–224.

De Beer, D., V. O'Flaharty, J. Thaveesri, and P. Lens. 1996. Distribution of extracellular polysaccharides and flotation of anaerobic sludge. Appl. Microbiol. Biotechnol. 46:197–201.

De Beer, D., A. Glud, E. Epping, and M. Kühl. 1997. A fast responding CO2 micro-electrode for profiling sediments, microbial mats and biofilms. Limnol. Oceanogr. 42(7):1590–1600.

De Beer, D., A. Schramm, C. M. Santegoeds, and M. Kühl. 1997. A nitrite microsensor for profiling environmental biofilms. Appl. Environ. Microbiol. 63(3):973–977.

De Beer, D., P. Stoodley, P., and Z. Lewandowski. 1997. Measurement of local diffusion coefficients in biofilms by microinjection and confocal microscopy. Biotechnol. Bioeng. 53:151–158.

De Beer, D., and M. Kühl. 1998. Interfacial processes, gradients and metabolic activity in microbial mats and biofilms. *In:* B. Boudreau and B. B. Jørgensen (Eds.) The Benthic Boundary Layer. Oxford University Press. Oxford, UK.

De Beer, D., A. Schramm, C. M. Santegoeds, and H. K. Nielsen. 1998. Anaerobic processes in activated sludge. Wat. Sci. Technol. 37(4–5):605–608.

De Beer, D. 1999. Use of microelectrodes to measure in situ microbial activities in biofilms, sediments and microbial mats. *In:* A. D. L. Akkermans. J. D. van Elsas, and F. J. de Bruin (Eds.) Molecular Microbial Ecology Manual. Kluwer.

De Beer, D. 1999. Micro-electrodes. *In:* R. Wijffels (Ed.) Immobilized Cells. Springer-Verlag. Heidelberg,

Decho, A. W. 1990. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. Oceanogr. Mar. Biol. Ann. Rev. 28:73–153.

Decho, A. W., and G. R. Lopez. 1993. Exopolymer microenvironments of microbial flora: multiple and interactive effects on trophic relationships. Limnol. Oceanogr. 38(8):1633–1645.

Eighmy, T. T., D. Maratea, and P. L. Bishop. 1983. Electron microscopic examination of wastewater biofilm formation and structural components. Appl. Environ. Microbiol. 45:1921–1931.

Elvers, K. T., K. Leeming, C. P. Moore, and H. M. Lappin-Scott. 1998. Bacterial-fungal biofilms in flowing water photo-processing tanks. J. Appl. Microbiol. 84:607–618.

Escher, A., and W. G. Characklis. 1990. Modeling the initial events in biofilm accumulation. *In:* W. G. Characklis and K. C. Marshall (Eds.) Biofilms. John Wiley and Sons. New York, NY. 445–486.

Fattom, A., and M. Shilo. 1984. Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. Appl. Environ. Microbiol. 47:135–143.

Flemming, H. C. 1998a. Extracellular polymeric substances—the key to understand biofilms. *In:* First International Workshop on Biofilms in Aerobic Wastewater Treatment: An Interdisciplinary Approach. Garching, Germany. 28–30.

Flemming, H. C., T. Griebe, and G. Schaule. 1998b. Antifouling strategies in technical systems: A short review. Wat. Sci. Technol. 34(5–6):517–524.

Fowler, H. W. 1988. Microbial adhesion to surfaces. *In:* J. W. T. Wimpenny (Ed.) Handbook of Laboratory Model Systems for Microbial Ecosystems. CRC Press. Boca Raton, FL. 139–153.

Frolund, B., R. Palmgren, K. Keiding, and P. H. Nielsen. 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin. Wat. Res. 30(8):1749–1758.

Frund, C., and Y. Cohen. 1992. Diurnal cycles of sulfate reduction under oxic conditions in Cyanobacterial mats. Appl. Environ. Microbiol. 58(1):70–77.

Gjaltema, A., P. A. M. Arts, M. C. M. van Loosdrecht, J. G. Kuenen, and J. J. Heijnen. 1994. Heterogeneity of biofilms in rotating annular reactors: Occurrence, structure, and consequences. Biotechnol. Bioeng. 194–204.

Gjaltema, A., L. Tijhuis, M. C. M. van Loosdrecht, and J. J. Heijnen. 1995. Detachment of biomass from suspended nongrowing spherical biofilms in airlift reactors. Biotechnol. Bioeng. 46(3):258–269.

Gjaltema, A., J. L. Vinke, M. C. M. van Loosdrecht, and J. J. Heynen. 1997. Abrasion of suspended biofilm pellets in airlift reactors: importance of shape, structure, and particle concentrations. Biotechnol. Bioeng. 53:88–99.

Gorris, L. G. M., J. M. A. van Deursen, C. van der Drift, and G. D. Vogels. 1988. Influence of waste water composition on biofilm development in laboratory methanogenic fluidized bed reactors. Appl. Microbiol. Biotechnol. 29:95–102.

Greenberg, E. P. 1997. Quorum sensing in gram-negative bacteria. ASM News 63(7):371–377.

Griebe, T., G. Schaule, J. Secker, and H. C. Flemming. 1995. Bestimmung der stoffwechselaktiven Bakterien im belebten Schlamm. Wasser Abwasser 136(12):608–615.

Griebe, T., and H. C. Flemming. 1998. Biocide-free antifouling strategy to protect RO membranes from biofouling. Desalination 118:153–157.

Gunning, P. A., A. R. Kirby, M. L. Parker, A. P. Gunning, and V. J. Morris. 1996. Comparative imaging of Pseudomonas putida bacterial biofilms by scanning electron microscopy and both DC contact and AC non-contact atomic force microscopy. J. Appl. Bacteriol. 81(3):276–282.

Haigler, C. H., R. M. Brown, and M. Benzman. 1980. Calcofluor White ST alters the in vivo assembly of cellulose microfibrils. Science 210:903–906.

Hall-Stoodley, L., and H. M. Lappin-Scott. 1998. Biofilm formation by the rapidly growing mycobacterial species Mycobacterium fortuitum. FEMS Microbiol. Lett. 168:77–84.

Hall-Stoodley, L., J. C. Rayner, P. Stoodley, and H. M. Lappin-Scott. 1999. Establishment of experimental biofilms using the modified Robbins device and flow cells. *In:* C. Edwards (Ed.) Methods in Biotechnology, Volume 12: Environmental Monitoring of Bacteria. Humana Press. Totowa, NJ. 307–319.

Harada, H., S. Eumura, and K. Momonoi. 1994. Interaction between sulfate reducing bacteria and methane-producing bacteria in UASB reactors fed with low strength wastes containing different levels of sulfate. Wat. Res. 28:355–367.

Harmer, C., and P. Bishop. 1992. Transformation of AZO dye AO-7 by waste water biofilms. Wat. Sci. Technol. 26:627–636.

Haugland, R. P. 1996. Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes Inc.. Eugene, OR.

Heijnen, J. J. 1984. Biological industrial waste-water treatment minimizing biomass production and maximizing biomass concentration. Technical University Delft. Delft, The Netherlands.

Heyndrickx, H. J. M., L. Vauterin, P. Vandamme, K. Kersters, and P. De Vos. 1996. Applicability of combined ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. J. Microbiol. Meth. 26:246–259.

Hinke, J. 1969. Glass microelectrodes for the study of binding and compartmentalisation of intracellular ions. *In:* M. Lavallee, O. F. Schanne, and N. C. Herbert (Eds.) Glass Microelectrodes. John Wiley and Sons. 349–375.

Horn, H., and D. C. Hempel. 1997. Substrate utilization and mass transfer in an autotrophic biofilm system: Experimental results and numerical simulation. Biotechnol. Bioeng. 53(4):363–371.

Hsu, Y., and W. K. Shieh. 1993. Startup of anaerobic fluidized bed reactors with acetic acid as the substrate. Biotechnol. Bioeng. 41:347–353.

Hulshoff Pol, L. W. 1989. The phenomenon of anaerobic sludge. Wageningen Agricultural University. Wageneningen, The Netherlands.

Hunik, J. H., M. P. van den Hoogen, W. de Boer, M. Smit, and J. Tramper. 1993. Quantitative determination of the spatial distribution of Nitrosomonas europea and Nitrobacter agilis cells immobilized in kappa carrageenan gel beads by a specific fluorescent antibody labeling technique. Appl. Environ. Microbiol. 59(6):1951–1954.

Jahn, A., and P. H. Nielsen. 1995. Extraction of extracellular polymeric substancesfrom biofilms using a cation exchange resin. Wat. Sci. Technol. 32(8):157–164.

Jensen, K., N. P. Revsbech, and L. P. Nielsen. 1993. Microscale distribution of nitrification activity in sediment determined with a shielded microsensor for nitrate. Appl. Environ. Microbiol. 59(10):3287–3296.

Jeroschewski, P., C. Steukart, and M. Kühl. 1996. An amperometric microsensor for the determination of H2S in aquatic environments. Anal. Chem. 68:4351–4357.

Jørgensen, B. B., and N. P. Revsbech. 1985. Diffusive boundary layers and the oxygen uptake of sediments and detritus. Limnol. Oceanogr. 30(1):111–122.

Jørgensen, B. B., and D. Des Marais. 1990. The diffusive boundary layer of sediments: oxygen microgradients over a microbial mat. Limnol. Oceanogr. 35:1343–1355.

Jørgensen, B. B. 1994. Diffusion processes and boundary layers in microbial mats. *In:* L. J. Stal and P. Caumette (Eds.) Microbial Mats: Structure, Development and Environmental Significance. Springer-Verlag. Berlin, 243–253.

Karsten, U., and M. Kühl. 1996. Die Mikrobenmatte—das kleinste Ökosystem der Welt. Biologie Unserer Zeit 26(1):16–26.

Klimant, I., M. Kühl, R. N. Glud, and G. Holst. 1997. Optical measurements of oxygen and temperature in microscale: Strategies and biological applications. Sensors and Actuators B 38–39:29–37.

Kobayashi, H. A., E. C. De Macario, R. S. Williams, and A. J. L. Macario. 1988. Direct characterization of methanogens in two high-rate anaerobic biological reactors. Appl. Environ. Microbiol. 54(3):693–698.

Koehl, M. A. R. 1984. How benthic organisms withstand moving water. Am. Zool. 24:57–70.

Koster, I. 1989. Toxicity in anaerobic digestion. Wageningen Agricultural University. Wageningen, The Netherlands.

Krekeler, D., P. Sigalevich, A. Teske, H. Cypionka, and Y. Cohen. 1997. A sulfate reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), Desulfovibrio oxyclineae sp. nov. Arch. Microbiol. 167(6):369–375.

Kugaprasatham, S., H. Nagaoka, and S. Ohgaki. 1992. Effect of turbulence on nitrifying biofilms at non-limiting substrate conditions. Wat. Res. 26(12):1629–1638.

Kühl, M., and B. B. Jørgensen. 1992. Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. Appl. Environ. Microbiol. 58(4):1164–1174.

Kühl, M., and N. P. Revsbech. 1998. Microsensors for the study of interfacial biogeochemical processes. *In:* B. Boudreau and B. B. Jørgensen (Eds.) The Benthic Boundary Layer. Oxford University Press. Oxford, UK.

Larsen, L. H., N. Revsbech, and S. J. Binnerup. 1996. A microsensor for nitrate based on immobilized denitrifying bacteria. Appl. Environ. Microbiol. 62(4):1248–1251.

Larsen, L. H., T. Kjaer, and N. P. Revsbech. 1997. A microscale NO3- biosensor for environmental applications. Anal. Chem. 69(17):3527–3531.

Lauwers, A. M., W. Heijnen, L. G. M. Gorris, and C. van der Drift. 1990. Early stages in biofilm development in methanogenic fluidized-bed reactors. Appl. Microbiol. Biotechnol. 33:352–358.

Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell. 1991. Optical sectioning of microbial biofilms. J. Bacteriol. 173(20):6558–6567.

LeChevallier, M. W., C. D. Lowry, and R. G. Lee. 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol. 54:2492–2499.

Lee, W., and D. de Beer. 1995. Oxygen and pH microprofiles above corroding mild steel covered with a biofilm. Biofouling 8:273–280.

Lens, P. N., M.-P. De Poorter, C. C. Cronenberg, and W. H. Verstraete. 1995. Sulfate reducing and methane producing bacteria in aerobic wastewater treatment systems. Wat. Res. 29(3):871–880.

Lettinga, G. 1995. Anaerobic digestion and wastewater treatment. Ant. v. Leeuwenhoek 67:3–28.

Lewandowski, Z., and G. Walser. 1991. Influence of hydrodynamics on biofilm accumulation. *In:* Environ. Eng. Proc. EE Div. ASCE. Reno, NV. 619–624.

Lewandowski, Z., G. Walser, and W. G. Characklis. 1991. Reaction kinetics in biofilms. Biotechnol. Bioeng. 38:877–882.

Lewandowski, Z., S. A. Altobelli, and E. Fukushima. 1993. NMR and microelectrode studies of hydrodynamics and kinetics in biofilms. Biotechnol. Prog. 9:40–45.

Libicki, S. B., P. M. Salmon, and C. R. Robertson. 1988. The effective diffusive permeability of a nonreacting solute in microbial cell aggregates. Biotechnol. Bioeng. 32:68–85.

Little, B., P. Wagner, R. Ray, R. Pope, and R. Scheetz. 1991. Biofilms, an ESEM evaluation of artifacts introduced during preparation. J. Ind. Microbiol. 8(4):213–222.

Liu, W. T., T. L. Marsh, and L. J. Forney. 1997. Determination of the microbial diversity of anaerobic-aerobic activated sludge by a novel molecular biological technique. 2nd International Conference on Microorganisms in Activated Sludge and Biofilm Processes. Berkeley, CA. 247–254.

Mack, W. N., J. P. Mack, and A. O. Ackerson. 1975. Microbial film development in trickling filters. Microbial Ecology 2:215–316.

Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese. 1994. The ribosomal database project. Nucleic Acids Res. 22:3485–3487.

Massol-Deya, A. A., J. Whallon, R. F. Hickey, and J. M. Tiedje. 1995. Channel structure in aerobic biofilms of fixed-film reactors treating contaminated groundwater. Appl. Environ. Microbiol. 61(2):769–777.

Maximilien, R., R. de Nys, C. Holmstrom, L. Gram, K. Crass, S. Kjelleberg, and P. D. Steinberg. 1998. Chemical mediation of bacterial surface colonisation by secondary metabolites from the red alga Delisa pulchra. Aquat.Microb. Ecol. 15(3):233–246.

McCoy, W. F., J. D. Bryers, J. Robbins, and J. W. Costerton. 1981. Observations of fouling biofilm formation. Can. J. Microbiol. 27:910–917.

McCoy, W. F. 1987. Effects of fouling biofilms on systems performance. *In:* M. W. Mittelman and G. G. Geesey (Eds.) Biological Fouling of Industrial Water Systems: A Problem Solving Approach. Water Micro Associates. San Diego, CA. 234–246.

Møller, S., D. R. Korber, G. M. Wolfaardt, S. Molin, and D. E. Caldwell. 1997. Impact of nutrient composition on a degradative biofilm community. Appl. Environ. Microbiol. 63:2432–2438.

Morck, D. W., K. Lam, S. G. Mckay, M. E. Olson, B. Prosser, B. D. Ellis, R. Cleeland, and J. W. Costerton. 1994. Comparative evaluation of fleroxacin, ampicillin, trimethoprim-sulfamethoxazole, and gentamicin as treatments of catheter-associated urinary tract infection in a rabbit model. Int. J. Antimicrob. Agents 4(2):521–527.

Morin, P., A. Camper, W. Jones, D. Gatel, and J. C. Goldman. 1996. Colonization and disinfection of biofilms hosting coliform-colonized carbon fines. Appl. Environ. Microbiol. 52(12):4428–4432.

Müller, B., K. Buis, R. Stierli, and B. Wehrli. 1998. High spatial resolution measurements in lake sediments with PVC based liquid membrane ion-selective electrodes. Limnol. Oceanogr. 43(7):1728–1733.

Muyzer, G., and E. C. de Waal. 1994. Determination of the genetic diversity of microbial communities using DGGE analysis of PCR-amplified 16S rDNA. NATO ASI Series, G35:207–214.

Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Ant. v. Leeuwenhoek 73:127–141.

Neu, T., and J. R. Lawrence. 1997. Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. FEMS Microbiol. Ecol. 24:11–25.

Nichols, P. H. 1988. Susceptability of biofilms to toxic compounds. *In:* W. G. Characklis and P. A. Wilderer (Eds.) Structure and Function of Biofilms. John Wiley and Sons. New York, NY. 321–331.

Nielsen, P. H., B. Frolund, and K. Keiding. 1996. Changes in composition of extracellular polymeric subtances in activated sludge during anaerobic storage. Appl. Microbiol. Biotechnol. 44(6):823–830.

Nielsen, P. H., A. Jahn, and R. Palmgren. 1997. Conceptual model for production and composition of exopolymers in biofilms. Wat. Sci. Technol. 36(1):11–19.

Nielsen, P. H., K. Andreasen, M. Wagner, L. L. Blackall, H. Lemmer, and R. J. Seviour. 1998. Variability of type 021N in activated sludge as determined by in situ substrate uptake pattern and in situ hybridization with fluorescent rRNA targeted probes. Wat. Sci. Technol. 37(4–5):423–430.

Okabe, S., K. Hirata, Y. Ozawa, and Y. Watanabe. 1996. Spatial microbial distributions of nitrifiers and heterotrophs in mixed population biofilms. Biotechnol. Bioeng. 50:24–35.

Okabe, S., Y. Ozawa, K. Hirata, and Y. Watanabe. 1996. Relationship between population dynamics of nitrifiers in biofilms and reactor performance at various C:N ratios. Wat. Res. 30(7):1563–1572.

Okabe, S., T. Yasuda, and Y. Watanabe. 1997. Uptake and release of inert fluorogenic particles by mixed-population biofilms. Biotechnol. Bioeng. 53(5):459–469.

Overmeire, A., P. Lens, and W. Verstraete. 1994. Mass transfer limitation of sulfate in methanogenic aggregates. Biotechnol. Bioeng. 44:387–391.

Paterson, D. M. 1995. Biogenic structure of early sediment fabric visualized by low-temperature scanning electron microscopy. J. Geol. Soc. London 152:131–140.

Peters, A., and J. W. T. Wimpenny. 1988. A constant depth laboratory model film fermenter. *In:* W. T. Wimpenny (Ed.) Handbook of Laboratory Model Systems for Microbial Ecosystems. CRC Press. Boca Raton, FL. 175–195.

Picologlou, B. F., N. Zelver, and W. G. Characklis. 1980. Biofilm growth and hydraulic performance. J. Hydraulic Div. Am. Soc. Civ. Eng. 106, HY5:733–746.

Ploug, H., M. Kühl, B. Buchholz-Cleven, and B. B. Jørgensen. 1997. Anoxic aggregates—an ephemeral phenomenon in the pelagic environment? Aquat. Microb. Ecol. 13:285–294.

Ploug, H., and B. B. Jørgensen. 1998. A net-jet flow system for mass transfer and microsensor studies of sinking aggregates. Mar. Ecol. Prog. Ser. 176:270–290.

Qian, Z., P. Stoodley, and W. G. Pitt. 1996. Effect of low-intensity ultrasound upon biofilm structure from confocal scanning microscopy observation. Biomaterials 17:1975–1980.

Ramsing, N. B., M. Kühl, and B. B. Jørgensen. 1993. Distribution of sulfate-reducing bacteria, O_2, and H_2S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. Appl. Environ. Microbiol. 59:3840–3849.

Rasmussen, K., and Z. Lewandowski. 1998. Microelectrode measurements of local mass transport rates in heterogeneous biofilms. Biotechnol. Bioeng. 59(3):303–309.

Revsbech, N. P., and B. B. Jørgensen. 1983. Photosynthesis of benthic microflora measured with high spatial resolution by the oxygen microprofile method: Capabilities and limitations of the method. Limnol. Oceanogr. 28(4):749–756.

Revsbech, N. P., and D. M. Ward. 1983. Oxygen microelectrode that is insensitive to medium chemical composition: Use in an acid microbial mat dominated by Cyanidium caldarum. Appl. Environ. Microbiol. 45:755–759.

Revsbech, N. P., B. B. Jørgensen, T. H. Blackburn, and Y. Cohen. 1983. Microelectrode studies of the photosynthesis and O_2, H_2S and pH profiles of a microbial mat. Limnol. Oceanogr. 28(6):1062–1074.

Revsbech, N. P., and B. B. Jørgensen. 1986. Microelectrodes: their use in microbial ecology. Adv. Microbial Ecol. 9:293–352.

Revsbech, N. P., B. Madsen, and B. B. Jørgensen. 1986. Oxygen production and consumption in sediments determined at high spatial resolution by computer simulation of oxygen microelectrode data. Limnol. Oceanogr. 31(2):293–304.

Revsbech, N. P., L. P. Nielsen, P. B. Christensen, and J. Sorensen. 1988. A combined oxygen and nitrous oxide microsensor for denitrification studies. Appl. Environ. Microbiol. 45:2245–2249.

Revsbech, N. P. 1989. An oxygen microelectrode with a guard cathode. Limnol. Oceanogr. 55:1907–1910.

Revsbech, N. P., L. P. Nielsen, and N. B. Ramsing. 1998. A novel microsensor for determination of apparent diffusivity in sediments. Limnol. Oceanogr. 43(5):986–992.

Rittmann, B. E., and J. A. Manem. 1992. Development and experimental evaluation of a steady-state, multispecies biofilm model. Biotechnol. Bioeng. 39:914–922.

Roberts, J., and C. W. Keevil. 1992. Immunogold and fluorescein immunolabelling of Legionella-pneumophila within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. Appl. Environ. Microbiol. 58(7):2326–2330.

Robinson, R. W., D. E. Akin, R. A. Nordstedt, M. V. Thomas, and H. C. Aldrich. 1984. Light and electron microscopic examinations of methane producing biofilms from anaerobic fixed bed reactors. Appl. Environ. Microbiol. 48(1):127–136.

Rodbard, D., and A. Chranbach. 1970. Unified theory for gel electrophoresis and gel filtration. Proc. Natl. Acad. Sci. USA 65:970–977.

Sanden, B., C. Grunditz, Y. Hansson, and G. Dalhammar. 1994. Quantification and characterization of Nitrosomonas and Nitrobacter using monoclonal antibodies. Wat. Sci. Technol. 29(7):1–6.

Sanford, B. A., V. L. Thomas, S. J. Mattingly, M. A. Ramsay, and M. M. Miller. 1995. Lectin-biotin assay for slime present in in situ biofilm produced by Staphylococcus epidermidis using transmission electron microscopy (TEM). J. Ind. Microbiol. 15(3):156–161.

Santegoeds, C. M., T. G. Ferdelman, G. Muyzer, and D. de Beer. 1998. Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. Appl. Environ. Microbiol. 64(11):3731–3739.

Santegoeds, C. M. 1999. Structure and function analysis of sulfate reducing communities in wastewater treatment systems: A combined molecular and microsensor study. Bremen University. Bremen, Germany.

Santegoeds, C. M., L. R. Damgaard, G. Hesselink, J. Zopfi, P. Lens, G. Muyzer, and D. de Beer. 1999. Distribution of sulfate reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular techniques. Appl. Environ. Microbiol.

Schmidt, J. E., and B. K. Ahring. 1994. Extracellular polymers in granular sludge from different upflow anaerobic sludge blanket (UASB) reactors. Appl. Microbiol. Biotechnol. 42:457–462.

Schopf, J. W., and C. Klein. 1992. The Proterozoic Biosphere: A Multidisciplinary Study. Cambridge University Press. New York, NY.

Schramm, A., L. H. Larsen, N. P. Revsbech, N. B. Ramsing, R. Amann, and K.-H. Schleifer. 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. 62:4641–4647.

Schramm, A. 1998. In situ structure and function analysis of nitrifying/denitrifying biofilms. Bremen University. Bremen, Germany.

Schramm, A., D. de Beer, H. van den Heuvel, S. P. P. Ottengraf, and R. Amann. 1998. In situ structure/function studies in wastewater treatment systems. Wat. Sci. Technol. 37(4–5):413–416.

Schramm, A., D. de Beer, M. Wagner, and R. Amann. 1998. Nitrosospira and Nitrospira sp. as dominant populations in a nitrifying fluidized bed reactor: identification and activity in situ. Appl. Environ. Microbiol. 64(9):3480–3485.

Schramm, A., and R. Amann. 1999. Nucleic acid based techniques for analyzing the diversity, structure, and dynamics of microbial communities in wastewater treatment. *In:* J. Winter (Ed.) Environmental Processes: Wastewater and Waste Treatment. John Wiley-VCH. Weinheim, Germany. 1–24.

Schulthess, P., Y. Shijo, H. V. Pham, E. Pretsch, D. Ammann, and W. Simon. 1981. A hydrogen ion-selective liquid-membrane electrode based on tri-n-dodecylamine as neutral carrier. Anal. Chim. Acta 131:111–116.

Shaw, D. A., and T. J. Hanratty. 1977. Turbulent mass transfer to a wall for large Schmidt numbers. A.I.Ch.E.J. 23:28–37.

Shieh, W. K., and Y. Hsu. 1996. Biomass loss from an anaerobic fluidized bed reactor. Wat. Res. 39(5):1253–1257.

Siebel, M. A., and W. G. Characklis. 1990. Observations of binary biofilms. Biotechnol. Bioeng. 37:778–789.

Siegrist, H., and W. Gujer. 1985. Mass transfer mechanisms in a heterotrophic biofilm. Wat. Res. 19:1369–1378.

Sly, L. I., M. C. Hodgkinson, and V. Arunpairojana. 1990. Deposition of manganese in a drinking water distribution system. Appl. Environ. Microbiol. 56(3):628–639.

Sonne Hansen, J., and B. K. Ahring. 1997. Anaerobic microbiology of an alkaline Icelandic hot spring. FEMS Microbiol. Ecol. 23(1):31–38.

Späth, R., H. C. Flemming, and S. Würtz. 1998. Sorption properties of biofilms. Wat. Sci. Technol. 37(4–5):207–210.

Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of rumenal ecology. Appl. Environ. Microbiol. 54:1079–1084.

Staley, J. T., and A. Konopka. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Ann. Rev. Microbiol. 39:321–346.

Stewart, P. S., W. J. Drury, and R. Murga. 1993. Quantitative observations of heterogeneities in Pseudomonas aeruginosa biofilms. Appl. Environ. Microbiol. 59(1):327–329.

Stewart, P. S., R. Murga, R. Srinivasan, and D. de Beer. 1995. Biofilm structural heterogeneity visualized by three microscopic methods. Wat. Res. 29(8):2006–2009.

Stewart, P. S., M. A. Hamilton, B. R. Goldstein, and B. T. Schneider. 1996. Modeling biocide action against biofilms. Biotechnol. Bioeng. 49:445–455.

Stewart, P. S., A. K. Camper, S. D. Handran, C. T. Huang, and M. Warnecke. 1997. Spatial distribution and coexistence of Klebsiella pneumoniae and Pseudomonas aeruginosa in biofilms. Microb. Ecol. 33(1):2–10.

Stewart, P. S. 1998. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. Biotechnol. Bioeng. 59(3):261–272.

Stoodley, P., de Beer, D., and Lewandowski, Z. 1994. Liquid flow in biofilm systems. Appl. Environ. Microbiol. 60:2711–2716.

Stoodley, P., D. de Beer, and H. M. Lappin-Scott. 1997. Influence of electric fields and pH on the biofilm structure as related to the bioelectric effect. Antimicrob. Agents Chemother. 41(9):1876–1879.

Stoodley, P., Z. Lewandowski, J. Boyle, and H. M. Lappin-Scott. 1998. Oscillation characteristics of biofilm streamers in turbulent flowing water as related to drag and pressure drop. Biotechnol. Bioeng. 57(5):536–544.

Stoodley, P., I. Dodds, J. D. Boyle, and H. M. Lappin-Scott. 1999. Influence of hydrodynamics and nutrients on biofilm structure. J. Appl. Microbiol. 85:19s–28s.

Stoodley, P., F. Jørgensen, P. Williams, and H. M. Lappin-Scott. 1999. The role of hydrodynamics and AHL signalling molecules as determinants of the structure of Pseudomonas aeruginosa biofilms. *In:* R. Bayston, M. Brading, P. Gilbert, J. Walker, and J. W. T. Wimpenny (Eds.) Biofilms: The Good, the Bad, and the Ugly. BioLine. Cardiff, UK. 323–330.

Stoodley, P., Z. Lewandowski, J. D. Boyle, and H. M. Lappin-Scott. 1999. Structural deformation of bacterial biofilms caused by short term fluctuations in flow velocity: an in-situ demonstration of biofilm viscoelasticity. Biotechnol. Bioeng. 65:83–92.

Stoodley, P., Z. Lewandowski, J. D. Boyle, and H. M. Lappin-Scott. 1999. The formation of migratory ripples in a mixed species bacterial biofilm growing in turbulent flow. Environ. Microbiol. 1:447–457.

Surman, S. B., J. T. Walker, D. T. Goddard, L. H. G. Morton, C. W. Keevil, W. Weaver, A. Skinner, K. Hanson, D. Caldwell, and J. Kurtz. 1996. Comparison of microscope techniques for the examination of biofilms. J. Microbiol. Meth. 25(1):57–70.

Sutherland, I. W. 1994. Structure-function relationships in microbial exopolysaccharides. Biotech. Adv. 12:393–447.

Sutherland, I. W. 1996. A natural terrestrial biofilm. J. Ind. Microbiol. 17:281–283.

Sweerts, J.-P. R. A., and D. de Beer. 1989. Microelectrode measurements of nitrate gradients in the littoral and profundal sediments of a meso-eutrophic lake (lake Vechten, The Netherlands). Appl. Environ. Microbiol. 55(3):754–757.

Thaveeshi, J., D. Daffonchio, B. Liessens, P. Vandermeren, and W. Verstraete. 1995. Granulation and sludge bed stability in UASB reactors in relation to surface thermodynamics. Appl. Environ. Microbiol. 61(10):3681–3686.

Thomas, R. C. 1978. Ion-sensitive intracellular microelectrodes, how to make and use them. Academic Press. London.

Tijhuis, L., W. A. J. van Benthum, M. C. M. van Loosdrecht, and J. J. Heijnen. 1994. Solids retention time in spherical biofilms in a biofilms airlift suspension reactor. Biotechnol. Bioeng. 867–879.

Tijhuis, L., M. C. M. van Loosdrecht, and J. J. Heijnen. 1994. Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. Biotechnol. Bioeng. 44:595–608.

Tijhuis, L., B. Hijman, M. C. M. van Loosdrecht, and J. J. Heijnen. 1996. Influence of detachment, substrate loading and reactor scale on the formation of biofilms in airlift reactors. Appl. Microbiol. Biotechnol. 45:7–17.

Torsvik, V., J. Goksoyr, and F. L. Daae. 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. 56:782–787.

Tsien, R. Y., and T. J. Rink. 1981. Ca^{2+}-selective electrodes: A novel PVC-gelled neutral carrier mixture compared with other currently available sensors. J. Neurosc. Meth. 4:73–86.

Van Der Kooij, D., H. S. Vrouwenvelder, and H. R. Veenendaal. 1995. Kinetic aspects of biofilm formation on surfaces exposed to drinking water. Wat. Sci. Technol. 32(8):61–65.

Van Houten, R. T., L. W. Hulshoff Pol, and G. Lettinga. 1994. Biological sulphate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. Biotechnol. Bioeng. 44:586–594.

Van Loosdrecht, M. C. M., J. Lyklema, W. Norde, and A. J. B. Zehnder. 1990. Influence of interfaces on microbial activity. Microbiol. Rev. 54(1):75–87.

Van Loosdrecht, M. C. M., D. Eikelboom, A. Gjaltema, A. Mulder, L. Tijhuis, and J. J. Heijnen. 1995. Biofilm structures. Wat. Sci. Tech. 32:35–43.

van Loosdrecht, M. C. M., C. Picioreanu, and J. J. Heijnen. 1997. A more unifying hypothesis for biofilm structures. FEMS Microbiol. Ecol. 24:181–183.

Vogel, S. 1994. Life in Moving Fluids (2nd ed.). Princeton University Press. Princeton, NJ.

Wagner, M., R. Amann, D. Lemmer, and K.-H. Schleifer. 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependant methods for describing microbial community structure. Appl. Environ. Microbiol. 59(5):1520–1525.

Wagner, M., B. Armus, A. Hartmann, P. Hutzler, and R. Amann. 1994. In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy. J. Microscopy 176(3):181–187.

Wanner, O., and W. Gujer. 1986. A multispecies biofilm model. Biotechnol. Bioeng. 28:314–328.

Wanner, O., and P. Reichert. 1996. Mathematical modeling of mixed culture biofilms. Biotechnol. Bioeng. 49:172–184.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, C. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463–464.

Westrin, B. A. 1991. Diffusion Measurements in Gels. University of Lund. Lund, Sweden.

Whiteley, M., E. Brown, and R. J. C. McLean. 1997. An inexpensive chemostat apparatus for the study of microbial biofilms. J. Microbiol. Meth. 30(2):125–132.

Widdel, F. 1988. Microbiology and ecology of sulfate and sulfur reducing bacteria. *In:* A. D. J. Zehnder (Ed.) Biology of anaerobic microorganisms. John Wiley and Sons. New York, NY. 469–585.

Wieland, K. 1999. Microsensor analysis of oxygen and sulfide turnover in hypersaline microbial mats. Bremen University. Bremen, Germany.

Wilderer, P. A., and W. G. Characklis. 1989. Structure and function of biofilms. *In:* W. G. Characklis and P. A. Wilderer (Eds.) Structure and Function of Biofilms. John Wiley and Sons. New York, NY. 5–17.

Williams, J. W., and J. W. T. Wimpenny. 1978. Exopolysaccharide production by Pseudomonas NCIB 11264 grown in continuous culture. J. Gen. Microbiol. 104:47–57.

Wimpenny, J. W. T. 1996. Development of a steady state oral microbial biofilm community using the constant depth film fermenter. Microbiology 142(3):631–638.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51(2):221–271.

Wolfaardt, G. M., J. R. Lawrence, R. D. Robarts, and D. E. Caldwell. 1995. Bioaccumulation of the herbicide diclofop in extracellular polymers and its utilization by a biofilm community during starvation. Appl. Environ. Microbiol. 152–158.

Wolfaardt, G. M., J. R. Lawrence, R. D. Robarts, and D. E. Caldwell. 1998. In situ characterization of biofilm exopolymers involved in the accumulation of chlorinated organics. Microb. Ecol. 35:213–223.

Xu, X., P. S. Stewart, and X. Chen. 1995. Transport limitation of chlorine disinfection of Pseudomonas aeruginosa entrapped in alginate beads. Biotechnol. Bioeng. 49:93–100.

Yang, S., and Z. Lewandowski. 1995. Measurement of local mass transfer coefficient in biofilms. Biotechnol. Bioeng. 737–744.

Zellner, G., E. Feuerhake, H.-J., J., A. J. L. Macario, and E. Conway De Macario. 1995. Denitrifying and methanogenic bacteria in the biofilm of a fixed-film reactor operated with ethanol-nitrate demonstrated by immunofluorescence and microscopy. Appl. Microbiol. Biotechnol. 43(5):566–571.

Zellner, G., A. J. L. Macario, and E. Conway De Macario. 1997. A study of three anaerobic methanogenic bioreactors reveals that syntrophs are diverse and different from reference organisms. FEMS Microbiol. Ecol. 22(4):295–301.

Zhang, T. C., and P. L. Bishop. 1994. Density, porosity, and pore structure of biofilms. Wat. Res. 2267–2277.

Zhang, T. C., Y. C. Fu, and P. L. Bishop. 1994. Competition in biofilms. Wat. Environ. Res. 67(6):992–1003.

Zhang, T. C., and P. L. Bishop. 1996. Evaluation of microelectrode performance and applicability in biofilm systems. *In:* 69th Annual Conference Water Environment Federation. Dallas, TX. 967–2007.

Index

B

C

D

E

F

G

H

I

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z