# 1 Exciting Times: The Challenge to be a Bacterial Systematist

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A comparison of the molar proportions reveals certain striking, but perhaps meaningless, regularities. *Vischer, Zamenhof and Chargaff (1949)*

Of all natural systems, living matter is the one which, in the face of great transformations, preserves inscribed in its organisation the largest amount of its own history. *Zuckerkandl and Pauling (1965)*

The species is man-made, and since it cannot be defined, the creation of taxa of higher categories based on species makes an absurd situation. *Cowan (1951)*

## 1.1 Introduction

In his overview "*Anaerobic life – a centennial view*" Ralf Wolfe (1999), referring to the dawn of complete genome sequencing of prokaryotes, states that "there has never been a more exciting time for the study of phylogeny and evolution". This citation complements the one by Hugenholtz and Pace (1996), referring to the encouraging development in microbial ecology, which is quoted by Neufeld and Mohn at the beginning of Chap. 7 in this book. These summaries are certainly more than personal opinions and highlight the enthusiasm that accompanies and drives microbiologists at unprecedented rates to new shores of understanding the biology of microorganisms. Can the history of microbiology be viewed as a series of isolated periods in which microbiologists considered themselves working in an exciting time? Is not the history of microbiology from the mid-nineteenth century a continuum of scientific achievements, in which scientists of any generation found it rewarding to contribute? When one considers not the short time periods, but the average generation time of 30–40 years as the productive years of a microbiologist, then this statement is correct (I am aware that the productive period of some microbiologists is significantly longer).

Looking backwards, there were times in which microbiologists must have been similarly impressed about developments in their own disciplines

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as we are today. In retrospect, these events are named *milestones*, mainly single events which most probably are the crystallization of a much longer preceding period. For the discipline of bacterial systematics, I could think of a few such milestones or milestone eras while a non-taxonomist will certainly define others although, in many cases, milestones cover more than a single discipline. Persons mentioned in the following chapter are recognized for their achievements in microbiology but do not comprise an exhaustive list: this is not a comprehensive chapter on the history of bacterial systematics but rather a short introduction to a subject which has caught the attention of microbiologists from its very early beginnings. We are fortunate to work in a time in which bacterial systematics has been elevated to a scientific multidisciplinary field. For me, the exciting time spanned from 1970 until today, but I fully agree with Ralph Wolfe that this period will be significantly extended with new emerging directions and techniques, several of which are summarized in this book. The two main achievements that influenced my perspective of modern bacterial systematics were: first, the introduction of DNA–DNA reassociation studies in the early 1970s and, second, 16S rRNA oligonucleotide cataloguing in the late 1970s. The following years witnessed the application of reverse transcriptase and PCR-mediated sequence analysis of 16S rRNA genes and the analysis of genes coding for proteins. The combination of molecular, chemotaxonomic, physiological and other cellular traits led to first insights into the relatedness among prokaryotic species, changing each textbook chapter on microbial systematics. This development also fertilized ecological studies, leading to the recognition of as-yet uncultured organisms and the linkage of function to structure. It revolutionized the scale on which to look at prokaryotic diversity (Venter et al. 2004) and it revived the discussion on the concept and definition of the taxon 'species', sharpening the awareness that species are populations rather than genomically coherent entities (Coenye et al. 2005).

Advancements achieved during the period of an exciting time are the basis for the exciting times to come and are a fundamental driving force of visions that still motivate young people to dedicate themselves to science. The knowledge that we are only passengers in the 'train of science', which we enter at a certain station and alight at another as the train continues down the tracks, puts the achievement of scientists into perspective: we use the scientific platform provided by our predecessors and we broaden the basis, modify and sometimes radically change existing developments. Occasionally, we even may break with existing dogmas. The accumulated knowledge will be passed on to our successors who will continue the process, starting from a much higher and broader knowledge platform than the preceding generation. The following paragraphs will briefly summarize four milestone eras that have influenced the direction of microbial system-

atics. The past 130 years have been shaped by developments originating in various other disciplines and, still today, microbial taxonomists are often the users rather than the architects of concepts.

As milestones and highly productive eras should be recognized as such through their merits, the following subdivision is somewhat artificial, a personal view influenced by teachers, literature and my own experience. In no way can a contribution such as an introduction to a series of recent achievements and developments be sufficiently comprehensive to fully acknowledge the contributions and the influence of key scientists on the development of their own and on neighbouring scientific fields. The reader is referred to their original literature and to monographs in order to pay full tribute to their achievements.

## 1.2 The Early Heroes (1860–1900)

Even though the beginning of bacterial systematics can be placed with the description of the first bacterial species in 1872 by Ferdinand Cohn, his conclusions, mainly based on his own observations, were also influenced by the concepts, accurate observations and misinterpretations of scientists working in the early decades of the nineteenth century. Several developments ran in parallel. Above all, the morphology of micro-organisms was observed by light microscopy in combination with the application of specific staining procedures. Although stains were introduced as early as 1770 in the study of the structure of wood, it was not until 1839 that Christian Gottfried Ehrenberg (1795–1876) used stains to study microbes. At that time, the isolation of micro-organisms in pure culture had not been achieved. Although Louis Pasteur (1822–1895) and other scientists from that era described micro-organisms which fermented and caused diseases of sheep, cattle and other farm animals, as well as human illnesses, it was Robert Koch (1843–1910) who developed the technique of growing pure bacterial cultures. Most of the cultivation [on potato, gelatine, agar medium; later done in glass dishes introduced by Richard J. Petri (1852–1921)] and staining techniques were developed in the mid- to late 1800s by Robert Koch, Paul Ehrlich (1854–1915) and Hans Christian Gram (1853–1938). These various fundamental procedures were necessary to turn bacteriology into a respected science; and at that time the improvement of the health of livestock and man had absolute priority.

Pasteur established the view that microbes could be classified into fixed and unchangeable species and genera. Each species was believed to cause a specific disease. In contrast, Antoin Bechamp (1816–1908) declared that all animal and plant cells contained minuscule granules (granulations

moléculaires) that did not die when the organism died. These granules were believed to be the source of fermentation; and micro-organisms could arise from them as well. Several respected scientists believed that the morphological diversity of micro-organisms was due to variations of one and the same organism, e.g. Zopf (1846–1909), Wilhelm von Naegeli (1817–1891), Theodor Billroth (1829–1894), "missing the point that different stages of development, types of multiplication, the variety of size and form, and specific metabolic properties were associated with distinct species types" (Drews 1999).

Organisms that were observable under the microscope and later as pure cultures were named without guidelines (not to speak about rules). As summarized by Drews (2000) in his essay on the roots of microbiology, almost every scientist who observed micro-organisms gave them a new name without noticing that the same organisms may have already been named differently by another taxonomist. Synonyms accumulated as culture-dependent changes erroneously mirrored the existence of novel organisms (more than 40,000 invalid names and synonyms were counted at the end of the 1970s).

Called the 'father of systematics', Ferdinand Cohn studied algae, lichens and bacteria in media composed of defined mineral solutions complemented with different organic carbon sources. He was the first to propose a relationship among these organisms (Cohn 1867) and, summarizing his observations on shape, cellular structures, pigmentation and metabolic activities, he presented the first classification system of bacteria (Cohn 1872, 1876). He concluded that bacteria can be divided into distinct species with typical characteristics, which are transmitted to the following generations when bacteria multiply. Cohn also proposed that varieties exist within species, a notion that today plays an important role in the recognition of a bacterial species as a population, guiding scientists towards a new definition of this taxon more than 130 years later (Palys et al. 1997, 2000; Stackebrandt et al. 2002; Gevers et al. 2005).

The lack of recognizable characters other than morphological properties explains the superimposition of the botanical classification system to bacteria by the botanist Cohn (1872, 1876). Cohn, using the binominal nomenclature, affiliated the Schizomyceae (bacteria) and Schizophyceae (Cyanophyceae or cyanobacteria) to the group of Schizophyta (fission plants), but considered these micro-organisms as a group on their own. Bacteria were defined as chlorophyll-less cells of characteristic shape that multiply by cross-division and live as single cells, filamentous cell chains, or cell aggregates. [The fact that some Bacteria (sensu Woese et al. 1990) still carry the ending 'mycetes' is a reminder of the now discarded hypothesis that bacteria are fission fungi (schizomycetes). Note that even some of the archaeal taxa carry the ending 'bacteria', although the bacteria and archaea are members of two different Domains, indicating that nomen-

clature does not necessarily reflect phylogeny.]. The Schizomycetes contained four groups: 'Sphaerobacteria' (sphere-shaped, e.g. *Micrococcus*), 'Microbacteria' (rod-like, e.g. *Bacterium*), 'Desmobacteria' (filamentous, e.g. *Bacillus*, *Vibrio*), and 'Spirobacteria' (screw-like bacteria, e.g. *Spirillum*, *Spirochaeta*). On the basis of specific properties which were considered taxonomically less significant than morphology, Cohn divided some of his proposed genera, e.g. *Micrococcus*, into chromogenic (pigmented), zymogenic (fermenting) and pathogenic (contagious) species; and he described the purple bacteria in terms of their shape, pigments, gas vacuoles and sulfur globules.

It has to be stressed that Cohn already commented on the limited phylogenetic significance of the taxa he included in the morphology-based system: he was aware that the genera and species of bacteria have other meanings than for higher organisms, which reproduce sexually. He clearly stated that the proposed 'form-genera' and 'form-species' needed to be tested to determine whether they were indeed related in terms of descent. This, however, could not be achieved prior to 1970 at the level of genera (De Ley et al. 1970, Palleroni and Duodoroff 1971; Palleroni et al. 1973) and prior to 1977 at the level of higher taxa (Woese and Fox 1977; Woese et al. 1990). As a phylogenetic framework is still missing at the intraspecific level, appropriate methods need to be developed before systematists will be in a position to develop concepts.

## 1.3
## The Dawn of Microbial Ecology and the Continuing Struggle with Classification Systems (1900–1930)

At the beginning of the twentieth century, the morphological basis of bacterial systematics was considerably broadened by the addition of physiological traits to the list of taxonomically important properties. Based on comparative morphological analysis and the hitherto unrecognized diversity of end-products and relation to oxygen, Orla-Jensen (1909) defined the main lines of bacterial systematics on the basis of physiological characteristics. However, as the system remained artificial (only elements of it were later found to have a phylogenetically sound basis) and the degree of the polyphyletic origin was not determinable, neither morphology, physiology, motility, nor any other property selected as the basis for a taxonomic scheme gave a satisfactory answer to conflicting alternatives. Even today, some of these discrepancies still complicate taxonomy.

At the turn of the century, microbial ecology was emerging as a new field, when Beijerinck (1895) described the formation of hydrogen sulfide from sulfate by a species later reclassified as *Desulfovibrio desulfuricans* and when

Winogradsky (1890) discovered chemoautotrophy (also see Winogradsky 1998). He was able to cultivate iron bacteria, described earlier by Cohn (1872), using mineral substrates from which ferrous iron was oxidized to ferric iron, obtaining energy for $CO_2$ assimilation. Analogous to this finding was the isolation of ammonium- and nitrite-oxidizing lithotrophic bacteria. At this time, microbial ecology was promoted mainly by members of the Delf School, e.g. Martinus Beijerinck, Cornelius B. van Niel and Albert J. Kluyver (to name a few with the greatest influence). They introduced the methods of selected isolation, including baiting micro-organisms with the properties they wanted to know about, by selecting the appropriate culture medium. The detection of a new range of physiologies considerably broadened the spectrum of taxonomically meaningful properties.

It must be mentioned in the context of this brief historical summary that, based on his own observations which were later supported by the theory of mutation of De Vries (1901), Martinus Beijerinck (1899) initiated experiments on changing physiological properties through variation and mutation, claiming that bacteria and fungi were more suitable objects for studies on heredity than higher evolved organisms (Beijerinck et al. 1940). These studies, later continued by members of the Delft school, led to the development of the genetics of micro-organisms (Delbrück and Luria 1942).

Though confronted with a broad spectrum of observations, the underlying genetic basis of the phenotype was missing. As pointed out by Palleroni (2003), the scientific community accepted the simplicity of Cohn's morphological system over the physiology-based concept for decades to come. His system was modified by adding new 'form-genera' to the inventory (Lehmann and Neumann 1896; Migula 1900; Pringsheim 1923; Janke 1924; Prévot 1933). Morphology continued to play a dominating conceptual role, far beyond the first morphology-based description of Ferdinand Cohn.

While Europe was setting the pace in the early years of bacterial systematics, America adopted its own bacterial classification system (Buchanan 1918; Winslow et al. 1920) by publishing the first edition of Bergey's *Manual of determinative bacteriology* (Bergey et al. 1923). This standard textbook was updated about every decade until 1990, when the first edition of Bergey's *Manual of systematic bacteriology* (Krieg 1986) was released. As the release of the new edition overlapped with the recognition about the restricted taxonomic value of morphology, these four volumes were composed to cluster groups of organisms under headings reflecting superficial morphological and physiological properties. Nevertheless, the merits of Bergey's manual has been recognized and the accumulated, systematized and published taxonomic knowledge in a single coherent volume constituted the "the first formal co-operation in the history of bacterial taxonomy" (Kluyver and van Niel 1936).

Today, knowing the basic phylogenetic lineages of cultured organisms, we consider most morphological and many physiological traits as being polyphyletic. Only a few morphologically complex traits are so far considered monophyletic, e.g. those of myxobacteria and spirochetes, as well as the formation of endospores. Even the thickness of the peptidoglycan, the basis for the Gram-staining reaction used to classify bacteria into two main groups, is not a monophyletic trait, as seen in the presence of Gram-positive cell walls in Archaea and Bacteria and the placement of Firmicutes, Actinobacteria and deinococci in separate higher taxa. The notion that morphologically different organisms may produce the same set of fermentation products or react similarly towards the presence of oxygen and light was first elucidated by deciphering metabolic pathways and recently by molecular analysis. Though certain physiological properties are indeed monophyletic, this information was not available to workers in the pre-molecular era. Rather than criticizing them for something they could not possibly have detected, we should acknowledge their attempts and those of the many others that followed for developing a range of systems, each of them devised to better serve the community of users.

## 1.4 Encouragement and Frustration (The Era 1930–1950)

Several key scientists from the early twentieth century influenced the science of bacterial systematics. There were the above-mentioned members of the Delft School, Albert J. Kluyver and his student Cornelius B. van Niel, as well as Robert E. Hungate, a student of the latter, and Roger Stanier. All of them were either involved in the isolation of bacteria, shifting the emphasis from clinical to environmental strains, or they were influencing the concepts of taxonomy. Hungate, the pioneer of anaerobic microbial microbiology and ecology (Chung and Bryant 1997), provided the fundament for the discovery of a new spectrum of microbial diversity, including the archaeabacteria (archaea), described about 40 years later (Woese and Fox 1977). Kluyver and van Niel are also recognized for their criticism against the system(s) outlined in the successive editions of Bergey's *Manual of determinative bacteriology*. Above all, they were critical of the "utter disregard for mutual relationships between natural groups" (Kluyver and van Niel 1936) and the disregard of other voices in the field (e.g. Rahn 1929, 1937). They also detailed many errors that arose as a consequence of the arbitrary use of morphological, physiological, cultural and pathogenic properties in bacterial classification (Palleroni 2003). This author also highlights the European tradition of favouring morphology as the first and most reliable guide of taxonomic systems (Kluyver and van Niel 1936) and disregarding

the use of physiology unless physiological principles could be subordinated to morphology. In the system of Kluyver and van Niel, morphological characters included the shape and size of cells, type of motility, presence of flagella, their number and type of insertion, the mode of reproduction, occurrence of endospores and various structural peculiarities. Certain physiological properties were indeed recognized but the overall importance of reactions for the cell was not reflected by their importance on taxonomic ranks. Pathogenicity was considered of doubtful value and differentiation of genera and even species on its basis was objectionable as a taxonomic criterion. Considering the genetic instability of many pathogenicity factors this may be judged as a wise decision; but the decision of Kluyver and van Niel was certainly not guided by genetic principles. It was inevitable that the basis of a true natural classification of bacteria would remain unsteady "inasmuch as the course of phylogeny will always remain unknown" (Kluyver and van Niel 1936). A call for a more prudent consideration of taxonomic systems was proposed by White (1937) who phrased: "the present call is not for newer, more ingenious, more pretentious, systems of classification, but for patient and incisive investigation". Later, Stanier and van Niel (1941) and van Niel (1946) commented on the inflexibility of Bergey's classification system that was based on the arbitrary selection of properties that could not be changed without replacing the existing system. The main advantage of Bergey's system was its practicability, i. e. identification and classification, but only if the key characters were mutually exclusive. The 'indications of relationships' should better be replaced by 'means of identification' and a broad range of differentiation characters rather than a few key properties should guide classification. This history of this period has been covered more extensively by Palleroni (2003).

It was not until the mid-1940s that van Niel (1946) agreed to add physiology, pathogenicity, nutrition and other easily determinable properties, e. g. colour, to the morphological properties used to devise an empirical key for bacteria. Obviously, systems were mainly devised to facilitate the affiliation of strains to species. The problem was the early adoption of names of taxonomic ranks from botanical and zoological systems where (at least in the majority of taxa) a taxon within a hierarchic system should indeed indicate genomic coherence and common ancestry. In microbiology, the majority of taxa (including even the taxon 'species') constituted a collection of entities of vastly different phylogenetic origin. van Niel (1946) pointed out the inability of phenotype-based classification systems to deduce phylogenetic interrelationships, though evolutionary consideration should have their place in bacterial taxonomy. Considering the general disbelief towards the emerging phylogenetic framework in 1980, it must be assumed that most microbiologists will have believed that determination of phylogenies were inherently indeterminable, at least at the higher

taxonomic levels. Woese (1987) criticized Roger Stanier who considered speculations on microbial evolution as being metascientific, by stating that "microbiology had reduced evolutionary matters to the status of dalliance was indeed unfortunate, for much of what is important and interesting about evolution lay hidden in the microbial world".

Not foreseeable by scientists in the 1940s, it was another 20 years before the pioneering work of Zuckerkandl and Pauling (1962) provided the framework of a phylogeny-based classification system. Today, with the broad outline of the system increasingly stable, a situation similar to that in the 1940s is occurring with the discussion of the concept of bacterial 'species' and the change from an artificial and arbitrary species definition (Staley and Konopka 1985; Wayne et al. 1987; Vandamme et al. 1996; Dijkshoorn et al. 2000; Rosselló-Mora and Amann 2001) to a definition that recognizes and describes natural mechanisms of speciation (summarized by Gevers et al. 2005).

Though the older systems have nothing less than historical value, they are important to remember as milestones of systematist's hybris to attempt to circumscribe the 'true' nature of the path of evolution. The merits and the correct perspective of early classification systems are discussed comprehensively by Kluyver and van Niel (1936) and by van Niel (1946). Still today we squeeze populations of more or less genomically diverse organisms into the taxon 'species' and define borders for genera, families and higher taxa, comforting ourselves by acknowledging the arbitrary nature of our definitions. Today, 130 years after Cohn's first description of species, our knowledge about the make-up and expression of a cell is breathtaking, but still we struggle with the definition of certain ranks.

Parallel to the discussion on the inappropriateness of phenotypic properties in reflecting evolutionary relationships, a possible solution through the linking of systematics to genealogy was slowly emerging. Originating in the nineteenth century, the discipline of Biochemistry was established together with the basis for a deeper understanding of heredity. Nucleic acids were isolated (Miescher, 1811–1887), terms like 'gene' and 'macromolecule' were introduced, the extraction of the first enzyme was described (Buchner 1897) and biochemical reactions were linked to genetic phenomena. The advantage of working with micro-organisms was recognized, but it was not until the 1940s when Avery et al. (1944) identified DNA as the responsible agent for the transfer of genetic markers in bacterial cultures. *Neurospora crassa* (Beadle and Tatum 1941) and bacterial species (Luria and Delbrück 1943) were study objects on physiological changes due to mutations. The mechanisms of the transfer of genetic information was described in *Escherichia coli* (Chargaff et al. 1949) and the genomic world was open to new research avenues, following the elucidation of the macromolecular structure of proteins (Pauling and Corey 1951) and nucleic acids (Watson and Crick 1953).

## 1.5 Expanding the Range of Properties: The Genetic and Epigenetic Levels (1950–1980)

The criticism on Bergey's classification system published in the 1940s and 1950s was accepted in the last edition of the Manual in 1974. Studies on the base composition of DNA, DNA–DNA reassociation studies and comparative biochemical and physiological studies did indeed demonstrate the phylogenetic coherence of some morphologically defined genera. However, major discrepancies were already noticed at the level of families and orders. The foreword to the eighth edition stated the inability of the present data set to deduce a hierarchic system of bacteria, as the majority of the key properties may have been the result of convergent evolution. Thus, the presentation of a fully developed system was abolished and taxa were clustered in 17 groups, according to the morphology and physiology of their members. In a few cases only were genera arranged into orders and families, only a few of which have survived the close scrutiny of phylogenetic analyses in recent years.

With a considerable delay of several years, several other important milestones in microbial systematics were accomplished, with their technical origins arising from ideas expressed in other disciplines. The most outstanding was the discovery of DNA, the full importance of which was recognized when the structure became available (Watson and Crick 1953) and appropriate methods for its analysis and manipulation were introduced. A second milestone was the development of computers in the 1950s and their use in handling phenetic and molecular data. A third milestone with direct implications on the future of systematics remained unnoticed by microbial systematists, who were involved in the daily struggle of identification and species description. Moreover, at the time of publication, microbiologists were not in a position to fully acknowledge that the ideas of Zuckerkandl and Pauling (1962, 1965) could be applied to bacteria. These visionaries postulated that "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 the elements that have to be effected to bring about a significant change." They not only defined sematophoric molecules, i. e. genes and their transcripts [DNA (primary), mRNA (secondary), proteins (tertiary semantides)], as 'sense-carrying' units, i. e. the blueprint of an organisms' evolutionary history, but they also predicted that parts of the phylogenetic tree could be defined in terms of episemantic molecules, i. e. molecules that are synthesized under the control of proteins. Due to methodological constraints, the tertiary semantides (i. e. proteins) were the first molecules to be analysed, either by direct sequence analysis (e. g. cytochrome C, fibrinopeptides,

ferrodoxins), or by immunological approaches such as immunodiffusion and microcomplement fixation. Though protein sequencing lost its significance with the introduction of rapid sequencing techniques for DNA, its results already pointed towards the discrepancies between the outline bacterial classification schemes and the natural relationships of bacteria (Schwartz et al. 1975; Dickerson 1980; Ambler et al. 1987). Analysis of DNA and RNA was delayed for more than a decade by the lack of routine sequencing methods. In order to obtain at least general insights into the nucleotide similarities of primary and secondary semanides, hybridization techniques were introduced. DNA–DNA reassociation studies were the first to cluster organisms according to phylogenetic relationships and they played a decisive role in the definition of the taxon 'species' (Brenner et al. 1969; Palleroni et al. 1971; Johnson 1973; Grimont 1981); and still today it is considered the 'gold standard' for the delineation of species (see Chap. 2). The recommendation to use a 70% or so DNA–DNA reassociation value for defining species originated mainly from the experience made with numerous strains of enterobacterial species (Steigerwalt et al. 1976). Transferring the situation defined for a phylogenetically very shallow group of mainly eukaryote-associated organisms to all prokaryotes – which are the recent manifestations of different modes and times at which organisms evolve – is a dramatic underestimation of their phylogenetic status. But then one has to remember that the taxon thus delineated is an artificial construct, helpful in structuring the bacterial world at the level of species in a coherent way. Nevertheless, in times of whole genome sequencing approaches, the laborious DNA–DNA hybridization methodology seems to be out of date. As the number, identity and degree of conservatism of genes involved in the hybridization process remain unknown (even today), the ancestral genotype of a species cannot be determined. The obvious disadvantages (Stackebrandt et al. 2002), are more than compensated by the involvement of the majority of genes in the reassociation process. More recent attempts, concentrating on only a single or a few molecular markers, are significantly more biased, as one can only speculate whether these genes represent the evolutionary status of the complete genome. The artificial threshold value of about 70% reassociation (reflecting > 96% genome similarity; Schleifer and Stackebrandt 1983) indeed correlate well with those phenotypic properties of strains which are of general taxonomic value for the description of a species. DNA–DNA reassociation experiments confirmed the notion that a bacterial 'species' is not a genomically coherent entity but represents a population of highly related strains.

The recognition that translation mechanisms are highly conserved between species has opened a superior method of bacterial systematics (Dubnau et al. 1965). When methodologies to sequence RNA were not initially available, hybridization regimes between the rRNA gene and the gene prod-

uct were applied to groups of organisms known to be taxonomic dumping grounds, e.g. pseudomonads (Palleroni et al. 1973; De Smedt and De Ley 1977; De Vos and de Ley 1983) and clostridia (Johnson and Francis 1975). These bacteria lacked the chemical diversity found in many Gram-positive bacteria, such as actinobacteria and lactic acid bacteria. Within a few years, microbiologists noticed the phylogenetic unrelatedness of groups of bacteria which, based on morphological and metabolic grounds, has constituted well established genera for more than 80 years. For the first time in the history of microbiology, the failure of superficial properties to circumscribe natural relatedness became obvious. Results of DNA–rRNA reassociation studies unravelled deeper phylogenetic relationships than those obtained by DNA–DNA reassociation. While this finding alone was extremely satisfying, the restrictions of rRNA hybridization methods became apparent with the publication of the first results of rRNA oligonucleotide catalogue comparisons (Woese and Fox 1977). Phylogenetic analyses of catalogues, though limited at that time because of the lack of methods to sequence complete genes, were able to include any strain into a single dendrogram of relationship, including archaebacteria, eubacteria and eukaryotes.

Comparative studies highlighted the usefulness of the accumulated database of episemantic markers used in chemosystematics (chemotaxonomy, chemical taxonomy). Chemotaxonomy evolved as the by-product of biochemical and chemical work and developed in parallel with the introduction of chromatographic and other analytical methods. Without the support of peptidoglycan structure (Weidel and Pelzer 1964; Schleifer and Kandler 1967), isoprenoid quinones (Collins et al. 1977) and the lipid and fatty acid composition of cells (Lechevalier and Lechevalier 1970; Langworthy 1977; Lechevalier et al. 1977; Kates 1978), the acceptance of the phylogenetic uniqueness of many archaeal and bacterial taxa would have been delayed considerably. The determination of chemical markers, introduced during the 1950s, not only circumscribe the present state of a cell's chemical composition but indeed provide valuable properties used to critically analyse the phylogenetic clustering of groups of organisms at the genus level. This facet of systematics has not lost any of its attraction and, without its discriminatory power, many phylogenetically closely related species groups would not have been described as genera. Types and variation of peptidoglycan isoprenoid quinones, fatty acids, base composition of DNA, polar lipids, polyamines, pigments or mycolic acids and more are routinely used within the polyphasic approach to systematics. While single markers are rarely indicative of the phylogenetic coherence of a higher taxon, novel combinations of two or more of these properties are often highly correlated with the phylogenetic uniqueness of the respective organisms (Stackebrandt and Schumann 2000).

This period also witnessed the development of a third mainstream in bacterial systematics, numerical phenetic taxonomy (NT), introduced in the 1950s. Lasting for about 25 years, its influence on the recognition of coherence and lack thereof should not be underestimated, even if this approach is hardly in use anymore. This method is tightly connected with the development of algorithms, computers and the taxonomic concept that the reliability of the description of a taxon is improved by the provision of a comprehensive set of phenetic characters. Electronic computerization of microbiological data was first introduced by Sneath (1957) in order to handle the enormous amount of phenetic data collected during a taxonomic study of the genus *Chromobacterium*. This development ran in parallel with the work of Sokal and Michener (1958), who used an electric device to generate a classification of a eukaryotic taxon. Sokal and Sneath (1963) joined forces to develop the "*Principles of numerical taxonomy*" and they were among the first to develop and apply clustering and probabilistic distance coefficients in numerical taxonomy, e. g. single and average-linkage clustering, Jaccard's coefficient, scaling of multistate characters, parallelism and convergence, and equal weighting. Many of these algorithms and their modifications are still in use today in cluster analysis of the electrophoretic patterns of DNA and RNA digests (Riboprint, ARDRA, DGGE, AFLP, RFLP, etc.), protein patterns, fatty acid methyl ester patterns and the evaluation of ecological parameters, to name a few. Numerical analysis pointed out many inconsistencies in the classification at that time, leading to many taxonomic rearrangements. However, in the absence of a phylogenetic background, the resolving power of numerical analyses was overestimated, as the significance of individual properties remained unknown. Superficial characters were treated the same way as properties which indeed reflected the genealogy of the study object. With the advent of chemotaxonomy and a revised species definition, the numerical analysis lost its influence and present-day studies mainly target intraspecific variations.

## 1.6 Yet Another Exciting Time: Unravelling the Genealogy(ies) of Cultured and As-Yet Uncultured Prokaryotes

Being trained as a bacterial systematist during the late 1960s, I applied some of the key techniques of that period (determination of metabolic pathways, peptidoglycan structure and base composition of DNA, DNA–DNA reassociation studies) and witnessed the emergence of the breathtaking and historical development of molecular systematics. This era began, almost unnoticed by taxonomists, with a paper by Uchida et al (1974). 16S rRNA

oligonucleotide cataloguing changed the perception with which systematics was going to be executed in the future.

Though only a few species were investigated by this time-demanding technique before the advent of reverse transcriptase sequencing and, a few years later, PCR-based cycle sequencing, accelerating the analyses, the new approach of aligning systematics to the emerging tree of conservative macromolecules must be considered a powerful kickstart (Woese et al. 1985). While the power of these methods for the determination of intraspecific relationships was certainly overemphasized in the 1980s (which somehow discredited this method for some systematists), ribosomal RNA/rRNA gene sequencing remained the key to affiliate novel organisms to genera and to infer their phylogenetic novelty. After this short period of hesitation and disbelief that sequencing analysis of macromolecules would indeed benefit bacterial systematics other than as the provision of just another fragment in the general description of species, it was accepted so rapidly that, 20 years after its introduction, it is considered a routine and long-established method. The broad outline of higher taxa (Gibbons and Murray 1978) was not corrected but replaced. In 2001, the new editors of Bergey's Manual fully adopted the new system (Garrity et al. 2001, 2002) and are now, together with a new generation of systematists, actively involved in shaping the hierarchic structure of prokaryotes (Stackebrandt et al. 1997). The acceptance of molecular sequences to guide systematics has been facilitated by the availability of an enormous amount of phenetic data accumulated over the past decades. When superimposed on the phylogenetic clusters, many chemotaxonomic data gained new taxonomic significance as they were often the main criteria to delineate higher taxa. The fear that species and genera were described chiefly on the basis of 16S rDNA gene sequences (Palleroni 2003) is unjustified.

There were voices that considered the introduction of gene sequence comparison unfortunate, as it appeared the only method upon which phylogenetic relationships were based. However, soon after the analyses of 16S ribosomal RNA sequences began to influence systematics, scientists began wondering whether changes in nucleotide sequence of this single molecule solely represents its own evolution, rather than the evolution of a large portion of the genome, reflecting the genealogy of the host. However similar sequence analysis of the genes coding for 23S rDNA, elongation factors, ATPase, chaperons and many others demonstrated that the majority of the so-called housekeeping genes or core genes provided tree topologies that by and large matched that of the 16S rDNA tree (Gupta 1998, 2000), thus confirming the description of kingdoms and phyla in the two prokaryotic domains [the interested reader is referred to the scientific debate between Mayr (1998) and Carl Woese (1998) about "differing views as to what biology is and will be"]. Today, public databases contain sequences of hundreds

of fully sequenced genomes, offering a rich playground for studies on the micro- and macroevolution of genes and, crucial for systematists, providing information on the extent of horizontal gene transfer (Lawrence 2002). Like in previous times when taxonomists tried to avoid the use of genetically instable and plasmid-coded phenetic properties, the taxonomist of today will be prudent not to derive a phylogenetic framework on the basis of genes subjected to lateral gene transfer among members of the taxon concerned.

The discussion of the nature of the taxon 'species' has been provoked by the application of molecular tools, especially at the level of the species concept, i. e. the hypothetical basis of speciation. As a result of intensive multilocus enzyme electrophoreses (Selander et al. 1994), RAPDs (Istock et al. 1996) and multilocus sequence typing of housekeeping genes (Maiden et al. 1998), new ideas about speciation mechanisms have been expressed and mechanisms identified that contribute to the evolution of the genome. Some organisms are subjected to reticulate events or panmixis (Maynart-Smith et al. 1993, Istock et al. 1996) in which clonal relationships, due to mutational events and vertically transmitted accessoric genetic elements, are perturbated by horizontal genetic transfer, e. g. conjugation, phage transduction DNA transformation (Achtman 1998). Others, mostly endosymbionts and obligate pathogenic organisms, are mainly clonal because horizontal gene transfer appears to be a rare event. In an attempt to come to a biological species definition for bacteria, it has been proposed (Dykhuizen and Green 1991) to consider the following observations: (1) phylogenetic trees from different genes from members of a single species should be different and (2) phylogenetic trees from different genes from members of different species should be the same. What had been a challenge at the time when this definition was proposed has now become possible through high-throughput sequencing automation, allowing the analysis of five genes with a total of about 3,500 base pairs for each of about 2,000 strains of a single species. The intraspecific diversity recognizes centres of evolution leading to recognizable entities, named ecotypes (Cohan 2001, 2002). Their possible role in a redefined species description has been discussed in detail (Palys et al. 2000; Gevers et al. 2005).

The following chapters will highlight some of the key approaches used in microbial systematics and molecular ecology. These microbiological areas are somewhat related, as they originally evolved from the analysis of the same molecule, the 16S rRNA. Both disciplines will mutually benefit from progress made in either field. One set of approaches is based on the finding of taxon-specific signature sequences in the rapidly increasing database of rRNA catalogues and complete sequences from the late 1980s on (Brosius et al. 1987). Molecular probes are used in clinical diagnostic and most impressively in in-situ hybridization studies in ecology. The

database of more than 120,000 16S rDNA gene sequences results from the recognition of the unexplored microbial diversity that reinforces earlier notions about the inability of cultured organisms to represent diversity. The listing of exiting new developments in systematics will however not be complete without a mention of rapid DNA profiling methods, used routinely not only in bacterial identification and in the description of new taxa, but also in the assessment of the molecular diversity of populations in their natural environment. The handling and identification of the relatively small number of only about 6,000 validly described species (with an annual increase of 230 – 300 species) is manageable, but the situation may soon get out of hand once novel and innovative isolation methods have been devised. A prerequisite for the handling of a substantial increase in species numbers is the design of dynamic automated identification systems that access curated databases of molecular and non-molecular data, combined with advanced computational strategies and knowledge management. The search for novel organisms should run in parallel with the investment in reproducible authentification methods with a high resolving power, such as those based on mass spectrometry (MS) and mainly in use for clinical isolates and select agents (e. g. matrix adsorbed laser deionization/ionization time-of-flight MS, Fourier-transformed infrared MS).

These times are so rich in new techniques, new technical support, new insights and fresh ideas that not only students find it difficult to maintain an overview about advances in the field of microbial systematics and diversity. Most obviously, it is a good time to be part of this exciting avenue. I am confident that the next generation of microbiologists will benefit from the scientific progress achieved at the turn of the twenty-first century. It is the hope of the authors of this book that newcomers to the field of microbial diversity may have the enthusiasm to equip themselves with a sufficiently qualified background and experience to carry on the exploration of the microbial world. To quote somebody who knew what it is all about: "The best way to have a good idea is to have a lot of ideas" (Linus Pauling, "*The nature of the chemical bond*")

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