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Inclusions in Prokaryotes

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Inclusions in Prokaryotes

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Preface to the Series

Microbiology is a still rapidly growing field with impact to a variety of basic and applied areas. The increase in knowledge about microbial physiology, cell structure, biotechnological capabilities and other aspects of microorganisms still continues to increase dramatically despite all the tremendous and fascinating achievements made already in the past. The speed by which new knowledge is revealed seems even to increase due to the enormous amount of data unravelled by the many microbial genome projects, by new methods and sophisticated techniques recently developed and by exploring the diversity of microorganisms. The new Springer book series ***Microbiology Monographs*** aims to cover hot topics and fields in which considerable progress was made recently. The series will cover topics like inclusions in prokaryotes, predatory prokaryotes, magnetoreception and magnetosomes in bacteria, palaeomicrobiology and past infections, uncultivable microorganisms, microbial endosymbionts, bacterial resistance, extremophilic microorganisms, analyses of genome sequences and structures, microorganisms as cell factories for chemicals and fuels, metabolic engineering, gene transfer and expression systems or distinct physiological groups of bacteria to mention only a few topics that are in preparation.

Each volume will focus on a selected microbiological topic. A well known expert in this field, who was invited as volume editor, will carefully compile a team of expert scientists that will contribute about 10 to 15 comprehensive review articles. These articles will cover all relevant aspects of the respective volume and will be carefully refereed. Each year several volumes of *Microbiology Monographs* shall be published. It will be an 'open' book series, and suggestions from the scientific community for specific topics are highly welcome.

Publishing of this new book series would certainly not have been possible without the expertise and engagement of the volume editors and the authors who contributed to a particular volume. I am very grateful to them that they committed their valuable time, knowledge and enthusiasm to this project.

It has also to be acknowledged that the publisher Springer recognized the demand for this book series, and that the volume could be produced in such high technical quality including colour photographs. Furthermore, publishing all volumes online in addition to the print edition releasing the individual

chapters *Online First* before publication of the book, makes the contents of the chapter much more rapidly available to the scientific community. I have in particular to thank Christina Eckey, Jutta Lindenborn and Dieter Czeschlik for their initiative to start this new book series and for their very helpful suggestions and constructive ideas.

I am very much convinced that the readers of *Microbiology Monographs* will recognize the joint efforts of all contributors, that they will enjoy reading the books and that they will profit from the compiled knowledge.

Münster, Germany Alexander Steinbüchel

Preface

In prior coverage of prokaryote inclusions (Pankratz and Bowen 1963; Lang 1968; Shively 1974; Allen 1984; Shively et al. 1988; Jensen 1993; Shively et al. 1998) all of the authors appeared to use a broad definition of "inclusion", i.e., a discrete, particulate, separate body in the cytoplasm of the prokaryotic cell. As we carefully searched the literature and began considering topics for this volume, it became obvious that in order to stay within an appropriate length, a more restrictive definition of "inclusion" needed to be invoked. This necessity was brought about primarily by two factors, the expansion of the number of "discrete, particulate, separate bodies" described, as well as the apparent wealth of material published on both the earlier and more recently discovered structures in prokaryotes (see material in this Volume as well as Volume 2 of the series *Microbiology Monographs*).

Thus, for this volume, we redefined "inclusion" as "a discrete body resulting from synthesis of a metabolic product/reserve." Further explanation of the definition can be found in Chapter 1 and will become clearer as the reader examines ensuing chapters. Using this new definition, we selected eight topics for chapters: sulfur globules, polyphosphate granules and acidicalcisomes, glycogen inclusions, polyhydroxyalkanoate granules, wax ester and triacylglycerol inclusions, cyanophycin inclusions, insecticidal protein crystals, and protein inclusion bodies of recombinant bacteria. Many other, less common inclusions have been reported in a variety of prokaryotes, generally with only limited to moderate research relating to occurrence, structure and function (Pankratz and Bowen 1963; Jensen and Bowen 1970; Shively 1974; Allen 1984; Jensen 1993). It was deemed appropriate to call attention to a few of these inclusions by adding them as cameo chapters in Part 2 of this volume.

Four of our "primary" inclusions, sulfur, polyphosphate, glycogen, and cyanophycin, were discovered more than 100 years ago. Insecticidal protein crystals were seen in 1915, but their identity was not confirmed until 1953. Although lipid bodies were discovered in 1893, it was not until 1926, 1976, and 1985 that poly(3-hydroxybutyrate), triacylglycerols, and wax esters were firmly established as lipoidal inclusions, respectively. Gene cloning brought about the eventual expression of recombinant proteins, and the first of many inclusions was observed in 1982. Brief descriptions of the inclusions along with the documentation of their discoveries are presented in Chapter 1. Since

most of the inclusions have been known for such a long period of time many readers are likely to question why we need a review. Has there not already been adequate coverage? To our knowledge, a comprehensive review of all of these inclusions together, in one volume, has never been accomplished. The closest to achieving this would be that of Shively et al. (1974). Having them all together provides the opportunity to compare the structures and the research approaches used in their characterization. Also, as noted above, there is still a sizable research interest in the inclusions. This became even more obvious to us when Chapter 1 was developed. Thirty percent of the references used to document inclusion descriptions and discovery were published in or after 1990. The continuing interest in the inclusions is further substantiated if one examines the reference lists of the eight submitted manuscripts; references published in or after 1990 varied from 45–92% (average = 69%), in or after 2000 from 11–41% (average = 31%). What are the driving forces behind this continuing interest? First, the complete characterization of an inclusion including related areas, e.g. biosynthesis and reutilization of the accumulated material(s), is an arduous undertaking. In many cases it is still incomplete. Second, new discoveries relating to an inclusion are not uncommon. As an example, poly(3-hydroybutyrate) was firmly established as a polyhydroxyalkonate (PHA) in 1926; hydroxyvalerate was reported as a constituent in 1974; there are now approximately 150 PHAs, and even a new class of biopolymers related to PHAs, the polythioesters, was recently discovered. There are numerous other examples. Finally, new approaches relating to the potential use of the biopolymers have been developed for many of the biopolymers as well as for their modification and biosynthesis. We look forward to viewing the results of the research on prokaryotic inclusions for many years to come.

We are very grateful to the authors who contributed excellent chapters to this volume of *Microbiology Monographs*. Their expertise and enthusiasm, which they devoted to their chapters, is highly appreciated. They did this in a timely manner in spite of many other obligations and duties. Without the engagement of the authors such a book could never have been prepared. We would also like to thank the publisher Springer for publishing this book. Special thanks are due to Christina Eckey and Jutta Lindenborn who gave us constant support and many good ideas.

Clemson and Münster, January 2006

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Alexander Steinbüchel

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Prokaryote Inclusions: Descriptions and Discoveries

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Abstract Spherical inclusions were observed in a "colorless egg-shaped alga" in 1786, but were not identified as sulfur until 1875. The discovery of additional inclusions required improvements to the light microscope. Thus, cyanophycin, polyphosphate, lipid, and glycogen inclusions were not observed until late in the nineteenth century and insecticidal protein crystals in 1915. Polyhydroxybutyrate was identified as a lipid inclusion in 1926, but the confirmed identity of the others as well as the discovery of new inclusions required the introduction of the electron microscope as well as other technological advances. Glycogen, polyphosphate, and insecticidal inclusions were confirmed in 1944, 1947, and 1953, respectively. Cyanophycin was not identified until 1971. Although lipid bodies were seen in 1893, triacylglycerols were not identified as accumulated materials in prokaryotes until 1976, and wax ester accumulation, discovered in 1960, was not confirmed as an inclusion until 1985. Recombinant protein inclusions, requiring the development of gene cloning technology, were not identified until 1982. The acidicalcisome, a polyphosphate accumulation in eukaryotes, was demonstrated to be present in a prokaryote in 2003. Characterization of the known inclusions, their structure, production and utilization, as well as the discovery of additional inclusions continue today.

1 Introduction

There are numerous structures reported in the prokaryotic literature that are called inclusions. As different intracellular structures were considered for this

volume, with one goal being that of keeping the volume to a reasonable length, it became necessary to more carefully define and limit what would be referred to as an inclusion. As a basic premise an inclusion was considered to be a localized accumulation of some material inside the cell wall; stipulating cell wall allows for those inclusions that reside outside the cytoplasmic membrane. This broad statement essentially described a variety of accumulated materials, many of which might not be most appropriately referred to as inclusions. Thus, it was necessary to further explain "inclusion" as a granule, a globule, a crystal, or just an amorphous mass that might or might not have a surrounding limiting barrier. The material may be principally inorganic or organic. It might be an element or a compound(s), a peptide or a protein, a carbohydrate or some type of a fatty material. It may function essentially as a storehouse of an element, of carbon and/or energy, nitrogen, or of the material itself. As an example of the latter, some organisms produce a toxic protein that kills a host, thereby providing a nutrient source. The accumulation is, in most instances, the result of nutrient imbalance during the growth/life cycle, but in other cases it is an integral part of the cell's metabolism, the result of genetic manipulation, or possibly an attempt by the cell to reduce the material's toxicity.

The "primary" inclusions to be presented in this monograph volume are sulfur globules, polyphosphate granules and acidicalcisomes, glycogen inclusions, polyhydroxyalkanoate granules, wax ester and triacylglycerol inclusions, cyanophycin inclusions, insecticidal protein crystals, and protein inclusion bodies of recombinant bacteria. This introductory chapter gives a brief description and the historical background for each inclusion. Subsequent chapters (this volume) authored by Dahl and Prange, Docampo, Preiss, Pötter and Steinbüchel, Wältermann and Steinbüchel, Obst and Steinbüchel, Federici et al., and Neubauer et al. provide in-depth coverage.

There are a considerable number of references in the literature that document unusual/uncommon inclusions in a variety of prokaryotes without additional research relating to their occurrence, structure, and function (Pankratz and Bowen 1963; Jensen and Bowen 1970; Shively 1974; Allen 1984; Jensen 1993). The scope of this volume does not allow coverage of the vast majority of these inclusions; however, in a few instances it was deemed appropriate to include some noteworthy inclusions that lack extensive research attention; see the cameo chapters in this volume authored by Schulz-Vogt, Gray, Langley, Park et al., and Sanchez-Amat. It is hoped that these cameos will call attention to these inclusions and stimulate research.

2 Technological Milestones

Although the instrumentation, methodologies, and technologies used in a variety of disciplines were/are essential, microscopy has played a cen-

tral role in the study of prokaryotes, and even more so in the elucidation of their inclusions (Fig. 1). Faber coined the term "microscope" in 1625 (Beck 2000). However, it was another 40 years (1665) before Hooke published the first drawing of microorganisms (molds) in *Micrographia* (Beck 2000). In 1676 Antonie van Leeuwenhoek (1677) transmitted, to the publisher, observations, made with a simple microscope of his own construction, on little animals (microorganisms) seen in rain, well-water, seawater, snow-water, and pepper-water infusion (Brock 1999). In a later communication van Leeuwenhoek (1684) described his microscopical observations, with drawings, about the animals in the scurf of teeth; these were the first published drawings of bacteria (Brock 1999). Although van Leeuwenhoek's findings were confirmed and extended little was done until the nineteenth century to make improvements to the light microscope and to understand the nature of microbial life.

Observing an inclusion depended, or for that matter still depends, upon a variety of factors. The earliest discoveries not only required the use of the simple light microscope and potentially on techniques related thereto, but also on the occurrence, size, and nature of the inclusion as well as an appropriate organism (Fig. 1). It is worthy to note that prokaryotes vary greatly in size, and that the majority of the largest prokaryotes are sulfur chemolithotrophs and cyanobacteria. Small inclusions could not be resolved by the light microscope and inclusions lacking optical properties different from those of the surrounding cytoplasm could not be seen unless preferentially stained. Seeing inclusions of uncommon occurrence depended on the serendipitous selection of the "right" organism for study. Those inclusions not seen using the "primitive" microscopes required the development of more refined light microscopes and vision-augmenting technology or the development of the electron microscope and related technologies.

3
Inclusions: Descriptions and Discoveries

Inclusions existing either naked or enveloped are inorganic as with sulfur and polyphosphate or organic as for carbohydrate, lipid, and peptide/protein. Inclusion discovery began in 1786 and their characterization as well as new discoveries continue today (Fig. 1).

3.1
Sulfur Globules

Sulfur globules, now known to be as large as 1.0 μm in diameter, were first observed by Müller in 1786 as spherical inclusions in, what he referred to as, a "colorless egg-shaped alga" (see Dahl et al. 2002). A number of sulfur bacte-

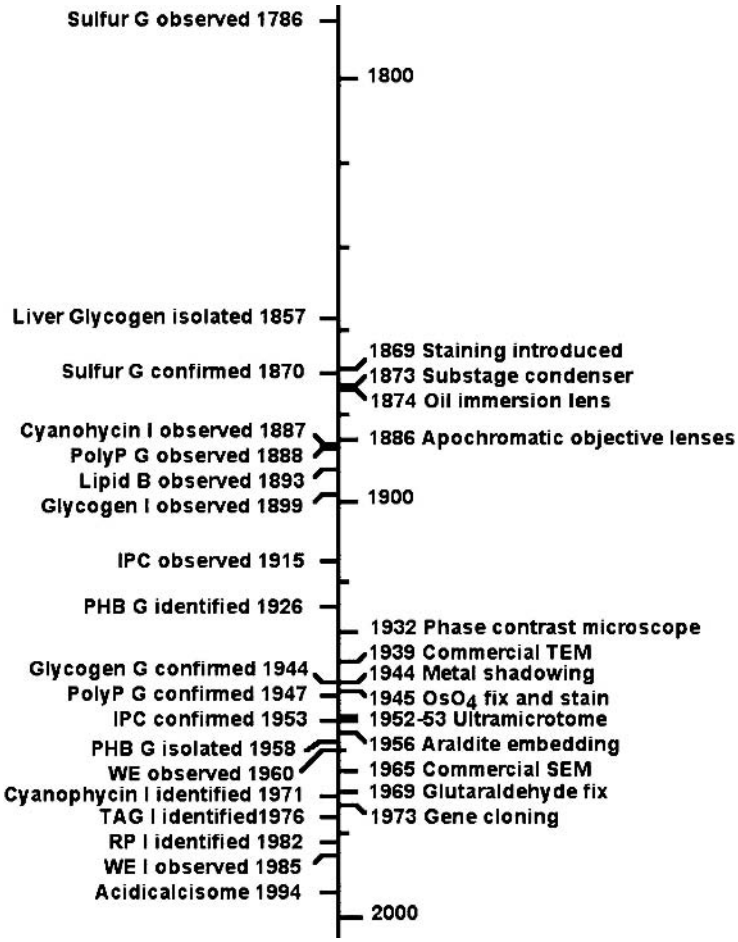

Fig. 1 Timeline of inclusion discoveries (*left*) and selected technological advances (*right*). *Sulfur G* sulfur globules, *cyanophycin I* cyanophycin inclusions, *PolyP G* polyphosphate granules, *lipid B* lipid bodies, *glycogen I* glycogen inclusions, *IPC* insecticidal protein crystals, *PHB G* polyhydroxybutyrate granules, *WE* wax esters, *TAG I* triacylglycerol inclusions, *RP I* recombinant protein inclusions, *WE I* wax ester inclusion, *TEM* transmission electron microscopy, *SEM* scanning electron microscopy. Technological advance dates taken from Alberts et al. (2002) and Beck (2000)

ria and their inclusions were described during the early and middle parts of the nineteenth century, but the inclusion material was not identified as elemental sulfur until it was shown to be extractable by carbon disulfide (Cohn 1875; Dahl et al. 2002). Thus, it took nearly 100 years before Müller's "alga" was recognized as a large sulfur bacterium with sulfur inclusions (Warming 1875). Winogradsky (1887) proposed calling the organisms that accumulated sulfur inside the cells "sulfur bacteria" and provided detailed descriptions of

the organisms and their "sulfur globules". Early research on sulfur globule structure reported the possible presence of a surrounding membrane on the sulfur globules of *Thiovulvum majus* (Faure-Fremiet and Rouiller 1958) and *Chromatium okenii* (Kran et al. 1963). In 1971, Nicolson and Schmidt (1971) demonstrated the presence of a unique proteinaceous monolayer membrane on isolated sulfur globules of *Chromatium vinosum*. On the basis of a variety of reports with a number of different organisms it seems likely that most, if not all, of the sulfur globules of chemotrophic sulfur-oxidizing bacteria are enclosed in a protein envelope (Shively 1974; Shively et al. 1988; Brune 1995; Pattaragulwanit et al. 1998; Dahl 1999; Dahl et al. 2002). X-ray absorption near-edge spectroscopy of intact cells provided evidence that the chemical structure of sulfur globule-sulfur varies among different metabolic groups (Prange et al. 2002). At least three different sulfur species were detected: sulfur rings, (cyclooctasulfur), polythionates and sulfur chains, probably present as organylsulfanes. A chapter by Dahl and Prange (this volume) provides in-depth coverage of sulfur globules.

3.2 Polyphosphate and Acidocalcisome Granules

Paul Ernst in 1888 and Victor Babes in 1889 independently observed granules in bacteria stained with methylene blue or toluidine blue (Beck 2000). Thus, in the literature one sees references to Babes–Ernst granules. Since the granules stained red, not blue, Babes (1895) called the granules seen in a variety of bacteria "metachromatic corpuscles", giving rise to the name metachromatic granules. Grimme (1902) called the granules of *Spirillum volutans* "volutankugeln" and reported their solubility in hot water. Realizing the chemical nature of the granule material, Meyer (1904) established tests for what he referred to as volutin. On the basis of these tests the granules were detected in a wide variety of both prokaryotes and eukaryotes (Shively 1974; Shively et al. 1988; Allen 1984; Docampo et al. 2005). However, it was not until nearly 50 years later that the granule material was clearly identified as polyphosphate (Wiame 1947; Ebel 1952). Although there had been reports suggesting the presence of a membrane (Shively 1974) it was generally accepted until recently that the granules were devoid of a limiting membrane (Harold 1966). The granules of several unicellular eukaryotes have now been shown to possess a functional membrane with pumps and exchangers, to be acidic in nature, and to be rich in, for example, calcium and polyphosphate (Vercesi et al. 1994; Docampo et al. 1995, 2005). Considering these characteristics the granules were renamed "acidocalcisomes". The polyphosphate granules of both *Agrobacterium tumefaciens* and *Rhodospirillum rubrum* have now been identified as acidocalcisomes (Seufferheld et al. 2003, 2004). A chapter by Docampo (this volume) provides in-depth coverage of polyphosphate granules and acidocalcisomes.

3.3 Bacterial Glycogen Inclusions

Whether it is glycogen, amylopectin, or starch, polyglucose is a common storage material in both eukaryotes and prokaryotes. Claude Bernard (1857) purified from liver tissue a substance he named glycogen. In 1899, Meyer stained granules with an iodine solution in a species of *Bacillus* and postulated the material to be glycogen (Beck 2000). In the early 1900s, on the basis of the "typical" brownish coloration of cells stained with iodine, several researchers surmised that cyanophycean storage material was either glycogen or glycoprotein (Lang 1968). Chargaff and Moore (1944) isolated a polyglucosan from avian tubercle bacilli with the properties of glycogen and during the next 25 years numerous reports documented the presence of glycogen-like polymers in both bacteria and cyanobacteria (Fredrick 1951; Dawes and Ribbons 1964; Lang 1968; Preiss 1969; Cheng et al. 1973; Dawes and Senior 1973; Wolk 1973). During this same period other researchers were demonstrating that the glycogen was being deposited as granules in the cytoplasm of bacteria and in the interlamellar (thylakoid) region of cyanobacterial cells (Gavard and Milhaud 1952; Barry 1953; Dagley and Johnson 1953; Niklowitz and Drews 1957; Cedergren and Holme 1959; Ris and Singh 1961; Pankratz and Bowen 1963; Fuhs 1963; Giesy 1964; Lang 1968; Wolk 1973; Shively 1974; Shively et al. 1988; Jensen 1993). The spherical uneven (rough) appearing granules vary from 20 to 100 nm in diameter. In some cases the granules of cyanobacteria appear as rods. The rods of *Nostoc muscorum*, isolated by Chao and Bowen (1971), are 30 nm × 65 nm; those of *Oscillatoria rubescens* are sometimes as long as 300 nm and are composed of 70 nm disks with a central pore (Jost 1965). In most instances the polyglucose polymers accumulate as non-membrane-bound inclusions; however, in several species of *Clostridium* the granules, 160–300 nm in diameter, are bounded by a single-layered, nonunit membrane coat (Laishley 1973; Shively 1974). Although the description of granules in a variety of organisms has continued, the primary emphasis has been on the enzymology, regulation, and molecular biology of the biosynthesis and degradation of the polymer (Ballicora et al. 2003; Bejar et al. 2004). A chapter by Preiss (this volume) provides in-depth coverage of glycogen inclusions.

3.4 Lipoid Inclusions

Fat bodies were observed in cyanobacteria by Palla in 1893 (Wolk 1973) and in bacteria in 1899 by Meyer using Sudan III as a stain (Beck 2000). Nearly all prokaryotes are capable of producing and sequestering large quantities of at least one of the following lipophilic compounds into cytoplasmic

inclusion bodies: poly(β-hydroxybutyrate (PHB) or other polyhydroxyalkanoates (PHAs), triacylglycerols (TAGs), and wax esters (WEs) (Wältermann and Steinbüchel 2005). All of these compounds are produced as storehouses of carbon and energy in response to periods of stress when carbon is in excess and another nutrient, for example, nitrogen, is limiting.

During the 30 plus years following the discovery of PHB (see later), numerous prokaryotes were reported to possess lipid inclusions. In many instances the inclusion lipid was identified as PHB. In other cases the lipid was not characterized, but was often hypothesized to be PHB. As analysis methods became more sophisticated, researchers began to more carefully assess the nature of stored lipids (see later).

3.4.1 Polyhydroxyalkonate Granules

Although PHAs have not been reported in eukaryotes, many prokaryotes are capable of their synthesis (Wältermann and Steinbüchel 2005). PHB, the most common PHA, was first reported by Lemoigne (1926) in *Bacillus megaterium*, and during the next 20 years Lemoigne reported on various aspects of PHB synthesis in several bacteria (Dawes and Senior 1973). In 1943 Lemoigne was first to observe the direct correlation between PHB synthesis and granule formation in members of the genus *Bacillus* (Dawes and Senior 1973). Macrae and Wilkinson (1958) made the important observation that PHB increased in *B. megaterium* as the ratio of carbon to nitrogen increased in the growth medium, indicating that the accumulation occurred in response to a growth imbalance. Williamson and Wilkinson (1958) isolated the Sudan Black staining granules from *B. megaterium* and demonstrated the material to be PHB. This finding was later confirmed and extended by Merrick and Doudoroff (1961). PHB granules isolated from *B. megaterium* were shown to be spherical, to be in the range 200–700 nm in diameter, and to contain several thousand PHB molecules (Ellar et al. 1968). The granules are surrounded by a nonunit membrane or shell 2–4 nm thick and composed of protein and phospholipids (Shively 1974; Dawes and Senior 1973). Numerous researchers demonstrated that structural proteins as well as the enzymes for polymerization and depolymerization were an integral part of the granule barrier (Shively 1974; Dawes and Senior 1973). Nearly 50 years after the discovery of PHB, Wallen and Rohwedder (1974) reported that heteropolymers in chloroform extracts of activated sewage sludge were composed of both 3-hydroxybutyrate and 3-hydroxyvalerate as major constituents. Since this initial discovery of a new PHA, approximately 150 different PHA constituents have now been detected (Anderson and Dawes 1990; Steinbüchel and Valentin 1995; Pötter and Steinbüchel 2005). A chapter by Pötter and Steinbüchel (this volume) provides in-depth coverage of PHA granules.

3.4.2 Wax Ester Inclusions

Storage WEs, oxoesters of primary long-chain fatty acids and alcohols, are known to occur in only one eukaryote, jojoba (Yermanos 1975), and are found in only a limited number of prokaryotes (Wältermann and Steinbüchel 2005). Stewart and colleagues (Stewart et al. 1959; Stewart and Kallio 1959) reported the production of WEs by a gram-negative soil isolate grown on alkanes. The WEs were not accumulated within the cell however, but were excreted into the medium. The next year Raymond and Davis (1960) reported alkane utilization and lipid production by *Nocardia*. Massive amounts of intracellular lipids were produced with as much as 70% of the cell material formed under optimal conditions extracted with fat solvents. The accumulated material consisted of 60% TAGs and 40% WEs. Gallagher (1971) was first to communicate the production of waxes by *Acinetobacter* and some years later Scott and Finnerty (1976) described hydrocarbon inclusions in *Acinetobacter* that were later identified as WE inclusions (Singer et al. 1985). In *Acinetobacter* species, the most studied WE-synthesizing organism, WEs have accumulated to 25% of the cellular dry weight (Fixter et al. 1986, Wältermann et al. 2005). Chain lengths of 32–48 carbon atoms have been reported, with C_{32} and C_{34} being most common. Saturated C_{16} and C_{18} fatty acid and alcohol are the most common constituents, but monoenic acids do occur (Gallagher 1971; Fixter et al. 1986). Three different WE inclusions have been reported in *Acinetobacter*: (1) Spherical inclusions with average diameters of 200 nm were seen in hexadecane-grown cells of *Acinetobacter calcoaceticus* HO1-N or carbohydrate-grown cells of *Acinetobacter calcoaceticus* ADP1 (Scott and Finnerty 1976; Stöveken et al. 2005; Wältermann et al. 2005); (2) *Acinetobacter calcoaceticus* HO1-N grown on hexadecanol exhibited electron-transparent, rectangular inclusions measuring 100–200 nm by 25–30 nm (Singer et al. 1985); (3) In hexadecane-grown cells of *Acinetobacter* sp. strain M-1 the WE bodies occurred as electron-transparent, smooth, disklike inclusions that reached diameters the same as that of the cell (Ishige et al. 2002). The presence of a surrounding barrier awaits further clarification; in the aforementioned cases the inclusions were reported to be surrounded either by just phospholipids, by a half-unit or bilayer membrane, or to possess no covering at all. WE synthesis requires three enzymes, acylcoenzyme A reductase, fatty aldehyde reductase, and a novel WE synthase/diacylgycerol acyltransferase (Ishige et al. 2002; Kalscheuer and Steinbüchel 2003; Stöveken et al. 2005; Wältermann and Steinbüchel 2005). A chapter by Wältermann and Steinbüchel (this volume) provides in-depth coverage of WE inclusions.

3.4.3
Triacylglycerol Inclusions

The presence of TAG storage bodies is widespread in plants, animals, and eukaryotic microorganisms, but their occurrence is rare in prokaryotes, essentially being limited to nocardioforms and streptomycetes and a few other organisms, for example, *Acinetobacter* species (Alvarez et al. 2000, 2001, 2002; Alvarez and Steinbüchel 2002; Wältermann and Steinbüchel 2005). Prior to 1970 a number of publications showed the presence of lipid inclusions in *Mycobacterium* (Brieger and Glauert 1956; Burdon 1946; Gale and McLain 1963; Knaysi et al. 1950) and during the 1970s the inclusion material was identified as TAGs (Akao and Kusaka 1976; Barksdale and Kim 1977). Unidentified lipid bodies were also reported in *Streptomyces* (Charter and Merrick 1979). The careful description of TAG bodies did not commence until 1995 when Packter and Olukoshi (1995) reported on the electron-transparent inclusions in the cytoplasm of *Streptomyces*. They found that the bodies accumulated in the stationary phase of growth, had no internal structure, appeared to possess a thin surrounding membrane, and filled a large amount of each cell. The first TAG inclusions isolated were from *Rhodococcus opacus* by Alvarez et al. (1996). The inclusions were 87% TAGs with lesser amounts of diacylglycerols, phospholipids, and proteins. The TAGs contained hexadecanoic acid, octadecanoic acid, as well as odd-numbered fatty acids. The spherical bodies range in size from 50 to 400 nm and the number accumulated per cell depends on the organism and the growth state and conditions (Wältermann and Steinbüchel 2005). Although it is still open to question, it appears likely that prokaryotic TAG inclusions do not possess a surrounding barrier, i.e., structural proteins, and thus resemble the oil bodies in yeasts and some plants (Wältermann and Steinbüchel 2005). A chapter by Wältermann and Steinbüchel (this volume) provides in-depth coverage of TAG inclusions.

3.5
Cyanophycin Inclusions

Cyanophycin granules, common if not ubiquitous entities of cyanobacteria, are highly refractile bodies with diameters in some instances greater than 1.0 μm. Borzi (1887) was the first to report these structures and although reports on the occurrence of the granules continued, it was over 40 years before their structural properties were investigated. Fritsch (1945) discovered that cyanophycin was soluble in dilute acid, and he and others used this property to identify the granules. Light microscopists found that the granules could be stained with some protein-specific reagents, for example, the Sakaguchi reaction for arginine (Fogg 1951), but not with others. As commercial electron microscopes became available, several investigators be-

gan examining cyanobacterial structure. Drews and Niklowitz (1956, 1957) labeled the large mottled inclusions they observed with the electron microscope structured granules. Whether structured granules were the electron microscope equivalent of the light microscope cyanophycin granules remained a controversy until after Simon (1971) isolated the granules and showed them to be composed of a copolymer of arginine and aspartic acid. Lang et al. (1972) studying the properties of isolated granules proved the correspondence between cyanophycin and structured granules. Until recently it was thought that cyanophycin was unique to the cyanobacteria. However it now appears that several eubacteria not belonging to the cyanobacterial group have the potential of synthesizing cyanophycin (Krehenbrink et al. 2002; Ziegler et al. 2002; Elbahloul et al. 2005). A chapter by Obst and Steinbüchel (this volume) provides in-depth coverage of cyanophycin inclusions.

3.6 Insecticidal Protein Crystals

In 1901, Shigetane Ishiwata (1901), while studying a bacterial disease of silkworms, isolated an aerobic spore-forming bacillus he called "Sotto-Bacillen", meaning "sudden-collapse bacillus". Although a scientific name was not given to the organism, this little-known report represents the first publication on *Bacillus thuringiensis*. Some years later, Berliner (1915) isolated a similar organism from flour moth larvae which he named *B. thuringiensis* after the German province Thuringia, which was the source of the insects. In addition, Berliner noted the presence of a Restkorper (parasporal body), inside the sporangium, but separate and distinct from the spore. Although French researchers using *B. thuringiensis* developed a product, Sporeine, during the 1930s for the control of caterpillar pests on vegetable crops little work was undertaken in the USA until Steinhaus (1951) reported on the use of the organism for the biological control of the alfalfa caterpillar. Hannay (1953) reported on the crystalline inclusions of the organism during spore formation noting the presence of a single crystal per cell and that the crystals varied a great deal in size, but always had the same diamond-shaped appearance. Years later the parasporal crystals were shown to be bipyrimidal octahedra of approximately 400 nm × 400 nm × 600 nm (Norris 1969). Angus (1954) showed that the parasporal crystals themselves were capable of killing silkworms. *B. thuringiensis* crystals, which separate from the spore after completion of sporulation, contain proteins of 125–140 kDa or 70–80 kDa (Hofte and Whiteley 1989; Schnepf et al. 1998). The proteins may be toxic to insects of the orders Lepidoptera, Coleoptera, or Diptera (Baumann et al. 1991). In *B. thuringiensis* susbp. *israelensis*, a subspecies highly toxic to mosquito and black fly larvae, the parasporal body is spherical rather than bipyramidal, and contains four major proteins held together in a fibrous envelope (Ibarra and

Federici 1986). Some strains of *B. sphaericus* also produce a parasporal crystal that contains proteins toxic for larvae of a variety of mosquito species (Kellen and Meyers 1964; Kellen et al. 1965; Payne and Davidson 1984; Yousten 1984; De Barjac et al. 1985). In the case of *B. sphaericus*, both the mature spore and the crystal remain in the exopsporium (Yousten 1984). Two proteins of 42 and 51 kDa, both of which are required for toxicity, are the major components of the crystal (Baumann et al. 1985). A chapter by Federici et al. (this volume) provides in-depth coverage of insecticidal protein crystals. For information on other crystals see Shively (1974), and a cameo chapter by Park et al. (this volume).

3.7
Recombinant Protein Inclusion Bodies

The synthesis of proteins by recombinant host systems developed rapidly following the report by Cohen et al. (1973) on the construction of biologically functional plasmids for *Escherichia coli* (Marston 1986). Although a variety of host systems are available today, *E. coli* or other prokaryotes are the host of choice especially if posttranslational modifications are not required for protein function (Fahnert et al. 2004). The expressed proteins in *E. coli* may accumulate to levels as high as 50% of the total cell protein, but in most cases the protein is in an insoluble form (Harris 1983, Fahnert et al. 2004). Goeddel et al. (1979) reported on the production of human insulin in *E. coli*; however, the discovery by Williams et al. (1982) of the concurrent appearance of inclusion bodies with the accumulation of proinsulin or insulin AB chains in *E. coli* was the first indication that the insoluble proteins might be accumulating as discrete entities. Marston et al. (1984) isolated the inclusion bodies from *E. coli* expressing prochymosin and found that the inclusions were indeed composed essentially of the recombinant protein. The inclusions are amorphous aggregates of various sizes, in some instances filling a significant portion of the cell, without a surrounding membrane (Williams et al. 1982; Cheng 1983; Schoemaker et al. 1985; Schoner et al. 1985). The knowledge that abnormal *E. coli* proteins were accumulated as intracellular inclusions (Prouty and Goldberg 1972; Prouty et al. 1975) led many to believe that the formation was simply a response to "foreign" proteins; however, Cheng (1983) showed that normal *E. coli* proteins synthesized in excess could also accumulate as inclusion bodies. During the last 20 years numerous researchers have concentrated their experimentation on various aspects of the production, destruction, utilization, isolation, and structure of recombinant protein inclusion bodies (Mar Carrió et al. 1998, 2000; Mar Carrió and Villaverde 2002; Villaverde and Mar Carrió 2003; Fahnert et al. 2004; Baneyx and Mujacic 2004). Very recently, as the structure and the formation processes of the inclusion bodies were studied in more detail, it became evident that bacterial inclusion bodies share characteristics with amyloid fibrils found in disorders

of humans and higher eukaryotes (Ventura 2005). A chapter by Neubauer et al. (this volume) provides in-depth coverage of recombinant protein inclusion bodies.

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Bacterial Sulfur Globules: Occurrence, Structure and Metabolism

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Dedicated to our esteemed academic teacher Prof. Dr. rer. nat. Dr. phil. Dr. rer. nat. h.c. Hans G. Trüper, Bonn, on the occasion of his 70th birthday on March 16th, 2006, for his fundamental work on microbial sulfur metabolism and encouraging support of his students.

Abstract Reduced sulfur compounds such as sulfide, polysulfides, thiosulfate, polythionates, and elemental sulfur are oxidized by a large and diverse group of prokaryotes, including the phototrophic sulfur bacteria, the thiobacilli and other colorless sulfur bacteria and some thermophilic *Archaea*. Typically, these sulfur compounds are oxidized to sulfate but in many cases globules of polymeric, water-insoluble sulfur accumulate as a transient and sometimes as the final product. While phototrophic bacteria of the families *Chlorobiaceae* and *Ectothiorhodospiraceae*, some *Rhodospirillaceae* and some thiobacilli form extracellular sulfur globules, sulfur is stored intracellularly in purple sulfur bacteria of the family *Chromatiaceae*, in *Beggiatoa* species and in the "morphologically conspicuous" sulfur bacteria (e.g., *Thioploca*, *Achromatium*, *Macromonas*,

Thiovulum). Our understanding of sulfur globule formation from sulfide, thiosulfate and tetrathionate (the latter occurs in acidophilic thiobacilli) is far from complete and suffers mainly from the uncertainties that exist about the exact chemical nature of the sulfur in the globules, the exact intracellular localization of internal sulfur deposits and the mechanisms to adhere to, attack and take up extracellular sulfur. The only fairly well described enzyme system involved in oxidative decomposition of intracellular sulfur globules is encoded by the 15 dissimilatory sulfite reductase (*dsr*) genes of the anoxygenic phototrophic purple sulfur bacterium *Allochromatium vinosum*.

1 Introduction

The first detailed description of sulfur bacteria and sulfur globules was given by Winogradsky (1887), who also demonstrated the oxidation of hydrogen sulfide to stored sulfur under microaerophilic conditions in the chemotrophic *Beggiatoa* (Winogradsky 1889). Pioneering studies on the oxidation of sulfur in bacterial photosynthesis were done by van Niel (1931, 1936), whose classic studies about phototrophic sulfur bacteria and accumulation of elemental sulfur can be considered as milestones and provided the basis for further studies about sulfur compounds in photosynthesis.

More than 100 years after Winogradsky's groundbreaking work, we now know that sulfur globules are formed by a large and diverse group of prokaryotes that metabolize reduced sulfur compounds such as sulfide, polysulfides, thiosulfate, polythionates, and external elemental sulfur. The immense diversity of sulfur-forming prokaryotes is reflected by the facts that the site of sulfur deposition (intracellular or extracellular) as well as the chemical nature of the deposited sulfur can vary and that a universal mechanism for the formation and degradation of sulfur deposits does not exist (Brune 1995b; Dahl et al. 2002; Friedrich 1998; Friedrich et al. 2005; Kelly 1989; Prange et al. 2002a; Shively et al. 1989). Whether the sulfur accumulates as a transient or the final product again varies depending on the organism, the culture conditions and the reduced sulfur substrate. Transiently formed sulfur deposits can serve as temporary energy reservoirs and it has been shown that the pool of intracellular sulfur plays a major role as a buffer of electron-donating potential in phototrophic sulfur bacteria (Overmann 1997).

Here, we attempt to give an overview about the different sulfur-forming prokaryotes, the structure and chemical nature of bacterial sulfur inclusions and the metabolic pathways related to sulfur globule formation and degradation. A strong focus will be laid on sulfur globules deposited within the confines of the cell wall, i.e., sulfur present as a bacterial inclusion *senso strictu*. For further detailed information the reader is referred to a number of reviews on storage compounds and oxidative sulfur metabolism (Brune 1989, 1995b; Dahl et al. 2002; Friedrich et al. 2001, 2005; Kelly et al. 1997; Mas and van Gemerden 1995; Pronk et al. 1990).

2
Occurrence and Localization of Sulfur Globules

Concerning their physiology, two large groups of sulfur-storing bacteria can be differentiated: the phototrophic and the chemotrophic sulfur bacteria. Within both groups, the site of sulfur deposition can vary, i.e., sulfur globules can be deposited either inside or outside of the cell.

2.1
Physiological Groups of Sulfur-Storing Bacteria

Sulfur can accumulate in the form of water-insoluble globules as a transient or the final product during the oxidation of reduced sulfur compounds (sulfide, polysulfides, thiosulfate, polythionates, and elemental sulfur) by either chemotrophic or phototrophic sulfur-oxidizing bacteria.

Chemotrophic (or "colorless") sulfur-oxidizing bacteria use the energy derived from the oxidation of sulfur compounds using either oxygen or nitrate as the electron acceptor to fix carbon dioxide. Free-living chemoautotrophic sulfur oxidizers are found within the *Bacteria* (mostly among the Gram-negatives) and the *Archaea* (within the order Sulfolobales) (Friedrich 1998; Kletzin et al. 2004). Sulfur compounds are also an important energy source for symbiotic associations between marine invertebrates and chemoautotrophic sulfur bacteria (Cavanaugh et al. 1981; Felbeck 1981; Nelson and Fisher 1995). Although first discovered at hydrothermal vents, "chemoautotrophic symbiosis" is not limited to these highly specialized environments but is found in an ever-increasing number of marine invertebrates of at least five animal phyla, including bivalve and gastropod mollusks as well as vestimeniferan, pogonophoran, annelid, and nematode worms (Distel 1998). The energy released from the oxidation of reduced sulfur compounds by the symbiontic bacteria is trapped in the form of ATP and used for CO_2 fixation, which provides the nutritional basis for the symbiosis. The bacteria responsible for primary production live intracellularly within specialized organs. These symbiont-bearing organs are typically the largest and most conspicuous organs in the animals (Cavanaugh et al. 1981; Felbeck 1981).

Bacteria able to use reduced sulfur compounds as electron donors for anoxygenic photosynthesis occur among all known groups of anoxygenic phototrophic bacteria: the purple bacteria (*Chromatiaceae*, *Ectothiorhodospiraceae*, and purple non-sulfur bacteria), the green sulfur bacteria (*Chlorobiaceae*), the green gliding bacteria (*Chloroflexaceae*) and the Gram-positive *Heliobacteria*. Even some species of the otherwise oxygenic cyanobacteria can oxidize sulfide, resulting in the formation of extraneous elemental sulfur (Brune 1995b).

The genera *Achromatium*, *Macromonas*, *Thiobacterium* (formerly *Thiodendron*), *Thiospira* and *Thiovulum* were originally grouped together as

"morphologically conspicuous" sulfur bacteria owing to the observed appearance and disappearance of sulfur inclusions which suggested the possession of at least the capacity to oxidize sulfide and sulfur (La Riviere and Schmidt 1999). While the capacity for chemolithotrophic growth on sulfide is now quite firmly established for *Thiovulum majus* (Robertson and Kuenen 1999; Wirsen and Jannasch 1978) and evidence has been provided that *Achromatium* can oxidize sulfide to sulfate via stored sulfur and probably gains energy from the process (Gray et al. 1999), the nutritional status of the other genera is uncertain and their relationship to reduced sulfur compounds may range from obligate chemolithotrophy to protective, detoxifying sulfide oxidation, or to merely gratuitous sulfide oxidation (La Riviere and Schmidt 1999). It may be noted that numerous other bacteria, including even *Escherichia coli* (Maier and Murray 1965), have also been reported to produce sulfur inside or outside of the cell as a protective, detoxifying reaction.

As noted earlier, sulfur-forming bacteria may exist as free-living large filaments or single cells (e.g., *Thioploca*, *Beggiatoa*, *Thiomargerita* spp., *Thiobacillus* spp., phototrophic sulfur bacteria) (Brune 1995b; Fossing et al. 1995; Nelson and Hagen 1995; Schulz et al. 1999) or as endosymbionts in metazoans (Nelson and Hagen 1995; Vetter 1985). In several cases, dual, triple and even multiple symbiosis have been described in which a single host harbors thiotrophic symbionts and in addition methanotrophic bacteria, sulfate-reducing bacteria and/or spirochetes (Blazejak et al. 2005; Dubilier et al. 2001; Dupperon et al. 2005). In addition, chemoautotrophic sulfur-oxidizing bacteria have been described as epibionts on protists (Buck et al. 2000) or metazoans (Gillan and Dubilier 2004; Polz et al. 1994). In general, sulfur-forming bacteria share environments characterized by elevated levels of hydrogen sulfide mainly produced by bacterial sulfate reduction in anoxic sediments rich in organic nutrients or originating from hydrothermal vents, cold seeps or silled anoxic sediments. Animals harboring sulfur-oxidizing bacterial symbionts have been found at hydrothermal vents, in mangrove swamps, in sea grass beds, in anoxic marine basins, in sewage outfalls and even in rotting whale carcasses (Distel 1998). Sulfur-depositing bacteria have played and are still playing an important role in the biogeochemical cycle of sulfur (Middelburg 2000). It is, for instance, generally accepted that the sulfur deposits that are mined today are biogenic and have been formed by sulfate reduction followed by an oxidation step that may have involved colorless sulfur bacteria.

2.2 Extracellular Sulfur Globules

Extracellular sulfur globules are formed by a diverse group of chemotrophic and phototrophic bacteria. Among the phototrophic bacteria this group includes the families *Ectothiorhodospiraceae* (Fig. 1c) and *Chlorobiaceae*, some species of the purple non-sulfur bacteria, the *Heliobacteriaceae* and the *Chlo-*

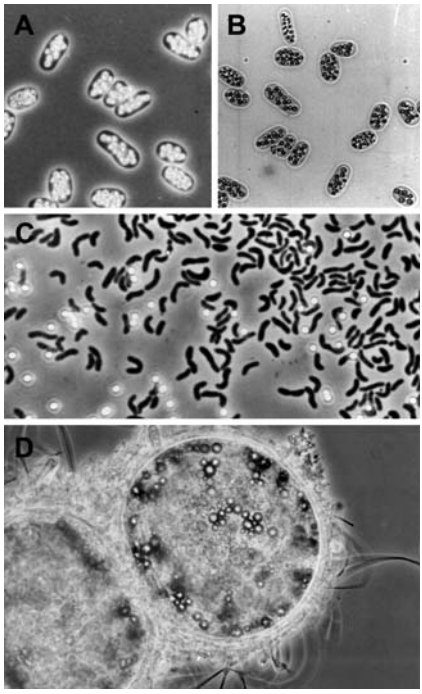

Fig. 1 Different bacteria with sulfur globules: *Chromatium buderi*, light microscopy, phase contrast (**a**); *Chromatium buderi*, light microscopy (**b**); *Ectothiorhodospira mobilis* (extracellular sulfur globules) (**c**) and *Thiomargerita namibiensis*, light microscopy (**d**); magnification ×1000. (Cells kindly provided by H.N. Schulz, Bremen; photos in **a**–**c** kindly provided by H.G. Trüper, Bonn)

roflexaceae and some cyanobacteria. Furthermore, a variety of chemotrophic sulfur oxidizers (e.g., some thiobacilli, *Thermothrix thiopara*) form extracellular elemental sulfur. For some of the latter, transient intracellular sulfur accumulation has also been reported (Hazeu et al. 1988; Schedel and Trüper 1980).

Comparable to intracellular sulfur globules (Sect. 5), externally deposited sulfur appears to be hydrophilic in nature. The extracellularly deposited sulfur globules produced by the acidophile *Acidithiobacillus ferrooxidans* grown aerobically on tetrathionate and pentathionate were concluded to consist of long-chain (up to 17 S atoms) polythionates attached through hydrophobic interactions to a nucleus of elemental sulfur containing mainly S_8 and some S_7 and S_6 rings (Prange et al. 2002a; Steudel et al. 1987a). The globules thus have a hydrophobic core and a hydrophilic surface. The chemical nature of the extracellular intermediary sulfur formed during sulfide or thiosulfate oxidation under oxic or anoxic conditions by other species has not been studied, except for the green sulfur bacterium *Chlorobium vibrioforme* (Sect. 4).

Proteinaceous envelopes have never been reported for extracellular sulfur globules. Accordingly, DNA from representatives of the *Ectothiorhodospiraceae* and *Chlorobiaceae* does not share detectable sequence similarity with the *sgpA* and *sgpC* genes from *Allochromatium vinosum*, encoding two of the three proteins present in the sulfur globule envelope of this purple sulfur bacterium (Pattaragulwanit et al. 1998).

2.3 Intracellular Sulfur Globules

Although a wide variety of prokaryotic microorganisms can metabolize elemental sulfur, including heterotrophic organisms, only a limited number of organisms deposit sulfur internally (Table 1). All of those for which the systematic affiliation has been unequivocally determined belong to the *Proteobacteria*. Sulfur deposition inside of the cells is typical for *Chromatiaceae*, a family of purple sulfur bacteria belonging to the γ-*Proteobacteria* (Figs. 1, 2). The γ branch of the *Proteobacteria* furthermore contains a number of chemotrophic bacteria storing sulfur within the confines of the cell wall. Among those are *Thiomargerita namibiensis* (Fig. 1) and the filamentous sulfur-oxidizing bacteria of the genera *Beggiatoa* and *Thioploca*, some of the largest and most conspicuous bacteria in nature (Schulz et al. 1999; Schulz and Jørgensen 2001). The chemoautotrophic symbionts examined to date also fall into the γ subdivision of *Proteobacteria* (Distel 1998). Within the β-*Proteobacteria* we find the genus *Macromonas* with uncertain physiology (La Riviere and Schmidt 1999) and *Thermothrix azorensis*, an aerobic, thermophilic, obligately chemolithoautotrophic, sulfur oxidizer that forms inclusions of sulfur under certain growth conditions (incomplete thiosulfate oxidation, pH above 7.0) (Odintsova et al. 1993). *Thiovulum* belongs to the ε branch of the *Proteobacteria*. The ovoid cells of this spectacular genus measure 5–25 μm in length. Sulfur globules are often concentrated at one end (La Riviere and Schmidt 1999). Sulfur inclusions have also been observed in several strains of magnetotactic bacteria (Bazylinski et al. 2004; Spring and Bazylinski 2000). All cultivated magnetotactic bacteria are autotrophic sulfide -oxidizing microaerophiles (Spring and Bazylinski 2000). The genus *Magnetospirillum* has been shown to belong to the α-*Proteobacteria* (Kawaguchi et al. 1992).

Sulfur globules appear highly refractile in light. Cultures of cells containing sulfur globules, therefore, exhibit a characteristic milky appearance (Fig. 3). Sulfur globules can reach diameters of up to 2 μm (Head et al. 1996; Remsen 1978; Williams et al. 1987) and are easily observed by light microscopy. The sulfur can comprise 20–34% of the cell dry mass of *Beggiatoa* sp. and purple sulfur bacteria, respectively (Nelson and Castenholz 1981; Overmann 1997). Deposition of sulfur by cells of *Allochromatium vinosum* and *Chromatium warmingii* increases their density from 1.150 to 1.2281 and from 1.0890 to 1.1321 g/cm^3, respectively (Guerrero et al. 1984; Mas et al. 1985; Mas and van

Table 1 Bacterial genera with microscopically visible sulfur inclusions

Organism/group	Main substrates for sulfur globules	Comments	Systematic affiliation	Refs.
Chemotrophic sulfur bacteria				
Magnetotactic bacteria	H_2S	Intracellular	α-*Proteobacteria* (*Magnetospirillum*)	Kawaguchi et al. (1992); Spring and Bazylinski (2000)
Thermothrix	$S_2O_3^{2-}$	Intra- and extracellular	β-*Proteobacteria*	Odintsova et al. (1996)
Macromonas	H_2S	Intracellular	β-*Proteobacteria*	La Riviere and Schmidt (1999) GenBank AB077037
Achromatium	H_2S	Intracellular	γ-*Proteobacteria*	Gray et al. (2004); Head et al. (1996)
Beggiatoa	H_2S, $S_2O_3^{2-}$	Periplasmic, *cyclo*-octasulfur	γ-*Proteobacteria*	Prange et al. (2002a); Teske and Nelson (2004)
Thiomargerita	H_2S	Periplasmic, *cyclo*-octasulfur	γ-*Proteobacteria*	Prange et al. (2002a); Schulz et al. (1999)
Thioploca	H_2S	Intracellular	γ-*Proteobacteria*	Otte et al. (1999); Teske et al. (1995)
Thiothrix	H_2S, $S_2O_3^{2-}$	Periplasmic	γ-*Proteobacteria*	Howarth et al. (1999)
Thioalkalivibrio	$S_2O_3^{2-}$	Extracellular, some strains intracellular in periplasm	γ-*Proteobacteria*	Sorokin et al. (2001)
Bacterial endosymbionts of invertebrates	H_2S	Intracellular	γ-*Proteobacteria*	Distel (1998)
Thiovulum	H_2S	Intracellular	ε-*Proteobacteria*	Lane et al. (1992)
Thiobacterium	H_2S	Intracellular	Unclear	La Riviere and Schmidt (1999)
Thiospira	H_2S	Intracellular	Unclear	La Riviere and Schmidt (1999)
Phototrophic bacteria				
Chromatiaceae	H_2S, S^0, polysulfide, some $S_2O_3^{2-}$	Intracellular, periplasmic, organic polysulfanes	γ-*Proteobacteria*	Pfennig and Trüper (1992); Prange et al. (2002a)

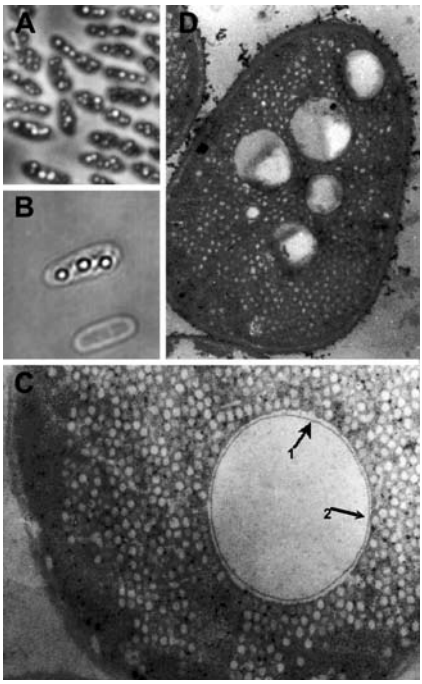

Fig. 2 *Allochromatium vinsoum*, living cells with sulfur globules, light microscopy, phase contrast (magnification ×1000) (**a**); *A. vinosum*, living cells with sulfur globules, light microscopy (magnification ×2000) (**b**); part of an *A. vinosum* cell, electron micrograph (magnification ×20 000) (**c**); *arrow 1* indicates the protein envelope, which surrounds the sulfur globule, *arrow 2* indicates that some chromatophores seem to be opening into the space enclosing the sulfur globules; *A. vinosum* cell, electron micrograph (magnification ×13 500) (**d**). As a result of the preparation of cells for electron microscopy, the localization of sulfur globules is visible as "holes" in the electron micrographs. (photo in **c** kindly provided by H.G. Trüper, Bonn; photo in **d** kindly provided by H. Engelhardt, Martinsried)

Gemerden 1987). These increased densities may have ecological as well as physiological consequences (Guerrero et al. 1984; Overmann 1997).

In thin-section electron micrographs, sulfur deposits appear as a conspicuous, empty and electron-lucent space as elemental sulfur readily dissolves in solvents used after chemical fixation and during alcohol dehydration and epoxy resin embedding of biological samples (Pasteris et al. 2001; Remsen and Trüper 1973; Strohl et al. 1981; Vetter 1985). Furthermore, sulfur is known for its instability under an electron beam, particularly while under vacuum; therefore, any remaining sulfur is subject to thermal degradation under the electron beam (Pasteris et al. 2001). These factors make it difficult to document the presence of sulfur in bacteria and, particularly, to identify exactly where it resides. As a consequence, the exact intracellular localization of sulfur globules has not been resolved in all cases. Differences observed have been

Fig. 3 Batch cultures of *A. vinosum* DSMZ 180^T containing sulfur globules after addition of sulfide (*left*) in comparison to starved cells (*right*)

interpreted as indicating that sulfur globules are the only bacterial inclusions that appear in markedly different morphological forms, depending on the organism (Shively et al. 1989). In several cases, especially in the investigation of the ultrastructural properties of sulfur-oxidizing endosymbiontic bacteria it has been very difficult to distinguish putative sulfur vesicles from other vesicle-like storage structures such as polyhydroxyalkanoate bodies. The situation in chemoautotrophic sulfur-oxidizing endosymbionts and their marine invertebrate hosts may serve as an example. The animals including, for example, *Rifita pachyptila* (Vestimenifera) (Cavanaugh 1983; Felbeck 1981) or Vesicomyid clams (Goffredi and Barry 2002) contain specialized symbiont-bearing organs (trophosome or specialized gills). The trophosome of vestimeniferan worms is made up by 24% of sulfur-oxidizing symbiontic bacteria, and 70% of the tissue consists of symbiont-containing cells (bacteriocytes) (Bright and Sorgo 2003). The trophosome tissue is loaded with sulfur globules. Biochemical measurements of elemental sulfur have revealed levels of 4–6% of the trophosome (deBurgh et al. 1989). The large spherical bacterial cells observed in the trophosome of *R. pachyptila* are structurally similar to the marine sulfur-oxidizing bacterium *Thiovulum*. The symbionts exhibit roundish to polymorphic electron-translucent vesicles whose membranes are infoldings of the cytoplasmic membrane, and the enclosed spaces are contiguous with the periplasmic space. These vesicles share common ultrastructural characteristics with sulfur-containing globules of other organisms, but it still remains to be established whether these structures are indeed related to sulfur storage (Bright and Sorgo 2003; Maina and Maloyi 1998). In three other vestimeniferan species very similar globular structures were interpreted to contain the lipid storage compound poly(β-hydroxybutyrate) although Vetter (1985) claimed that similar globules in gill symbionts of bivalves were sulfur deposits con-

taining elemental sulfur, polysulfides or polythionates (deBurgh et al. 1989). If the vesicles observed in *R. pachyptila* and Vesicomyid clams are indeed sulfur deposits, the sulfur would reside in the periplasm of the bacterial endosymbionts (Bright and Sorgo 2003; Vetter 1985). The bacterial symbionts in gills of the large tropical shallow water bivalve *Lucina pectinata* have also been described to contain periplasmic sulfur globules (Frenkiel et al. 1996). However, in this case a new technique (cryogenic energy-filtered transmission electron microscopy) revealed that the host cells containing the endosymbionts (bacteriocytes) also appear to be able to produce sulfur from sulfide and to deposit it in electron-dense vesicles near the basal pole of the cell (Lechaire et al. 2006). On the other hand, cytoplasmic sulfur globules have been described for the endosymbionts of gutless oligochaete worms (Krieger et al. 2000).

In studies with free-living filamentous sulfur bacteria, including *Thiothrix* (Bland and Staley 1978; Larkin and Shinabarger 1983; Williams et al. 1987), *Thioploca* (Maier and Murray 1965) and *Beggiatoa* (Larkin and Strohl 1983; Maier and Murray 1965), sulfur inclusions were found to be located within invaginated pockets of the cell membrane; thus, the inclusions were within the boundary of the cell wall but were external to the cytoplasm. In some cases, the sulfur globules appeared as a membrane-bound inclusion in the cytoplasm with no apparent connection to the cytoplasmic membrane (Strohl et al. 1981). As already pointed out by Shively et al. (1989) this may be a result of the sectioning plane. The sulfur globules of purple sulfur bacteria were originally assumed to be located in the cytoplasm (Brune 1995a, b; Remsen 1978; Remsen and Trüper 1973). Purple sulfur bacterial cells are packed with intracytoplasmic membranes harboring the photosynthetic apparatus (the so-called chromatophores), which makes interpretation of electron micrographs particularly difficult. The chromatophores are associated with sulfur globules in a highly organized manner (Fig. 2c). The sulfur globules are bounded by an electron-dense layer, the protein envelope (Nicolson and Schmidt 1971). Careful inspection of electron micrographs like that presented in Fig. 2 reveal that some chromatophores, the insides of which are extracytoplasmic or periplasmic (depending on whether the insides are continuous with the periplasm or not), seem to be opening into the space enclosing the sulfur globules, thus implying an extracytoplasmic location for the globules themselves. This interpretation is strongly supported by the finding that the sulfur globule proteins (Sgps) of *Allochromatium vinosum* are synthesized as precursors carrying amino-terminal signal peptides mediating transport across the cytoplasmic membrane (Pattaragulwanit et al. 1998). The proposed targeting process was experimentally confirmed with a *sgpA-phoA* fusion in *Escherichia coli* and in *Allochromatium vinosum* (Pattaragulwanit et al. 1998; Prange et al. 2004) and the controversies about the subcellular localization of the globules in *Chromatiaceae* are now finally resolved.

In summary, intracellular sulfur globules appear in most cases to be separated from the cytoplasm by a unit membrane which may be continuous

with the cytoplasmic membrane, depending on the organism (Pattaragulwanit et al. 1998; Shively et al. 1989; Vetter 1985). The location of the sulfur inclusions in the periplasmic space may be of consequence for the bacteria in several ways. Oxidation of sulfide at the outer surface of the cytoplasmic membrane, adjacent to the sulfur inclusions, may (1) establish a proton gradient necessary for ATP synthesis and (2) reduce the potential for sulfide toxicity within the cytoplasm.

As already mentioned for purple sulfur bacteria of the family *Chromatiaceae*, sulfur inclusions have been observed to be bounded by a protein envelope in many cases. The sulfur globules of *Chromatiaceae*, most *Beggiatoa* species and strains of *Thiothrix* are surrounded by a single-layered electron-dense envelope of 2–5 nm (Schmidt et al. 1971; Strohl et al. 1981; Williams et al. 1987). For *Beggiatoa alba* BL15D a pentalaminar envelope of 12–14-nm thickness has been described. This envelope consists of three electron-dense layers 3.5, 2.1 and 3.5-nm thick (Strohl et al. 1982). Interestingly, cells of this *B. alba* strain grown in the absence of sulfur compounds apparently contained small rudimentary sulfur inclusion envelopes. It was hypothesized that these envelopes were present in collapsed form until a reduced sulfur source became available. Upon exposure to sulfide, the envelopes would quickly expand to deposit the sulfur from the oxidation of sulfide (Strohl et al. 1982). Sulfur inclusion envelopes have been described as being fragile in fixatives used for transmission electron microscopy (Strohl et al. 1981); therefore, their lack in electron micrographs may not always be proof that they are indeed absent *in vivo*.

In the purple sulfur bacteria *Thiocapsa roseopersicina* and *Allochromatium vinosum*, the sulfur globule envelope consists of two or three different, extremely hydrophobic proteins of 8.5–10.5 kDa (Brune 1995a). From *B. alba* B18LD, a protein with an approximate molecular mass of 15 kDa was enriched with the sulfur inclusions and was the major protein in response to addition of sulfide (Schmidt et al. 1986). It may therefore be important to the structure of the *B. alba* sulfur globules or even represent a constituent of the protein envelope.

The specialized arrangement of sulfur inclusions in many sulfur-oxidizing bacteria suggests an important structure–function relationship. This is underlined by the fact that mutants of *Allochromatium vinosum* lacking SgpB and SgpC, two of the three Sgps, are no longer able to oxidize sulfide and to form sulfur inclusions from it (Prange et al. 2004).

3
Relevant Chemical Structures

Sulfur is the element with the highest number of allotropes; however, only a few occur in biological systems. Appearing in many different organic com-

pounds like in the amino acids (cysteine and methionine) and therefore in (poly)peptides, enyzme cofactors, sulfolipids, vitamins, carbohydrates and antibiotics (penicillin), sulfur is an abundant element in all living matter (Dick 1992; Falbe and Regitz 1995; Postgate 1968). Sulfate, (bi)sulfite, elemental sulfur, thiosulfate, (poly)thionate, elemental sulfur, (poly)sulfide and sulfide are the inorganic sulfur compounds of biological relevance (Table 2) which occur in the biological sulfur cycle (Brüser et al. 2000; Trüper 1984). The complexity of sulfur chemistry originates from the many oxidation states, ranging from – 2 to + 6, sulfur atoms can adopt. Furthermore, sulfur tends to catenate and forms various chain and ring sizes, especially in the zero oxidation state as elemental sulfur (Steudel 2000). At ambient pressure and temperature sulfur can exist as rings of different sizes (S_n) or as polymeric chains of high molecular mass (S_∞ or S_μ) (Steudel 1982, 2000).

For more detailed information on sulfur chemistry, the reader is referred to a number of excellent reviews (and references therein) and books (Müller and Krebs 1984; Nickless 1968; Steudel 1982, 1987, 1998, 2000, 2003b, c; Steudel and Kustos 1994).

Biologically produced sulfur deposited in bacterial sulfur globules (sometimes termed biosulfur, elemental sulfur or S^0) is a from of sulfur of the zero-valent oxidation state which is produced by so-called sulfur bacteria (Sect. 4). Table 3 gives an overview of different forms of "elemental sulfur species".

Although, about 30 solid allotropes exist, the homocyclic, orthorhombic crystalline α-sulfur (α-S_8) (*cyclo*-octasulfur) is the most thermodynamic-

Table 2 Biologically relevant inorganic sulfur compounds and their oxidation states

Compound	Chemical formula	Sulfur oxidation state
Sulfate	SO_4^{2-}	+ 6
Thiosulfate	$S_2O_3^{2-}$	+ 5 (sulfone S)/– 1 (sulfane S)
(Poly)thionates	$^-O_3S(S)_nO_3^-$	+ 5 (sulfone S)/±0 (inner S)
Sulfite	SO_3^{2-}	+ 4
Elemental sulfur	S_n (S rings), S_μ or S_∞ (polymeric S)[a]	±0
(Poly)sulfides	S_n^{2-}	– 1 (terminal S)/±0 (inner S)
Sulfide	HS^-/S^{2-}	– 2

[a] In the literature, the synonyms S_μ and S_∞ are used for "polymeric sulfur". S_μ is a synonym for polymeric or insoluble sulfur and is also used for the molecule catenapolysulfur in the solid phase and sometimes in the liquid phase (S_∞). S_∞ is generally used for the catenapolysulfur molecule; further synonyms are polymeric sulfur, S_n chains, and S_μ (adopted from Steudel and Eckert 2003); Steudel and Eckert (2003) recommend distinguishing between the polymeric molecules in the liquid (S_∞) and the insoluble, polymeric content of a solid material (S_μ).

Table 3 Elemental sulfur species (modified after Steudel 2000)

Sulfur	Chemical formula	Ring sizes or chain lengths
Homocycles	S_n	$n = 6–20$ (pure compounds) [a]
Polymeric sulfur or polysulfur	S_μ or S_∞	$n > 10^5$
Polysulfides	S_n^{2-}	$n = 1–8$
Polysulfanes	$H – S_n – H$	$n = 1–8$ (pure compounds) [a]
Organic polysulfanes	$R – S_n – R$	$n = 1–13$, R is an organic group (pure compounds) [a]
Polythionates	$^-O_3S – S_n – SO_3^-$	$n = 1–4$ (pure compounds) [a]

[a] Larger species are known, but detected in mixtures only

ally stable form at standard conditions (Roy and Trudinger 1970; Steudel 1996a, b). Other sulfur rings from S_6 to S_{20} have been synthesized as pure substances; of these, S_6, S_7 and S_{12} have also been detected in samples of biological origin (Steudel 1987, 2000). The typical solid "sulfur-yellow", customary in trade elemental sulfur consists typically mainly of S_8 rings as well as of some polymeric sulfur and traces of S_7 rings. The bright yellow color originates from the small amount of S_7 rings (Steudel and Holz 1988). In nature, S_8 rings occur in huge deposits (e.g., in the USA, Mexico, Poland) mostly together with calcite, clay, anhydrite or with gypsum and can be found in volcanic areas (Dahl et al. 2002; Falbe and Regitz 1995). Polymeric sulfur, in addition, consists of very long chains of almost all sizes and is insoluble in all solvents except liquid sulfur (Steudel 2000). Regardless of the molecular size, all sulfur allotropes are hydrophobic, are not wetted by water and barely dissolve in water (Steudel 1989). Polysulfide anions can exist only in neutral or alkaline solutions (pH> 6). The higher the pH of the polysulfide mixtures is, the shorter the chains lengths are. The chain length varies from two up to eight sulfur atoms depending on the pH. Aqueous polysulfide solutions are yellow/orange and are subject to rapid autoxidation when exposed to air; thiosulfate (colorless in solution) is formed in this reaction (Steudel et al. 1986; Steudel 2000). Polysulfanes are known as pure substances with up to eight sulfur atoms in the chain, organic polysulfanes have up to 13 sulfur atoms, but larger species are known, however only in mixtures (Steudel 2000). Polysulfane mixtures are almost insoluble in water, as pure substances slowly decompose to H_2S and S_8 rings. Mixtures of (bis)organic sulfanes with sulfur chains of different chain lengths are light yellow, strongly refractive oily liquids, especially in the presence of higher homologous compounds and they can exist in a lot of different conformational isomers, which makes crystallization more difficult (Steudel 1985). The solubility of the organic polysulfanes (chainlike or cyclic) in water or organic solvents depends on the organic part. Organic polysulfanes tend to form mixtures of homologous compounds

and finally disulfanes together with S_8 rings when nucleophiles (e.g., HS^- and S_n^{2-} anions) are present; a large number of organic polysulfanes with three or more sulfur atoms in a chain have been isolated from various organisms (a summary of naturally occurring polysulfanes can be found in Steudel 2000). Polythionates can only exist at acidic pH values (pH< 6) and in the absence of nucleophiles and reducing agents. The sulfur-rich polythionates are the main constituents of the hydrophilic sulfur sols which had been discussed as models for bacterial sulfur globules (Steudel 1985, 2000; Steudel and Albertsen 1999).

As in this short summary of elemental sulfur species only some "cornerstones" of properties relevant for the sulfur in bacterial globules can be presented, the reader is referred to the reviews of Steudel (Steudel 2000, 2003b, c) and references therein, which present the best comprehensive overviews.

4 Chemical Structures of Bacterial Sulfur Inclusions

The identification of the exact chemical nature of elemental sulfur in bacterial sulfur globules produced by sulfur-oxidizing bacteria has a very long history and was a topic of various studies in the last few decades. As early as the end of the eighteenth century, Müller (1786) reported "colorless, egg-shaped algae with spherical inclusions", which were later described as "*Monas muelleri*" (Warming 1875). In the nineteenth century, inclusions in the sulfur bacteria "*Monas okenii*" (*Chromatium okenii*), "*Ophidomonas jenensis*" (*Thiospirillum jenense*) and "*Monas vinosa*" (Ehrenberg 1838) [*Chromatium vinosum* (Perty 1852); renamed: *Allochromatium vinosum* (Imhoff et al. 1998)] and "*Beggiatoa*" (Beggiato 1838; Cohn 1865; Trevisan 1842) were observed and described. However, in the 1870s, the "inclusions" were identified as elemental sulfur by extraction with carbon disulfide (*Beggiatoa*: C. Cramer in Müller 1870; *Chromatium vinosum*: Cohn 1875; *Thiovolum muelleri*: Warming 1875).

The first detailed description of these bacteria, light microscopy investigations and the first physiological characterizations were given by Winogradsky (1887, 1889). He proposed the term "sulfur bacteria" for "bacteria, which store sulfur as small globules inside the cell". Furthermore, he postulated that living cells contain "weak, amorphous sulfur" because he was only able to observe crystalline sulfur in dead cells. Corsini (1905) argued and assumed on the basis of some chemical and microscopical experiments on cultures of *Beggiatoa* that the granules contain sulfur in a weak and oily modification and do not consist only of pure sulfur. Therefore, he proposed the term "sulfur droplets" instead of "sulfur granules". About 30 years later, van Niel (1931, 1936) provided deep insights into the physiology of phototrophic sulfur bacteria and performed the first quantiative investigations on the storage of "elemental sulfur".

Following these pioneering studies, a significant amount of research has been done and various techniques and approaches have been used to determine the exact chemical nature of the "elemental sulfur" in different sulfur bacteria. Different modifications of sulfur were detected or claimed in different bacteria and several models were proposed. Orthorhombic sulfur crystals in *Thiocystis violaceae* and other phototrophic bacteria were proposed by Trüper and Hathaway (1967). Hageage et al. (1970) determined by using X-ray diffraction the intracellular sulfur of *Chromatium okenii* as "spherically symmetrical aggregates of radially arranged arrays of S_8 molecules that were in a metastable, liquid modification". By determining and calculating the density of non-sulfur-containing bacteria and two sulfur-globule-containing *Chromatium* spp. in comparison with the density of elemental sulfur, Guerrero et al. (1984) postulated that the intracellularly deposited sulfur is complexed with another low-dense component and might be "hydrated sulfur". Furthermore, microscopic investigations (polarized microscopy, freeze etch microscopy) yielded evidence that the elemental sulfur in the globules is not crystalline orthorhombic sulfur (Hageage et al. 1970; Remsen 1978; Remsen and Trüper 1973; Strohl et al. 1981). Raman spectroscopic investigations showed that the sulfur globules of *Allochromatium vinosum* and *Halorhodospira* (formerly *Ectothiorhodospira*) *abedelmalekii* contain only small amounts of S_8 rings (Steudel 1985; Then 1984). In cultures of *Acidithiobacillus ferrooxidans*, polythionates with up to 17 sulfur atoms have been detected (Steudel et al. 1987b). In a model of Steudel et al. (1990), the sulfur in the sulfur globules consists of a nucleus of S_8 rings surrounded by water and long-chain sulfur species like polysulfides or polythionates act as an amphiphilic interface (Steudel 1989; Steudel et al. 1990). As another model for the modification of sulfur in sulfur bacteria, sulfur sols [small milky colored emulsions of elemental sulfur (sulfur rings) in water] were proposed (Steudel 1996b, 2003a; Steudel and Albertsen 1999).

Although the bacterial sulfur globules have now been known and investigated with different approaches, it was not possible to clarify the exact chemical nature of the "elemental sulfur" in intact bacterial sulfur globules. In almost all the investigations, the sulfur globules were extracted from the cells prior to analysis (e.g., X-ray diffraction), which causes changes in the chemical structure of the sulfur (Prange et al. 2002a). Thus, in the last few years X-ray absorption near-edge structure (XANES) spectroscopy at the sulfur K-edge using synchrotron radiation was used as an *in situ* approach to investigate the sulfur speciation in intact bacterial cells (Pickering et al. 2001; Prange et al. 1999, 2002a, b; Prange 2001), leading to the first *in situ* results of sulfur speciation. XANES spectroscopy is a nondestructive and sensitive method which yields information about the local geometric and electronic environment and the effective charge of a chosen atom within a molecule and therefore also of the formal oxidation state. The fact that the local environment of the absorbing atoms is probed implies that XANES spectra

are additive, i.e., the spectrum of a mixture of substances A and B can be composed from the separately measured spectra of A and B, respectively. Because of this additivity a "quantitative analysis" of XANES spectra can be performed, which means the decomposition of a sum spectrum into the components it is composed of. To achieve this decomposition, a "quality function" defined by the difference between experimental data and a linear combination of spectra contained in a basis set can be minimized (for further details and a recent review of its application in biological sciences see Prange and Modrow 2002).

XANES spectroscopy revealed at least three main different forms of sulfur in bacterial sulfur globules (Prange et al. 2002a):

1. *Organic polysulfanes*. Irrespective of whether the sulfur is accumulated in globules inside or outside the cells, it mainly consists of long sulfur chains very probably terminated by organic residues (monoorganyl/bisorganyl polysulfanes) in purple and green sulfur bacteria (*Allochromatium vinosum* (Fig. 2), *Thiocapsa roseopersicina*, *Marichromatium purpuratum*, *Halorhodospira halophila*, *Halorhodospira abdelmalekii*, *Chlorobium vibrioforme*). Most probably, the organic residue present at the end of the sulfur chains present in the sulfur globules is glutathione (Prange et al. 2002a). This hydrophilic residue (glutathione) can be responsible for keeping the sulfur in a "liquid" state at ambient pressure and temperature. Earlier speculations and proposals that reduced glutathione (probably in its amidated form) could act as carrier molecules of sulfur to and from the globules (Bartsch et al. 1996; Pott and Dahl 1998) were supported by XANES spectroscopy results (Prange 2001). Furthermore, XANES spectroscopy yielded evidence that the sulfur chains in globules of *Allochromatium vinosum* were gradually shortened during oxidation of intracellularly stored sulfur to sulfate (Prange et al. 2002b).
2. *Cyclo*-Octasulfur. This is the dominant sulfur species in the sulfur globules of the chemotrophic sulfur oxidizer *Beggiatoa alba* and the giant bacterium *Thiomargarita namibiensis* (Fig. 1d) (Prange et al. 2002a). The presence of S_8 rings in *B. alba* was also detected using Raman spectroscopy (Pasteris et al. 2001).
3. *Polythionates*. Theses are present in the sulfur globules of the aerobically grown (at pH 2) chemotrophic sulfur oxidizer *Acidithiobacillus ferrooxidans*, which is in good accordance with previous findings (Steudel et al. 1987b).

A clear correlation between sulfur speciation and occurrence of oxygen during growth of the bacteria is evident: sulfur chains as organic polysulfanes are present in the anaerobically grown phototrophic sulfur bacteria, while sulfur rings (*Beggiatoa alba*, *Thiomargerita namibiensis*) and polythionates (*Acidithiobacillus ferrooxidans*) are present in aerobically grown organisms. These results indicate that the speciation of sulfur in the sulfur globules re-

flects the different ecological and physiological properties of different "types" of bacteria (Prange et al. 2002a). Furthermore, sulfur of isolated sulfur globules from anaerobically grown *Allochromatium vinosum* was found as S_8 rings (Prange et al. 2002a), indicating the influence of oxygen and the necessity of *in situ* methods like XANES spectroscopy that can be applied to avoid destruction of the original sulfur environment. Some controversy has arisen from the interpretation of XANES measurements. The investigations of phototrophic sulfur bacteria using XANES spectroscopy (Pickering et al. 2001; Prange et al. 1999, 2002a) yielded partly comparable experimental data but were interpreted in quite a different way by the two research groups. Pickering et al. (2001) concluded on the basis of theoretical considerations that the sulfur is "simply solid S_8". The discrepancies and discussion around this topic focus on the measurement mode (George et al. 2002; Prange et al. 2002c). The model for the sulfur globules of *Allochromatium vinosum* that corresponds best with the available "biological" experimental data consists of long sulfur chains terminated by organic groups and was suggested by Prange et al. (Kleinjan et al. 2003).

5
A Model Organism: the Sulfur Globules of *Allochromatium vinosum*

Sulfur deposition and sulfur metabolism in purple sulfur bacteria are comparatively well characterized and have recently received increased attention, mostly owing to the availability of reverse genetics for one of their representatives, *Allochromatium vinosum* (Pattaragulwanit and Dahl 1995). The following discussion of the formation, degradation and envelope of bacterial sulfur globules therefore focuses on this organism. A schematic overview about the current model of sulfur globule formation and degradation in *Allochromatium vinosum* is given in Fig. 4.

5.1
Sulfur Globule Proteins: Properties and Regulation

As noted earlier, the sulfur globules in the *Chromatiaceae* are enclosed by a protein envelope, a feature shared by most if not all of the chemotrophic sulfur-oxidizing bacteria that form intracellular sulfur globules (Brune 1995a; Dahl 1999; Nicolson and Schmidt 1971; Pattaragulwanit et al. 1998; Schmidt et al. 1971; Strohl et al. 1981, 1982). In *Allochromatium* this envelope is a monolayer of 2–5 nm consisting of three different hydrophobic Sgps of 10.5, 10.6 (SgpA and SgpB) and 8.5 kDa (SgpC), while that of the related *Thiocapsa roseopersicina* contains only two proteins of 10.7 and 8.7 kDa (Brune 1995a; Pattaragulwanit et al. 1998). The two larger proteins of *Allochromatium vinosum* are homologous to each other and to the larger protein of *Thiocapsa roseopersicina* (Brune 1995a; Pattaragulwanit et al. 1998). The

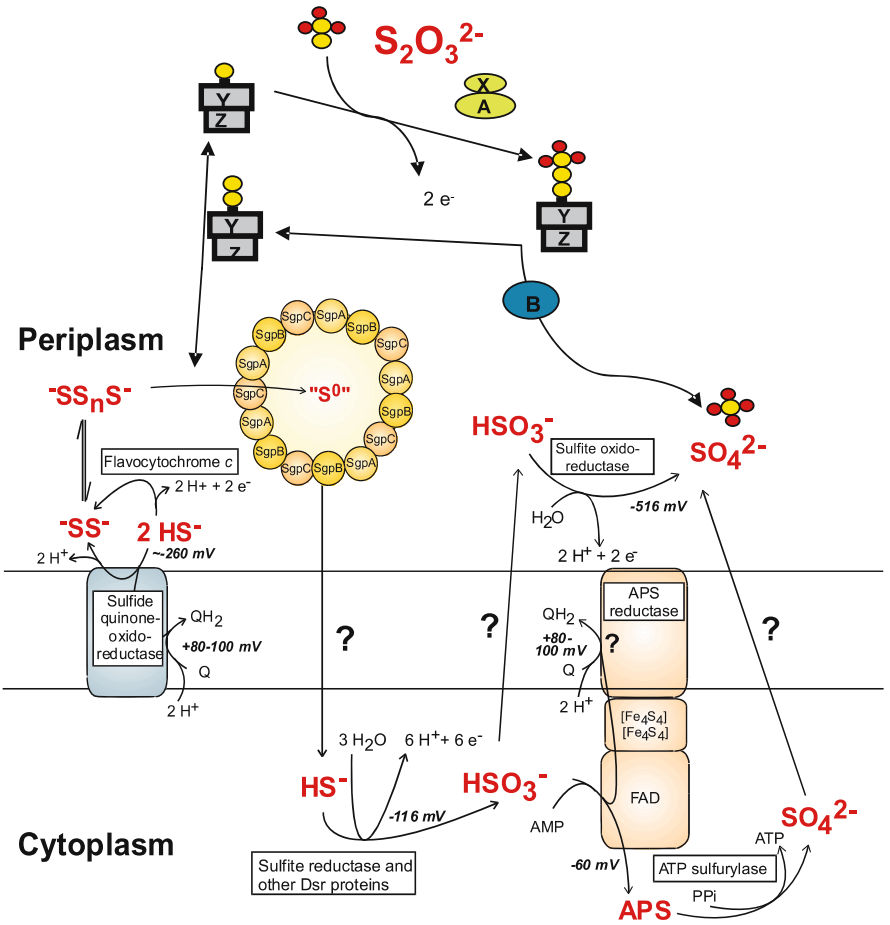

Fig. 4 Current model of sulfur globule formation and degradation in *Allochromatium vinosum*. *APS* adenosine 5′-phosphosulfate

smaller Sgps in *Allochromatium vinosum* and *Thiocapsa roseopersicina* are also homologous, indicating that these proteins are highly conserved between different species of the family *Chromatiaceae*. Interestingly, they share significant similarity with proteins like cytoskeletal keratin or plant cell wall proteins, suggesting that they are structural proteins rather than enzymes involved in sulfur metabolism (Brune 1995a). Topologically, the Sgps and the sulfur globules of *Allochromatium vinosum* and probably in other *Chromatiaceae* are located extracytoplasmically in the periplasm (Pattaragulwanit et al. 1998).

Comparatively little is known about the function of the Sgps. In bacteria forming extracellular sulfur globules Sgps do not appear to be present (Pattaragulwanit et al. 1998; Then 1984). Consistent with this observation,

the complete genome sequence of the green sulfur bacterium *Chlorobium tepidum* (Eisen et al. 2002) does not encode homologues of the purple bacterial Sgp genes. The sulfur speciation in sulfur globules of anoxygenic phototrophic bacteria is nearly identical irrespective of whether it is accumulated in globules inside or outside the cells (Prange et al. 1999, 2002a). It therefore appears that the Sgps themselves are not responsible for keeping the sulfur in a certain chemical structure. A direct/covalent attachment of chains of stored sulfur to the proteins enclosing the globules is unlikely as none of the Sgps sequenced so far contain cysteine residues (Brune 1995a; Pattaragulwanit et al. 1998). Furthermore ideas have been promoted that the protein envelope serves as a barrier to separate the sulfur from other cellular constituents (Shively et al. 1989) and/or that it provides binding sites for sulfur-metabolizing enzymes (Schmidt et al. 1971).

In *Allochromatium vinosum* mutants, SgpA and SgpB can replace each other in the presence of SgpC (Pattaragulwanit et al. 1998; Prange et al. 2004). A mutant possessing SgpA and SgpB but lacking SgpC can grow on sulfide and thiosulfate. As this mutant forms significantly smaller sulfur globules, SgpC probably plays an important role in sulfur globule expansion. SgpA and SgpB are not fully competent to replace each other as sulfur globule formation is not possible in mutants possessing solely SgpA or SgpB. Experiments with an *sgpBC*$^{-}$ double mutant clearly showed that an envelope is indispensable for the formation and deposition of intracellular sulfur (Prange et al. 2004). The construction of mutants lacking SgpA and SgpC or all three Sgps was not possible, leading to the conclusion that a basic level of Sgps is obligatory for cell survival even under conditions that do not allow sulfur globule formation (Prange et al. 2004).

Each of the three *sgp* genes of *Allochromatium vinosum* forms a separate transcriptional unit (Pattaragulwanit et al. 1998). All are constitutively expressed; however, the expression of *sgpB* and *sgpC* is significantly enhanced under photolithoautotrophic compared with photoorganoheterotrophic conditions. Interestingly, *sgpB* is expressed 10 times less than *sgpA* and *sgpC*, implying that SgpA and SgpC are the "main proteins" of the sulfur globule envelope (Prange et al. 2004).

5.2 Formation of Sulfur Globules

In *Allochromatium vinosum*, sulfur globules are formed from sulfide, thiosulfate, polysulfides and extraneous sulfur. In this organism, the formation of sulfur globules is obligatory during the oxidation of these substrates to the end product sulfate.

In phototrophic sulfur bacteria, the main enzymes currently being discussed as catalyzing the oxidation of sulfide are the periplasmic FAD-containing flavocytochrome *c* and the membrane-bound sulfide:quinone

oxidoreductase (SQR) (Brune 1995b) (Fig. 4), enzymes that have also been reported to occur in some chemotrophic sulfur oxidizers (Friedrich 1998; Visser et al. 1997). In the purple nonsulfur bacterium *Rhodovulum sulfidophilum* the so-called Sox enzyme system (Friedrich et al. 2001), which catalyzes the oxidation of thiosulfate to sulfate, is also indispensable for the oxidation of sulfide (Appia-Ayme et al. 2001). In *Allochromatium vinosum* mutants deficient in either flavocytochrome *c* (Reinartz et al. 1998) or *sox* genes or both (D. Hensen, B. Franz and C. Dahl, unpublished results) sulfide oxidation proceeds with wild-type rates, indicating that that SQR plays the main role in sulfide oxidation in this organism. In other phototrophic sulfur bacteria, *c*-type cytochromes have been proposed to mediate sulfide oxidation (Brune 1989).

In experiments using isolated spheroplasts from *Chlorobium vibrioforme* and *Allochromatium minutissimum*, soluble polysulfides have been detected as the product of sulfide oxidation (Blöthe and Fischer 2000). In accordance, polysulfides were detected as primary products of sulfide oxidation by whole cells of *Allochromatium vinosum* (Prange et al. 2004). Polysulfides have also been reported as intermediates in the oxidation of sulfide to extracellular sulfur by species of the purple sulfur bacterial family *Ectothiorhodospiraceae* (Then and Trüper 1983, 1984; Trüper 1978). While transient formation of polysulfide by these species was originally attributed to chemical reaction between H_2S and elemental sulfur promoted by the alkaline culture medium (Trüper 1978), it now appears more likely that they present biochemically generated intermediates. In agreement with the results obtained with whole cells and spheroblasts, the primary *in vitro* product of the SQR reaction is soluble polysulfide (Griesbeck et al. 2002). Very probably, disulfide is the initial product of sulfide oxidation, which is released from the enzyme. As already pointed out by Griesbeck et al. (2002), polysulfide anions of different chain lengths are in equilibrium with each other; therefore, longer-chain polysulfides can be formed by disproportionation reactions from the initial disulfide. It is currently unknown how polysulfides are converted into sulfur globules. Theoretically this could be a purely chemical, spontaneous process as longer polysulfides are in equilibrium with elemental sulfur (Steudel 1996b). However, *Allochromatium vinosum* sulfur globules do not contain major amounts of sulfur rings (Prange et al. 2002a). Instead, the organylsulfanes detected by XANES must eventually be formed by an unknown (enzymatic) mechanism.

Many phototrophic and chemotrophic sulfur oxidizers form sulfur globules from thiosulfate. In purple sulfur bacteria the intermediary formation of sulfur globules is an obligatory step during the oxidation of thiosulfate to sulfate (Pott and Dahl 1998). Only the sulfane group is transformed into storage sulfur, whereas the sulfone group is converted into sulfate and excreted (Brune 1989; Takakuwa 1992). Thus, the initial step in thiosulfate oxidation is a separation of the two sulfur atoms, which are then processed separately. The detection of thiosulfate-reducing enzyme activities, namely, rhodanese

and thiosulfate reductase, in phototrophic and chemotrophic sulfur bacteria led to the long-held assumption that the sulfane sulfur is not directly hooked up to growing sulfur globules but that the primary reaction is reductive cleavage of thiosulfate to sulfide and sulfite and that the H_2S formed is immediately oxidized to stored sulfur (Brune 1989, 1995b; Brüser et al. 2000; Dahl 1999; Fischer 1988). However, genetic proof for this conviction was missing and rhodanese as well as thiosulfate reductase occur in a wide range of organisms not able to metabolize thiosulfate (Brune 1989). Moreover, initial reductive cleavage of thiosulfate is unlikely in the light of more recent work: Gene inactivation and complementation clearly showed that the *soxBXA* and *soxYZ* genes are essential for thiosulfate oxidation in *Allochromatium vinosum* (Hensen and Dahl, unpublished results). The *soxY* gene product also appears to be important for thiosulfate oxidation in *Chlorobium tepidum*. A pleiotropic *Chlorobium tepidum* mutant (strain Ω::RLP), which lacks not only the Rubisco-like protein but also SoxY, is defective in thiosulfate oxidation (Hanson and Tabita 2003). Furthermore, formation of SoxA is upregulated by thiosulfate in *Chlorobium limicola* (Verte et al. 2002).

SoxBXAYZ-related proteins are part of the widely distributed periplasmic Sox multienzyme system that oxidizes thiosulfate directly to sulfate without the formation of sulfur globules as intermediates (Friedrich et al. 2001, 2005). The proteins SoxXA, SoxYZ, SoxB and $Sox(CD)_2$ are the essential components of the biochemically well characterized Sox system from the facultative chemolithoautotroph *Paracoccus pantotrophus*. Currently, a model is promoted that SoxXA intiates oxidation and covalent attachment of thiosulfate to cysteine 138 of SoxY. SoxB would then hydrolytically release sulfate leaving SoxY with a cysteine persulfide which is proposed to be oxidized by the hemomolybdoenzyme $Sox(CD)_2$ yielding a cysteine-S-sulfate. In the final step SoxB would again release sulfate and thereby recycle SoxY. *Chlorobium tepidum* and *Chlorobium limicola* both contain clusters of *sox* genes of which *soxXYZAB* encode components homologous to *Paracoccus pantotrophus* proteins essential for reconstitution of cytochrome *c* dependent thiosulfate oxidation activity *in vitro*. One very conspicuous difference between the non-sulfur-forming *Paracoccus pantotrophus* and the sulfur-globule-forming anoxygenic phototrophs is the absence of *soxCD* genes from the complete *Chlorobium tepidum* genome (Eisen et al. 2002), the *Chlorobium limicola sox* gene cluster (Verte et al. 2002) and also from *Allochromatium vinosum* (C. Dahl, unpublished results). In the absence of $Sox(CD)_2$ SoxY-bound sulfane sulfur cannot be further oxidized and may instead be transferred to growing sulfur globules (Fig. 4). It is currently unclear how this is achieved. Polysulfides are unlikely as intermediates in the process as they were never detected in thiosulfate-oxidizing *Allochromatium vinosum* cultures (Prange et al. 2004). A potential sulfurtransferase encoded at the *Allochromatium vinosum soxBXA* locus could be involved in transfer of sulfur atoms from SoxY to stored sulfur.

5.3 Degradation of Sulfur Globules

The oxidative degradation of sulfur deposits is one of the most poorly understood areas of sulfur metabolism. In the case of exogenous or extracellularly deposited sulfur, this process does not only involve oxidation of the sulfur but must also include binding, activation and transport inside the cells. Little information is available about adhesion to and attack of extracellular sulfur. In some thiobacilli a material similar to bacterial glycocalyx may participate in adherence to elemental sulfur (Blais et al. 1994). Cell envelope sulfhydryl groups have been proposed to be involved in cell–sulfur adhesion in *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* (Ohmura et al. 1996; Takakuwa 1992). In the green sulfur bacterium *Chlorobium limicola*, structures attached to the cell wall (the so-called spinae) probably mediate adhesion to extracellularly deposited sulfur (Pibernat and Abella 1996).

The only gene region known so far to be essential for oxidation of stored sulfur was localized by interposon mutagenesis in *Allochromatium vinosum* (Dahl et al. 2005; Pott and Dahl 1998). Fifteen open reading frames, designated *dsrABEFHCMKLJOPNRS*, were identified. The *dsrAB* products form the $\alpha_2\beta_2$-structured sulfite reductase. This protein is closely related to the dissimilatory sulfite reductases from sulfate-reducing bacteria and archaea (Hipp et al. 1997), enzymes which are generally located in the cytoplasm. The adjacent *dsrEFHC* genes encode small cytoplasmic proteins with hitherto unknown function. DsrE, DsrF, and DsrH were purified from the soluble fraction and constitute a soluble $\alpha_2\beta_2\gamma_2$-structured 75-kDa holoprotein. The *dsrM*-encoded protein is predicted to be a membrane-bound *b*-type cytochrome, and DsrK exhibits relevant similarity to heterodisulfide reductases from methanogenic archaea. DsrK is predicted to reside in the cytoplasm. The genes *dsrJ*, *dsrO*, and *dsrP* encode a triheme *c*-type cytochrome, a periplasmic iron–sulfur protein and an integral membrane protein, respectively. DsrK, DsrJ, and DsrO were purified from membranes pointing at the presence of a transmembrane electron-transporting complex consisting of DsrK, DsrM, DsrJ, DsrO, and DsrP. In accordance with the suggestion that related complexes from dissimilatory sulfate reducers transfer electrons to sulfite reductase, the *Allochromatium vinosum* Dsr complex is copurified with sulfite reductase, DsrEFH and DsrC. DsrL is an iron–sulfur flavoprotein with NADH:acceptor oxidoreductase activity (Y. Lübbe and C. Dahl, unpublished results). DsrN resembles cobyrinic acid *a*,*c*-diamide synthases and is probably involved in the biosynthesis of siro(heme)amide, the prosthetic group of the *dsrAB*-encoded sulfite reductase. DsrR and DsrS are cytoplasmic proteins of unknown function. With the exception of the constitutively present DsrC, the formation of Dsr gene products is greatly enhanced by sulfide.

Since the proteins encoded at the *dsr* locus are either cytoplasmic or membrane-bound and cannot act directly on the extracytoplasmic sulfur

globules (Fig. 4), it is proposed that the sulfur is reductively activated, transported to and further oxidized in the cytoplasm. Different models have been suggested to explain the roles of the *dsr*-encoded proteins in such a scenario (Dahl et al. 2005; Pott and Dahl 1998). In the final step, sulfite is oxidized to sulfate, either directly or via the nonessential enzymes adenosine 5′-phosphosulfate reductase and ATP sulfurylase (Fig. 4).

Sulfur globules can also serve as an electron acceptor reserve that allows a rudimentary anaerobic respiration with sulfur. Under anoxic conditions in the absence of light, purple sulfur bacteria like *Allochromatium vinosum* can reduce stored sulfur back to sulfide (Trüper 1978; van Gemerden 1968). *Beggiatoa* str. OH-75-2a used sulfur globules that were accumulated during aerobic thiosulfate oxidation, to sustain anaerobic metabolism and growth during several days of anoxia (Nelson and Castenholz 1981) Reduction of sulfur globules to sulfide, coupled to de novo synthesis of cell material, was also found in *Beggiatoa alba* B18LD during anoxic incubation (Schmidt et al. 1987). It has also been reported that in the absence of oxygen and nitrate, elemental sulfur stored as globules in the periplasmic space of thioautotrophic chemosymbionts can serve as an electron sink leading to production of sulfide (Arndt et al. 2001; Duplessis et al. 2004). Nothing is known about the enzymatic mechanisms underlying these processes.

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Acidocalcisomes and Polyphosphate Granules

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Abstract Polyphosphate (poly P) granules were one of the first subcellular structures described in bacteria and are characterized by their high content of phosphorus in the form of poly P. Recent work has shown that poly P granules have a limiting membrane and possess an enzymatic mechanism for their acidification. Their electron density and enrichment in pyrophosphate (PP_i), poly P, and cations such as calcium and magnesium are characteristics in common with those of the organelles described as acidocalcisomes in a number of eukaryotic cells, including human platelets, indicating that they have been conserved during evolution from prokaryotes to eukaryotes. Acidocalcisomes have multiple functions that are related to the functions of their main constituents, PP_i, poly P, and cations.

1 Introduction

The metachromatic or volutin granule was one of the first subcellular structures recognized in bacteria (Babes 1895; Meyer 1905). Its name was derived from its property to stain red when treated with toluidine blue and for its presence in the bacterium *Spirillum volutans*, respectively. Volutin granules were later shown to be present in algae, yeasts, and protists, and were named

polyphosphate (poly P) granules when it was found that their numbers in yeasts increased as the amount of this polymer increased (Wiame 1947). In recent years poly P granules were demonstrated to be similar to organelles first described in trypanosomatids and named acidocalcisomes (Vercesi et al. 1994; Docampo et al. 1995). Acidocalcisomes have been detected in a number of protist parasites, in green algae, in slime molds, and in human platelets (dense granules), which indicates that these organelles have been conserved from bacteria to man (Docampo et al. 2005).

Although early reports suggested the presence of a limiting membrane surrounding the granules (Jensen 1968; Friedberg and Avigad 1968), for many years they were assumed to lack an internal structure or limiting membrane (Shively 1974; Shively et al. 1988). More recent work has revealed a surrounding membrane (Seufferheld et al. 2003, 2004). Evidence for the presence of this limiting membrane was (1) its detection by electron microscopy of intact bacteria and subcellular fractions, (2) staining of the granules by dyes that accumulate in closed acidic compartments, and (3) detection by immunofluorescence and immunoelectron microscopy of a membrane-bound vacuolar pyrophosphatase (V-H^+-PPase) in the membrane of these organelles (Seufferheld et al. 2003, 2004) (Fig. 1b).

Most of the functions of acidocalcisomes are related to the presence in them of poly P, a linear chain of inorganic phosphate moieties linked by high-energy phosphoanhydride bonds. Poly P can be a phosphate store or an

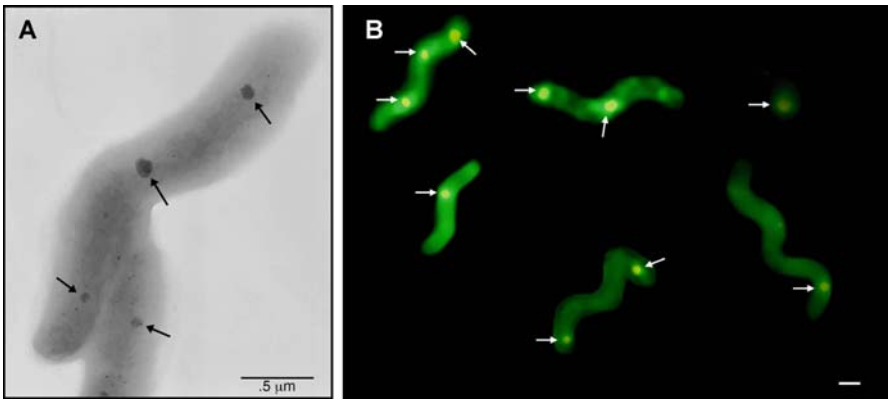

Fig. 1 Electron microscopy and immunofluorescence microscopy of *Rhodospirillum rubrum*. **a** Visualization of acidocalcisomes in whole unfixed cells allowed to adhere to a Formvar- and carbon-coated grid and then observed by transmission electron microscopy. Large granules appear located at bending sites and smaller granules of varying sizes appear distributed in the cytosol (*arrows*). *Bar*0.5 μm. **b** Confocal immunofluorescence analysis of the V-H^+-PPase of *R. rubrum* as detected using polyclonal antibodies against *Arabidopsis thaliana* V-H^+-PPase. *Bar* 0.5 μm. (Reproduced with permission from Seufferheld et al. 2004 *J. Biol. Chem.* 279:51193–51202)

energy source to replace ATP, and can have roles in cation sequestration and storage, cell membrane formation and function, transcriptional control, regulation of enzyme activities, response to stress and stationary phase, and in the structure of channels and pumps. The mobilization of poly P is mainly due to the action of enzymes that catalyze the synthesis and degradation of this polymer—the poly P kinases (PPKs) and the exopolyphosphatases (PPXs), respectively. Mutant strains of bacteria lacking these enzymes have helped in elucidating the structural and regulatory roles of this polymer.

2 Acidocalcisome Structure and Chemical Composition in Bacteria

Acidocalcisomes in bacteria are electron-dense, spherical, and from 15 to about 200 nm in diameter. Their number varies in different species. *Agmenellum quadruplicatum* contains an average of five granules per cell (Nierzwicki-Bauer et al. 1983), *Rhodospirillum rubrum* contains two to three granules per cell (Seufferheld et al. 2004), while *Agrobacterium tumefaciens* usually contains only one granule in one of the polar ends of the cell (Seufferheld et al. 2003) (Fig. 2). They usually occupy about 1% of the cell volume although in extreme cases, such as in metal-treated *Anabena variabilis*, this proportion can rise to 23% of the cell volume (Rachlin et al. 1985). Stress conditions increase the percentage of the cell volume occupied by these organelles. In *Helicobacter pylori* they accumulate under anaerobic conditions (Shirai et al. 2000).

The organelle contains an amorphous and electron-dense material, but the amount seen is dependent on the method of preparation of the sample for

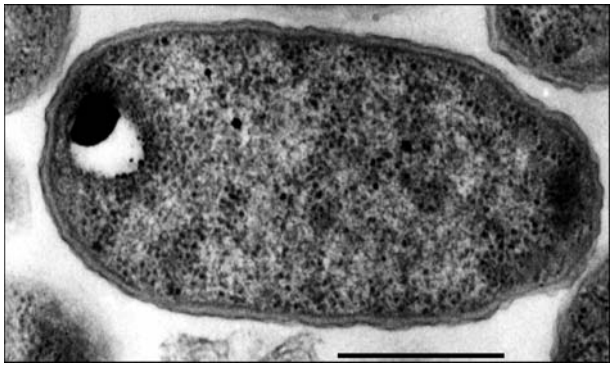

Fig. 2 Electron microscopy of *Agrobacterium tumefaciens*. Electron micrograph of a thin section of a whole bacterium showing an acidocalcisome containing electron-dense material in its periphery. *Bar* 0.5 μm. (Reproduced with permission from Seufferheld et al. 2002 *J. Biol. Chem.* 278:29971–29978)

electron microscopy. When standard staining methods for transmission electron microscopy are used, part of the dense material can be lost, either leaving an empty vacuole or a thin layer of dense material that sticks to the inner face of the membrane. In some bacteria the dense material adheres to one side of the membrane, as an inclusion (Fig. 2). Another useful method to observe acidocalcisomes is to allow whole cells to dry onto carbon- and Formvar-coated grids in the transmission electron microscope. When this technique is used, they appear as electron-dense spheres (Fig. 1a). The dense material appears to volatilize when submitted to the electron beam, giving them a spongelike appearance (Seufferheld et al. 2004).

At the optical microscope level, staining with 4′-6′-diamino-2-phenylindole (DAPI), which stains poly P (Fig. 3), and cycloprodigiosin or Lysosensor blue DND-167, which accumulate in acidic compartments, has also been shown to be effective (Seufferheld et al. 2002, 2004).

Acidocalcisomes of *Agrobacterium tumefaciens* and *R. rubrum* have been shown to possess short-chain and long-chain poly P, as well as pyrophosphate (PP_i) and a number of cations such as calcium, magnesium, and potassium. In other species they have been shown to accumulate large amounts of other metals such as cadmium, cobalt, copper, mercury (Jensen et al. 1982), strontium, barium, manganese (Baxter and Jensen 1980), nickel (Gonzales and

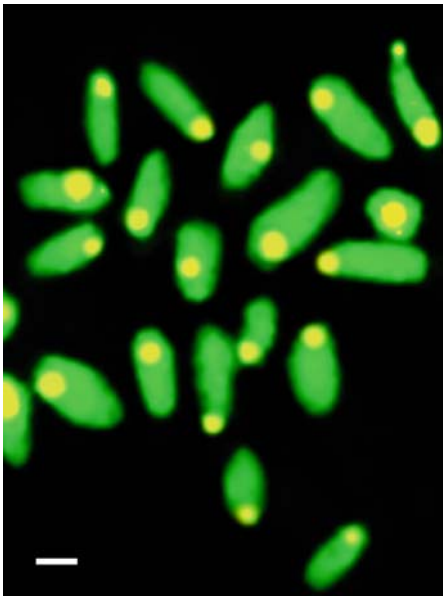

Fig. 3 Staining of acidocalcisomes from *Agrobacterium tumefaciens* with 4′-6′-diamino-2-phenylindole (*DAPI*). Cells were incubated with DAPI as described by Seufferheld et al. (2002), and were observed by fluorescence microscopy. Note the yellow staining at one pole of the cells. *Bar* 0.5 μm

Jensen 1998), lead (Jensen et al. 1982) and aluminum (Torres et al. 1998) when cultivated in their presence. The granules of *Micrococcus lysodeikticus* have been chemically analyzed and shown to contain 24% protein, 30% lipid, 27% poly P, as well as sodium, magnesium, calcium, potassium, manganese, iron, and copper. Zinc was found in the granules from *Plectonema boryanum* (Baxter and Jensen 1980) and is accumulated in *Anabena* spp. (Rachlin et al. 1985). In *Desulfibrio gigas* they were shown to contain a novel metabolite that was identified as α-glucose 1,2,3,4,6-pentakis(diphosphate) (Hensgens et al. 1996).

At least one proton pump has been identified in the membrane lining the acidocalcisomes of *Agrobacterium tumefaciens* and *R. rubrum*: a V-H^+-PPase. The acidocalcisomal V-H^+-PPase of *Agrobacterium tumefaciens* and *R. rubrum* is K^+-insensitive (type 2) and was used as a marker for acidocalcisome purification. The enzyme is also present in the chromatophore membranes of *R. rubrum* (Baltscheffsky et al. 1966). The low sulphur content detected by elemental analysis indicates that few proteins are present in bacterial acidocalcisomes.

3
Acidocalcisome Functions

Most of the functions of acidocalcisomes are those of their main constituents, cations and phosphorus compounds, which are stored in large quantities.

3.1
Phosphorus Storage

Acidocalcisomes are the major storage compartment for phosphorus compounds (orthophosphate, denoted hereafter as P_i, PP_i, and poly P) in several bacteria.

3.1.1
Pyrophosphate

PP_i is a by-product of biosynthetic reactions (e.g., synthesis of nucleic acids, coenzymes, and proteins, activation of fatty acids and isoprenoid synthesis) and is also synthesized in phototrophic bacteria by a reaction catalyzed by the V-H^+-PPase (H^+-pyrophosphate synthase) present in chromatophore membranes. The V-H^+-PPase of *R. rubrum* is capable of PP_i synthesis in the light (Baltscheffsky et al. 1999). Synthesis is driven by the energy derived from the electrochemical H^+ gradient generated across the membrane of the chromatophores during illumination (Baltscheffsky et al. 1999).

PP_i can be used in a number of reactions in bacteria, such as the PP_i-dependent phosphoenolpyruvate carboxykinase (Wood et al. 1977), the pyru-

vate phosphate dikinase (Evans and Wood 1968; Reeves 1968), the PP_i-dependent 6-phosphofructokinase (O'Brien et al. 1975), and in the direct phosphorylation of serine to *O*-phospho-L-serine (Cagen and Friedmann 1972). Hydrolysis of PP_i is catalyzed by soluble pyrophosphatases (sPPases) and vacuolar pyrophosphatases (V-PPases). sPPases are divided into different groups (family I and family II) according to their molecular properties and phylogeny. Family I sPPases are the most widespread, require Mg^{2+}, and are present in the cytosol of archaea, bacteria, fungi and metazoa, as well as in organelles (mitochondria, plastids) (Serrano 2004). The distribution of family II sPPases, first discovered in *Bacillus subtilis* (Young et al. 1998; Shintani et al. 1998), is restricted to archaea and bacteria, and they require Mn^{2+} or Co^{2+} for activity. V-PPases are also divided into two groups, type I, which is K^+-sensitive, and type II, which is K^+-insensitive. Both types are present in archaea, bacteria, protists, and plants and their K^+ sensitivity depends on a single amino acid substitution (Belogurov and Lahti 2002).

Little is know about how PP_i is transported into and out of acidocalcisomes and the reasons for its storage.

3.1.2 Polyphosphate

Poly P is ubiquitous from bacteria to mammals (Kornberg et al. 1999; Kulaev and Kulakovskaya 2000). Poly P has several functions in bacteria.

As a *phosphate store* poly P reduces the osmotic effect of large pools of this important compound. The amount of poly P in bacteria depends on the phosphate content in the medium. Bacteria from wastewaters, such as *Acinetobacter johnsonii* (Deinema et al. 1985), *Microlunatus phosphovorus* (Nakamura et al. 1995), and *Microthrix parvicella* (Erhart et al. 1997) can accumulate large amounts of poly P that can account for to up 30% of their dry biomass (Deinema et al. 1985). There is great interest in the role of poly P accumulation in biological P_i removal from wastewaters (Ohtake et al. 1998). Algal blooms reduce water quality by producing an offensive odor and taste, render boating and fishing difficult, and discourage swimming. When aerobic bacteria consume algae in decomposition there is an increase in dissolved oxygen consumption, causing mass mortality of fish and other aquatic organisms. Algal toxins are also a problem for water supplies. Since P_i is a limiting factor for algal growth in nature, its removal by microorganisms that accumulate it as poly P becomes very important (Ohtake et al. 1998) and efforts have been made to genetically improve bacteria to remove P_i from wastewaters (Kato et al. 1993; Morohoshi et al. 2002). The ability of *Escherichia coli* to accumulate poly P has been enhanced by manipulating the genes involved in the transport and metabolism of P_i and those encoding the enzymes involved in poly P metabolism (Kulaev et al. 1999). A decrease in the level of the PPX and an increase in the level of PPK could lead to an increase in the amount of poly P in

bacteria (Ohtake et al. 1994). When *E. coli* recombinants accumulate high levels of poly P, they release poly P into the medium concomitantly with P_i uptake (Hardoyo et al. 1994), but the mechanism for poly P release is still unclear.

On the other hand, phosphate starvation drastically reduces the amount of poly P in bacteria (Nesmeyanova et al. 1974). When P_i is added to some bacteria, such as *Klebsiella aerogenes*, previously subjected to P_i starvation, they rapidly accumulate poly P, and this is called the poly P overplus (Harold 1966). The mechanism underlying this poly P accumulation is not clearly understood. In *E. coli*, poly P accumulation is not induced by P_i starvation stress alone but takes place as a result of the stringent response (Kuroda and Kornberg 1997). The stringent response is a physiological response caused by a failure in the capacity for transfer RNA aminoacylation to keep up with the demands of protein synthesis (Kuroda and Ohtake 2000). Guanosine 5′-triphosphate 3′-diphosphate (pppGpp) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp) accumulate during the stringent response. ppGpp, which is the major regulatory signal for the stringent response, inhibits the PPX activity without affecting the PPK activity. This leads to slow accumulation of poly P in response to amino acid starvation (Kuroda and Kornberg 1997). Mutants that fail to produce stringent factors are deficient in poly P accumulation in response to amino acid and P_i starvation. As a result of its accumulation, poly P can form a complex with the ATP-dependent protease Lon, and can promote ribosomal protein degradation, thereby supplying the amino acids needed to respond to starvation (Kuroda et al. 2001). It has been shown that poly P first binds to Lon, which stimulates Lon-mediated degradation of ribosomal proteins. Poly P can also compete with DNA for binding Lon, suggesting that poly P may control the cellular activity of Lon not only as a protease but also as a DNA-binding protein. Poly P binding is hypothesized to activate Lon by freeing it from DNA (Nomura et al. 2004). If poly P competes with DNA or RNA for binding sites to other proteins, this may explain how poly P levels influence many different cellular processes, including DNA replication (Stumpf and Foster 2005).

Poly P in bacterial acidocalcisomes could potentially have similar roles to those demonstrated in eukaryotic acidocalcisomes, such as a buffer against alkaline stress or in recovery of cell volume after hypo-osmotic stress (Docampo et al. 2005), but these functions have not been investigated in bacteria.

In addition to its functions within acidocalcisomes, bacterial poly P is a component of the cell capsule in *Neisseria* species (Tinsley et al. 1993). Poly P also forms a complex with Ca^{2+} and poly(β-hydroxybutyrate) in the membrane of *E. coli* that is believed to act as a channel allowing DNA passage through the cells during transformation (Huang and Reusch 1995) or to act as an ion channel (Reusch and Sadoff 1988; Zakharian and Reusch 2004). It has been postulated that the complex is a double helix, in which the outer chain is formed by poly(β-hydroxybutyrate) and the inner chain by poly P molecules linked to each other by Ca^{2+} ions (Reusch 1999).

Poly P can also be used as an energy donor in a number of reactions in bacteria. The PPK can convert poly P to ATP using ADP (Kornberg 1995) (Eq. 1):

$$\text{Poly P}_n + n\text{ADP} \rightarrow n\text{ATP}\,. \quad (1)$$

An AMP phosphotransferase could use AMP and poly P to generate ADP (Eq. 2), which is then converted to ATP by coupling with PPK (Eq. 3) or with adenylate kinase (Eq. 4) (Ishige and Noguchi 2000, 2001):

$$\text{Poly P}_n + \text{AMP} \rightarrow \text{Poly P}_{n-1} + \text{ADP}\,, \quad (2)$$

$$\text{Poly P}_n + \text{ADP} \rightarrow \text{Poly P}_{n-1} + \text{ATP}\,, \quad (3)$$

$$2\text{ADP} \longleftrightarrow \text{AMP} + \text{ATP}\,, \quad (4)$$

$$\text{Poly P}_n + \text{glucose} \rightarrow \text{Poly P}_{n-1} + \text{Glucose 6-P}\,. \quad (5)$$

AMP phosphotransferase has been identified in *Acinetobacter* (Bonting et al. 1991), *E. coli*, and *Myxococcus xanthus* (Kornberg 1995), while adenylate kinase is a ubiquitous enzyme (Kornberg 1995). Poly P can also replace ATP in the phosphorylation of glucose catalyzed by glucokinases (Eq. 5) (Hsieh et al. 1993), and can also phosphorylate proteins in *Sulfolobus acidocaldarius* (Skorko 1989). Enzymes with both poly P- and ATP-dependent NAD kinase activities were isolated from *Micrococcus flavus* and *Mycobacterium tuberculosis* (Kawai et al. 2000), The gene for a poly P- and ATP-dependent NAD kinase from *B. subtilis* has been cloned, expressed, and characterized (Garavaglia et al. 2003). A novel poly P- and ATP-dependent glucomannokinase was isolated from the bacterium *Arthrobacter* sp., which also has several poly P- and ATP-dependent kinases, including glucokinase, NAD kinase, mannokinase, and fructokinase (Mukai et al. 2003). The gene encoding this enzyme was found to be homologous to glucokinases of other bacteria and to proteins of unknown function, and the crystal structure of its protein product has been resolved recently (Mukai et al. 2004).

Since the metabolic turnover of ATP is considerably higher than that of poly P (Chapman and Atkinson 1977), it has been suggested (Rao and Kornberg 1996) that poly P is not an efficient supply of energy and that it has a mainly a regulatory role.

3.2 Cation Storage

Bacterial acidocalcisomes are the main storage compartment for calcium, magnesium, sodium, potassium, and other cations, which are combined with poly P. Poly P in acidocalcisomes can also accumulate heavy-metal cations when they are present in the environment (Kulaev et al. 1999). As indicated earlier poly P within acidocalcisomes can sequester nickel, cadmium, lead, and other heavy metals. Their accumulation into the cells also stimulates the

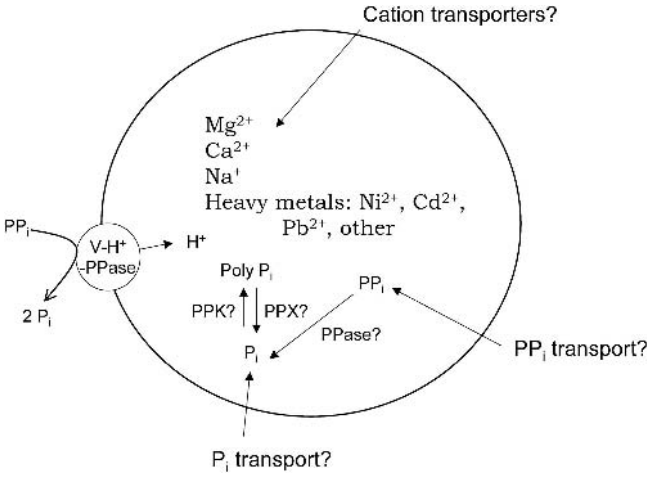

Fig. 4 Representation of a bacterial acidocalcisome. An aminomethylene diphosphonate sensitive vacuolar H^+-PPase is responsible for proton uptake. Other transporters (e.g., cation, P_i, and PP_i transporters) are probably present. The acidocalcisome is rich in PP_i, poly P, magnesium, calcium, and sodium and can accumulate heavy metals. Enzymes involved in PP_i and poly P metabolism (PPX, PPK, PPase) could also be present. See text for an explanation of the abbreviations

PPX activity, releasing P_i from poly P and the metal–phosphate complexes can be transported out of the cells (Keasling and Hupf 1996; Keasling 1997). It has been shown that poly P generated in recombinant bacteria confers mercury resistance (Pan-Hou et al. 2002).

In some cases the chelating properties of poly P could play a significant role in cell metabolism. For example, *Lactobacillus plantarum*, which is devoid of a superoxide dismutase, an important enzyme for the detoxification of superoxide anion, contains very high concentrations of Mn^{2+} (30 mM) that is chelated to 60 mM poly P and effectively replaces the function of a superoxide dismutase (Archibald and Fridovich 1982). Figure 4 shows a schematic drawing of the structure of a bacterial acidocalcisome.

4 Enzymes Involved in Poly P Synthesis and Degradation

In bacteria the mobilization of poly P is performed primarily by the action of enzymes that catalyze the synthesis and degradation of this polymer. The most important enzymes involved in the synthesis of poly P in bacteria are the PPKs. Two PPKs have been described: PPK1, which catalyzes the reversible transfer of phosphate residues from ATP to poly P and from poly P to ADP (Eq. 1); and PPK2, which catalyzes the synthesis of poly P from GTP

or ATP (Zhang et al. 2002; Ishige et al. 2002). The gene encoding PPK1 was first cloned from *E. coli* (Akiyama et al. 1992), and was later found in several bacteria. The gene encoding PPK2 was first found in certain mutants of *Pseudomonas aeruginosa* that lacked PPK1 and still had persistent levels of poly P (Zhang et al. 2002). BLAST searches have detected homologs to PPK2 in 32 species that contained both PPK1 and PPK2, only three species contained only PPK2, and 43 species contained only PPK1 (Zhang et al. 2002). Interestingly, mutants deficient in PPK1 in one of these species, *E. coli*, have been shown to still possess poly(β-hydroxybutyrate)–calcium–poly P membrane complexes (Castuma et al. 1995). This indicates that another PPK with ability to synthesize poly P chains of about 60 P_i residues (those present in the complexes) must be present in *E. coli*.

PPK1 is membrane-bound and has been studied in more detail. The first step of PPK1-catalyzed poly P synthesis involves the autophosphorylation of its histidine residues, and PPK is therefore a histidine kinase. The crystal structure of *E. coli* PPK1 has been resolved recently (Zhu et al. 2005). PPK forms an interlocked dimer, with each 80-kDa monomer containing four structural domains. The PPK active site is located in a tunnel, which contains a unique ATP-binding pocket and may accommodate the translocation of synthesized poly P (Zhu et al. 2005). The two closely related carboxy-terminal domains (C1 and C2 domains) of PPK are structurally similar to the catalytic domain of phospholipase D (PLD). It is interesting to note that the first step of the PLD reaction is the transfer of a phosphate moiety to a PLD histidine residue (Zhu et al. 2005). PPK1 can catalyze, in addition to the synthesis of poly P from ATP, the conversion of ADP back to ATP, and the conversion of other nucleotides, especially GDP to GTP (nucleoside diphosphate kinase activity, Eq. 6). Another reaction is the transfer of a pyrophosphoryl group from poly P to GDP to form the linear guanosine 5′-tetraphosphate (Eq. 7) (ppppG; Kornberg et al. 1999).

$$\text{Poly P}_n + n\text{GDP} \rightarrow \text{Poly P}_{n-1} + n\text{GTP} \quad (6)$$

$$\text{Poly P}_n + \text{GDP} \rightarrow \text{Poly P}_{n-2} + \text{ppppG} \quad (7)$$

PPK1 associates to the *E. coli* degradosome (Blum et al. 1997). This is a multienzyme complex with four major components: the endoribonuclease RNase E, the exoribonuclease polynucleotide phosphorylase (PNPase), the RNA helicase RhlB, and enolase. The first three of these proteins have important functions in mesenger RNA (mRNA) processing and degradation. By virtue of its RNA binding activity PPK1 might promote the assembly of the degradosomes or its interaction with the RNA to be degraded. In addition, since it converts poly P to ATP using ADP, it would remove poly P, which is known to be a potent inhibitor of mRNA degradation, and ADP, which is a potent inhibitor of PNPase, in the degradosome. Mutations in PPK1 increased mRNA stability. However, since PPK1 is not essential it probably

plays a modulatory role rather than a core role in mRNA degradation (Blum et al. 1997).

PPK2 has been shown to differ from PPK1 is several aspects: synthesis of poly P from GTP or ATP, a preference for Mn^{2+} over Mg^{2+}, and stimulation by poly P. The reverse reaction, a poly P-driven nucleoside diphosphate kinase synthesis of GTP from GDP, is 75-fold faster than the forward reaction (Zhang et al. 2002; Ishige et al. 2002).

PPX is one of the most important enzymes involved in poly P degradation in bacteria. The PPX of *E. coli* is a homodimer of a 58-kDa subunit and, as PPK, is membrane-bound (Akiyama et al. 1993). The gene encoding PPX (*ppx*) is in the same operon as the gene encoding PPK (*ppk*) in *E. coli* and in other but not all bacteria (Kornberg et al. 1999). PPX splits P_i from the end of long-chain poly P in a processive manner (Eq. 8):

$$\text{Poly } P_n + (n-2)H_2O \rightarrow (n-2)P_i + PP_i\,. \tag{8}$$

Another *E. coli* PPX was shown to be responsible for the conversion of pppGpp to the tetraphosphate (ppGpp), which is the most active nucleotide involved in the stringent cellular response to deficiencies in amino acids and P_i (Keasling et al. 1993). The structural characterization of *Aquifex aeolicus* PPX/guanosine pentaphosphate phosphohydrolase (GPPA) has been determined (Kristensen et al. 2004). The protein has a two-domain structure with an active site located in the interdomain cleft. A calcium ion was observed at the center of the active site, substantiating that PPK/GPPA enzymes use metal ions for catalysis.

A specific tripolyphosphatase was purified from *Methanobacterium thermoautotrophicum* (van Alebeek et al. 1994), and the *S*-adenosyl methionine synthetase, first isolated from *E. coli*, is known to catalyze cleavage of tripolyphosphate (poly P_3), a reaction that is stimulated by adenosylmethionine (AdoMet). Both enzymatic activities require a divalent metal ion and are markedly stimulated by certain monovalent cations. AdoMet synthesis also takes place if adenyl-5′yl imidodiphosphate (AMP-PNP) is substituted for ATP. The imidotriphosphate (PPNP) formed is not hydrolyzed, permitting dissociation of AdoMet formation from poly P_3 cleavage (Markham et al. 1980). Two isofoms were found in *Sulfolobus solfataricus* but the tripolyphosphatase activity of only only one of them is stimulated by AdoMet (Porcelli et al. 1988).

5
Functions of Poly P Discovered by the Manipulation of the Expression of Genes Involved in Their Synthesis and Degradation

Disruption of the *ppk* gene in *E. coli* led to a disruption of the *ppx* gene (located in the same operon) and to a drastic reduction in the poly P content.

Poly P deficient cells exhibited a striking phenotype characterized by their failure to survive in the stationary phase and loss of resistance to heat, oxidants, and osmotic challenge (Crooke et al. 1994; Rao and Kornberg 1996). High poly P levels were also associated with reduced survival (Crooke et al. 1994). Lack of *ppk* in *E. coli* also impaired the induction of amino acid biosynthetic enzymes and the cells were defective in their adaptation to nutritional deprivation (Kuroda et al. 1999). Poly P levels in *E. coli* have also been reduced by overexpression of yeast PPX, leading to decreased resistance to H_2O_2 (Shiba et al. 1997). The expression of *rpoS*, the gene encoding the stationary-phase-specific RNA polymerase σ factor that governs the expression of many genes is affected by the lack of poly P (Shiba et al. 1997). The demonstration of a functional interaction between *E. coli* RNA polymerase and poly P has led to the suggestion that poly P may play a role in the promoter selectivity control of RNA polymerase (Kusano and Ishihama 1997).

Disruption of the *ppk* gene in six bacterial pathogens greatly reduced their motility on semisolid agar plates likely owing to altered functioning of the flagella (Rashid et al. 2000). In *Pseudomonas aeruginosa* this disruption rendered them unable to form a thick and differentiated biofilm, caused alterations in quorum sensing and virulence (Rashid et al. 2000b), and affected their swimming, swarming, and twitching motilities (Rashid and Kornberg 2000). Disruption of *ppk* in *Porphyromonas gingivalis*, one of the agents causing periodontitis, also resulted in deficient biofilm formation (Chen et al. 2002). Disruption of *ppk* in *Shigella* sp. and *Salmonella* spp. produced growth defects, defective responses to stress and starvation, intolerance to acid and heat, and diminished invasiveness in epithelial cells (Kim et al. 2002), while disruption of *ppk* in *B. cereus* resulted in addition to defects in motility and biofilm formation, in a defective sporulation (Shi et al. 2004). The *null* mutant of *ppk1* in *Myxococcus xanthus* was defective in social motility, overproduced pilin protein on the cell surface, was delayed in fruiting body formation, produced fewer spores, was delayed in germination, and had reduced predation, while the *null* mutant in poly P : AMP phosphotransferase (*pap*) showed only slightly reduced abilities in development and predation (Zhang et al. 2005). In agreement with all these results it has been shown that a modification in the *ppk* gene of *H. pylori* that led to higher enzymatic activity of PPK resulted in a better capacity of the bacteria to colonize mice, suggesting that PPK is an important virulence factor (Ayraud et al. 2003). In contrast with these results, although *ppk1* inactivation caused the expected near-complete absence of poly P in all strains, it had phenotypic effects that differed markedly among unrelated strains of *H. pylori* (Tan et al. 2005).

Recent work has shown that an insertion mutation in *ppk* casues a decrease in adaptive mutation in *E. coli* strain FC40. The results suggest a novel mechanism involving poly P that directly or indirectly regulates DNA polymerase activity or fidelity (Stumpf and Foster 2005).

6 Conclusions

Acidocalcisomes or poly P granules were found in bacteria more than 100 years ago but their study, as well as the study of the main constituent, poly P, has been neglected for many years. The conservation of this organelle from bacteria to humans implies important functions that await discovery. Further studies are necessary to understand the biogenesis and function of acidocalcisomes in bacteria, and how widely distributed the organelle is.

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Bacterial Glycogen Inclusions: Enzymology and Regulation of Synthesis

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Abstract Many bacteria accumulate amylopectin-type/glycogen-type polymers as inclusion bodies. The synthesis of bacterial glycogen occurs with synthesis of its glycosyl donor, adenosine diphosphate–glucose (ADP-Glc), and then its utilization for synthesis of the α-1,4 glucosidic bond in the glycogen molecule. This reaction is catalyzed by glycogen synthase. After extensive elongation of the α-glucosidic chain, branching enzyme then catalyzes formation of α-1,6 linked branch chains. The synthesis of ADP-Glc is catalyzed by ADP-Glc pyrophosphorylase. This is the first committed step in bacterial glycogen synthesis and is allosterically regulated. The enzyme is activated by glycolytic intermediates. The nature of the activator is dependent on the major carbon assimilatory pathway dominant in the organism. For example, the enterics that utilize glycolysis for glucose utilization have fructose-1,6-bisphosphate as the activator, while cyanobacteria, being oxygenic photosynthesizers, have

3-phosphoglycerate as the activator. The allosteric inhibitors are adenosine monophosphate, orthophosphate or ADP, indicating that ADP-Glc and glycogen synthesis are also controlled by the energy status of the cell. The enzymatic and structural properties of the three glycogen biosynthetic enzymes are reviewed and the regulatory properties of the various bacterial ADP-Glc pyrophosphorylases are compared.

1
Introduction: Is Glycogen a Reserve Storage Compound?

A good number of bacteria accumulate glycogen, a polysaccharide containing only glucose with α-1,4 linkages and α-1,6 linked branched oligosaccharide chains (Fig. 1). It has a molecular weight of about 10^7–10^8 and is considered to be a storage compound, acting as a carbon source and providing energy for the organisms that accumulate them. Wilkinson (1959) proposed that for a compound to be classified as having energy-storage function, it must satisfy three criteria:

1. It must be accumulated intracellularly under conditions when the energy supply for growth of the organism is in excess.
2. It must be utilized when exogenous supplies of energy carbon are no longer available for maintenance of growth or other processes necessary to sustain viability. Examples of processes sustained by glycogen presence in the cell were energy needed for osmotic regulation, for maintenance of intracellular pH, for motility, for turnover of proteins and nucleic acids, or for physiological processes such as sporulation and en-

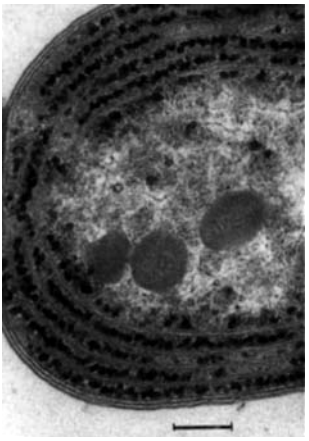

Fig. 1 *Anacystis nidulans* cell showing numerous dark-staining glycogen-type storage granules between the photosynthetic membranes (cells were fixed in glutaraldehyde followed by osmium tetroxide, and the section was poststained with lead citrate). The *bar* represents 0.25 μm. (Courtesy of Elisabeth Gantt, University of Maryland)

cystment. The energy needed for the latter processes may not be necessarily involved in cell division and growth, and were defined as energy of maintenance.

3. Probably the most important criterion is that the proposed storage compound is degraded to an energy form that is utilized by and enables the cell to survive or attend better in the media or environment.

Studies indicating whether glycogen fits these criteria will be briefly discussed. However, the main purpose of this review is to present the current information known about the various enzymatic processes involved in bacterial glycogen synthetic pathways and the associated allosteric and genetic regulatory phenomena. Previous reviews (Preiss 1969, 1973, 1978, 1984, 1989, 1996a, b, 2000, 2002; Ballicora et al. 2003; Dawes and Senior 1973; Krebs and Preiss 1975; Preiss et al. 1983; Preiss and Romeo 1989, 1994; Preiss and Walsh 1981) on bacterial glycogen synthesis and its regulation have appeared and will be referred to with respect to the material discussed here.

2
Occurrence, Characterization and Structure of Glycogen Inclusions

Many bacteria accumulate glycogen particularly under conditions of carbon and energy excess during growth. The structure of bacterial glycogen is similar to that observed for mammalian glycogen.

2.1
Occurrence

In a previous review (Preiss 1989), the occurrence of glycogen -like reserves was reported in over 40 different bacterial species. A number of these have been reported as inclusion bodies (Chao and Bowen 1971; Elbein and Mitchell 1973; Lang 1968) and a previous review (Shively 1974) classified these polyglucoside-containing inclusion bodies as either non-membrane-enclosed or membrane-enclosed. These bodies can be 20–100 nm in diameter in non-membrane-enclosed inclusions (Shively 1974). Membrane-enclosed polyglucose inclusions are found in some *Clostridia* strains (Shively 1974) and were found to be 160–300 nm in diameter in *Clostridium pasteuranium* (Laishley et al. 1973). The enzyme granulose synthetase was associated with the amylopectin granule (Robson et al. 1972). Thus, enzymes involved in the synthesis of the polyglucose polymer may be associated with the inclusion body. Indeed, the glycogen bodies of both cytoplasmic membranes and intracellular fractions of cellulose-grown *Fibrobacter succinogenes* were shown to contain endoglucanase and cellobiase activities (Gong and Forsberg 1993). It was proposed that cellobiase binding was functional in that the bound cel-

lobiase activity was stable to air and did not require reduced sulfhydryl group activation as did the nonbound cellobiase. Some other later publications of prokaryotic glycogen inclusions are noted in a review (Shively et al. 1988), in Lang et al. (1987) and in Iiiffe-Lee and McClarty (2000). Figure 1 shows the presence of dark staining glycogen inclusions in *Anacystis nidulans* interspersed among the photosynthetic membranes.

Glycogen synthesis usually occurs at periods of slow growth or no growth in the presence of an excess carbon source. Thus, glycogen accumulation will occur preponderantly in the stationary phase (Holme 1957). For many bacteria accumulation of glycogen occurs when growth ceases because of either depletion of an essential nutrient such as phosphate (Zevenhiuzen 1966), nitrogen (Holme 1957; Holme and Palmstierna 1956; Madsen 1963; Segel et al. 1965; Sigal et al. 1953; Strange et al. 1961) or sulfur (Segel et al. 1965; Zevenhiuzen 1966). In *Escherichia coli* B glucose-grown cells limited with nitrogen (Holme 1957), there is an inverse relationship in the rate of growth and the quantity of glycogen accumulated. Some exceptions have been reported for *Streptococcus mitis* (Gibbons and Kapsirnalis 1963) and *Rhodopseudomonas capsulata* (Eidels and Preiss 1970), where glycogen accumulates to optimal levels in an exponential phase of growth.

Glycogen does not appear to be required for bacterial growth as glycogen -deficient mutants of *E. coli* (Cattaneo et al. 1969; Creuzat-Sigal et al. 1972; Govons et al. 1973; Schwartz 1966), *Salmonella typhimuriurn* (Steiner and Preiss 1977) and *Clostridium pasteurianum* (Darvill et al. 1977), grow as well as their normal wild-type strains.

Glycogen accumulates in a number of organisms during sporogenesis as seen in *Clostridia* strains (Mackey and Morris 1971; Strasdine 1968, 1972). In species of *Anabaena*, glycogen accumulation is dependent on photosynthesis (Sarma and Kanta 1979). Large quantities accumulated in those cells also undergoing sporulation and glycogen did not accumulate in the dark or in the light in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). DCMU is an inhibitor of photophosphorylation, suggesting glycogen was accumulating under conditions where energy was abundant.

2.2 Characterization of Bacterial Glycogen Structure

Bacterial glycogen is similar to mammalian glycogen in having mainly α-(1,4)-glucosyl linkages and having about 8–12% of linkages as α-(1,6) linkages. Thus, glycogen is a branched polysaccharide and Fig. 2 depicts both linkages. In *Mycobacterium smegmatis* the degree of branching decreases as the glycogen granule increases in size (Antoine and Tepper 1969a). The molecular weights of glycogen from different bacteria can vary. For example, the molecular weight of *Mycobacterium phlei* is about 1×10^8–2×10^8 (Antoine and Tepper 1969a) and is higher than that of *E. coli*, which is 8.2×10^7 (Holme et al. 1957),

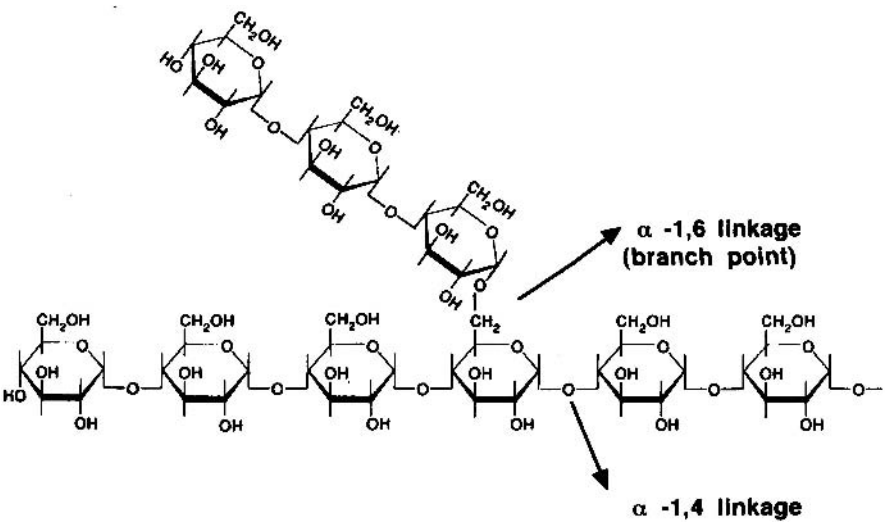

Fig. 2 Structure of a branched oligosaccharide showing the α-1,4- and α-1,6-glucosyl linkages seen in glycogen

or of that of *Enterobacter aerogenes*, which is 9.2×10^6 (Levine et al. 1953). However, it should be pointed out that these preparations were isolated by different methods and may be the reason for the molecular weight difference. The *M. phlei* glycogen was isolated by cold-water extraction, while isolation of *E. coli* utilized hot-alkali extraction. The glycogen isolated by the more drastic methods such as hot alkali or cold trichloroacetic acid may have its molecular weight reduced by chemical cleavage of glycosidic bonds. Thus, it may not be valid to compare the various reported molecular weights of the isolated bacterial glycogens when different procedures are used.

Many times the characterization of the glycogen from an organism is limited and the identification of the polysaccharide is based solely on its I_2 staining property (iodophilic polysaccharide) and sensitivity to either α-amylase or β-amylase digestion. However, some studies have determined the average chain length and percentage of α-(1,6) branching. Most bacterial glycogens studied in this manner have chain lengths of about 10–13 glucose units and I_2 spectra with a maximum absorption of 410–480 nm. The glycogens isolated from mycobacteria (Antoine and Tepper 1969a, b), *Arthrobacter* (Zevenhuizen 1966) and from thermoacidophilic archaebacteria (Konig et al. 1982), however, exhibit shorter chain lengths of about seven to nine. Other organisms (Darvill et al. 1977; Kamio et al. 1981; Nakamura et al. 2005; Slock and Stahly 1974; Strasdine 1968) have on the basis of the I_2 spectra wavelength maximum longer chain lengths and would appear to have structures closer to that of amylopectin. Thus, the bacterial α-glucans may differ in their structural properties. This could be due to the different methods used for

isolation of the glucan or may be attributable to the organism's branching enzyme or debranching enzyme activities.

3 Physiological Functions of Glycogen

Glycogen-containing cells of *E. coli* (Strange 1968), *Enterobacter aerogenes* (Strange et al. 1961), and *Streptococcus mitis* (Van Houte and Jansen 1970) present in media with no exogenous carbon source have been reported to have prolonged viability compared with their glycogen -deficient strains under starvation conditions. Under starvation conditions, *E. coli* and *Enterobacter aerogenes* (Ribbons and Dawes 1963; Strange 1968) that contain glycogen do not degrade their RNA and protein components to NH_3, whereas those cells having no glycogen do. The presence of glycogen is therefore hypothesized to preserve cellular components susceptible to turnover in the stationary phase for production of energy during starvation. However, in media containing 0.5–1.0 mM $MgCl_2$, both glycogen-containing and glycogen-deficient cells had the same survival rate (Strange 1968; Tempest and Strange 1966). $MgCl_2$ is known to increase the stability of the ribosomal components in the cell, thus decreasing the RNA and protein turnover rates (Mallete 1963; Marr et al. 1963; Pirt 1965). It is quite possible that glycogen may also preserve and maintain the intracellular Mg^{+2} concentration.

In the case of *Clostridia* species the glycogen equivalent granulose can be accumulated up to 60% of the organism's dry weight prior to the onset of sporulation (Mackey and Morris 1971; Strasdine 1968, 1972). The accumulated polysaccharide is rapidly degraded with resultant spore formation, suggesting that the granulose serves as a carbon and energy source for spore formation and maturation. Glycogen granules also appear in sporulating hyphae of *Streptomyces viridochromogenes* and reach a maximum number during early stages of maturation (Brana et al. 1980). During later stages of maturation the number of granules decreased, and granules were seen in mature spores. Glycogen also accumulates in *Bacillus cereus* early in sporulation and degrades at the time of spore maturation (Slock and Stahly 1974). Therefore, in sporulating microorganisms, it has been hypothesized that glycogen or granulose serves as an endogenous source of carbon and energy for spore maturation. Thus, many experiments suggest that glycogen plays a role in the survival of bacteria during starvation or provides energy and carbon for spore formation. The precise function of glycogen in these roles remains unclear. Also other observations seem to conflict with the concept that glycogen is an energy-storage compound. As mentioned earlier, the survival rates of magnesium-rich *E. coli* and *Enterobacter aerogenes* cells are not affected whether they contain glycogen or not (Strange 1968; Tempest and Strange 1966). Also glycogen-rich *Sarcina lutea* dies at a faster rate than cells containing no polysaccharide when starved

in phosphate-containing media (Burleigh and Dawes 1967). Further studies should be done to clarify the function of glycogen in bacteria.

Of interest is that glycogen in oral bacteria such as *Streptococcus mutans* (Huisintveld and Dirks 1978) or *Streptococcus mitis* (Gibbons and Kapsimalis 1963) plays a role in the development of dental caries (Tanzer et al. 1976; Van Houte and Saxton 1971). Consistent with the suggestion is the finding that these organisms capable of glycogen synthesis produce more acid when exogenous carbohydrate is present and produce acid in the absence of exogenous carbohydrate. This may be of significance in production of dental caries, and may be responsible for the lower resting pH that has been observed in plaque from individuals having active caries (Huisintveld and Dirks 1978). Of interest is a report showing that a mutant of *Streptococcus mutans* that synthesizes elevated levels of intracellular polysaccharide is also hypercariogenic (Spatafora et al. 1995).

4
Enzymatic Reactions Leading to Glycogen Synthesis

Bacterial glycogen synthesis requires three enzymes. One catalyzes the synthesis of the sugar nucleotide glucosyl donor, the second causes formation of the α-1,4-glucosyl linkage and the last catalyzes synthesis of the branched α-1,6-glucosyl linkage.

4.1
Adenosine Diphosphate–Glucose Pathway to Glycogen Synthesis

It is well established that the glycosyl donor for bacterial glycogen synthesis is adenosine diphosphate–glucose (ADP-Glc). ADP-Glc is synthesized from adenosine triphosphate (ATP) and α-glucose-1-phosphate (α-Glc-1-P) via a reaction catalyzed by the enzyme ADP-Glc pyrophosphorylase (PPase) (reaction 1, ATP: α-Glc-1-P adenylyltransferase, EC 2.7.7.27) (Ballicora et al. 2003; Preiss 1969, 1973, 1978; Preiss and Walsh 1981; Shen and Preiss 1965).

$$\text{ATP} + \alpha\text{-Glc-1-P} \longleftrightarrow \text{ADP-Glc} + \text{pyrophosphate} \quad (1)$$

The next reaction involves the transfer of the glucosyl unit ADP-Glc to a glucan or a maltodextrin primer to form a new α-1,4-glucosyl linkage. An ADP-Glc specific glycosyl transferase, glycogen synthase (ADP-Glc: 1,4-α-D-glucan 4-α-glucosyl transferase, EC 2.4.1.21) catalyzes this reaction and it is seen as reaction 2 (Fox et al. 1976; Greenberg and Preiss 1964).

$$\text{ADP-Glc} + \alpha\text{-1,4-glucan} \rightarrow \alpha\text{-1,4-glucosyl glucan} + \text{ADP} \quad (2)$$

As indicated before, 10% of the total linkages found in glycogen are α-1,6-glucosyl bonds. They are formed in a reaction catalyzed by branching enzyme

(α-1,4-glucan:α-1,4-glucan-6 glycosyltransferase, EC 2.4.1.18). Branching-enzyme activity (reaction 3) has been observed in many bacteria; in enterics (Boyer and Preiss 1977; Holmes et al. 1982; Preiss et al. 1976; Sigal et al. 1965; Steiner and Preiss 1977), in *Arthrobacter globiformis* (Zevenhuizen 1964) and in photosynthetic bacteria (Greenberg et al. 1983; Preiss and Greenberg 1981; Preiss et al. 1980).

$$\text{Linear } \alpha - 1,4 \text{ chain} \rightarrow \text{branched } \alpha\text{-}1,4, \alpha\text{-}1,6 \text{ chain} \quad (3)$$

4.2 Alternative Pathways Towards Synthesis of α-1,4-Glucan

Some α-glucans similar to glycogen in structure are formed either from sucrose or maltose in reactions catalyzed, respectively, by amylosucrase (reaction 4) and amylomaltase (reaction 5).

$$\text{Sucrose} + (1,4\text{-}\alpha\text{-D-glucosyl})_n \rightarrow \text{D-fructose} + (1,4\text{-}\alpha\text{-D-glucosyl})_{n+1} \quad (4)$$

$$\text{Maltose} + (1,4\text{-}\alpha\text{-D-glucosyl})_n \rightarrow \text{D-glucose} + (1,4\text{-}\alpha\text{-D-glucosyl})_{n+1} \quad (5)$$

Neisseria strains, having amylosucrase, accumulate large amounts of α-1,4-glucan when grown on sucrose (Hehre and Hamilton 1946, 1948; Hehre et al. 1949; Hestrin 1960; MacKenzie et al. 1977; Okada and Hehre 1974). Amylosucrase, however, is present in only a few bacterial species and is only induced if sucrose is available as a carbon source. *Neisseria* does not synthesize sucrose. Thus, the role of amylosucrase in the synthesis of α-1,4-glucans in bacteria is limited.

Amylomaltase, the enzyme that catalyzes reaction 5, is induced by the presence of maltose in the media along with a number of other enzymes involved in maltose metabolism in various strains of *E. coli* and *Aerobacter aerogenes* (Palmer et al. 1973), *Streptococcus mitis* (Walker 1966), *Streptococcus pyogenes* (McFarland et al. 1981), *Streptococcus mutans* (Palmer et al. 1973), *Diplococcus pneumoniae* (Lacks 1968), and *Pseudomonas stutzeri* (Wober 1973). Glucose in the media will repress synthesis of amylmaltase (Chao and Weathersbee 1974) and thus is not active when glycogen accumulates in glucose-grown organisms.

α-1,4-Glucans are also formed in reactions catalyzed by maltodextrin phosphorylase or glycogen phosphorylase (reaction 6). These enzymes are present in many different bacterial cell extracts (Hestrin 1960; Preiss and Walsh 1981).

$$\alpha(\text{-}1,4\text{-glucosyl})_{n+1} + \text{orthophosphate} \longleftrightarrow \alpha\text{-Glc-1-P} + \alpha(\text{-}1,4\text{-glucosyl})_n \quad (6)$$

Maltodextrin phosphorylase is only induced by maltose or maltodextrins and the enzyme has as its function to degrade maltodextrins to Glc-1-P. *E. coli* mutants, deficient in maltodextrin phosphorylase, accumulate maltodextrins and this lends support to maltodextrin phosphorylase being a degradative enzyme of α-1,4-glucans rather than a synthetic one (Schwartz 1965). Moreover, the activity of glycogen phosphorylase present in bacterial extracts is usually quite low to account for the rate of glycogen accumulation seen (Chen and Segal 1968a, b; Khandelwal et al. 1973).

Thus, most of the glycogen accumulated in bacteria is through the ADP-Glc pathway. This view is supported by data showing that glycogen-deficient or glycogen-excess mutants of *E. coli* (Cattaneo et al. 1969; Damotte et al. 1968; Govons et al. 1973; Krebs and Preiss 1975; Preiss 1969, 1973, 1978, 1984, 1989, 1996a, b; Preiss et al. 1970, 1971, 1975, 1976; Preiss and Walsh 1981) or of *Salmonella typhimurium* (Steiner and Preiss 1977) are mutants affected in ADP-Glc PPase, glycogen synthase or branching enzyme activities.

Forty-two different bacterial strains whose extracts contained substantial levels of ADP-Glc PPase and glycogen synthase were listed in a previous review (Preiss and Walsh 1981). Since that review other studies (Greenberg et al. 1983; Yung et al. 1984) and other reviews (Ballicora et al. 2003; Preiss 1984, 1989, 1996a, b, 2000, 2002) have reported an increased number of bacterial strains shown to contain the glycogen biosynthetic enzymes (e.g., *Aeromonas liquefaciens*, *Edwardsiella tarda*, *Enterobacter hafniae* and *Rhodopseudmonas blastica*).

5
Properties of the Glycogen Biosynthetic Enzymes

Present studies indicate that ADP-Glc PPase is similar to other sugar nucleotide pyrophosphorylases with respect to catalytic mechanism and catalytic sites. The catalytic and substrate sites of glycogen synthase have been determined to be similar to those of other retaining glycosyl transferases and branching enzyme is a member of the α-amylase family.

5.1
ADP-Glc PPase

The ADP-Glc PPase is an allosteric enzyme activated by glycolytic intermediates and inhibited by either adenosine monophosphate (AMP), ADP or orthophosphate (P_i).

5.1.1 Subunit and Quaternary Structure

ADP-Glc PPase has been purified from a number of microorganisms and has been found to be homotetrameric in structure with a subunit molecular mass of about 50 kDa. The only exception noted is with *Bacillus stearothermophilus* ADP-Glc PPase (Takata et al. 1997). It has been determined that in *B. subtilis* and *B. stearothermophillus* the genes for glycogen synthesis are clustered in one operon, *glgBCDAP* (Kiel et al. 1994; Takata et al. 1997). A comparative analysis of the gene cluster showed that *glgC* and *glgD* encode for proteins homologous to ADP-Glc PPases from prokaryotes. Thus, the putative GlgC protein from *B. stearothermophillus* has 387 amino acids, with a predicted molecular mass of 43.3 kDa and shares 42–70% identity with bacterial ADP-Glc PPases. The GlgD product is a shorter protein (343 amino acids and a predicted molecular mass of 38.9 kDa) with a lower homology to ADP-Glc PPase (20–30% identity) (Takata et al. 1997). The expression of the *glgC* gene from *B. stearothermophillus* rendered an active recombinant enzyme; whereas GlgD exhibited negligible activity. However, when *glgC* and *glgD* genes were expressed together, the resulting GlgCD protein exhibited higher affinity for substrates and twofold higher V_{max} in catalyzing ADP-Glc synthesis than GlgC by itself.

The *Bacillus* enzyme is therefore similar to higher-plant ADP-Glc PPases which are also heterotetrameric, with an $\alpha_2\beta_2$ heterotetrameric structure (Preiss and Sivak 1998a, b). The plant small subunit has been shown to be the catalytic subunit and the large subunit, the regulatory subunit (Okita et al. 1990; Preiss and Sivak 1998a, b). The potato tuber ADP-Glc PPase catalytic subunit expressed in *E. coli* is active, while the large subunit by itself shows no activity. Expression of both subunits results in a heterotetramer having higher affinity for the allosteric activator and lower affinity for the inhibitor than seen. Therefore, the large subunit is regarded as the regulatory subunit. The *B. stearothermophilus* GlgC subunit shows a 42–70% similarity with other ADP-Glc PPases and when expressed in *E. coli* alone has catalytic activity. The *Bacillus* GlgD subunit has no activity and its function is unknown; however, it seems to increase the V_{max} of the GlgC activity and slightly increases the apparent affinity of the enzyme to its substrates.

It is of interest to compare further the plant enzyme subunit and quaternary structure with the bacterial enzymes. The spinach leaf ADP-Glc PPase was shown to have two distinct subunits (Morell et al. 1987). Other immunological studies with maize endosperm indicated that both endosperm and leaf tissues had ADP-Glc PPases with two different subunits that are products of two genes (Preiss and Sivak 1998a, b). ADP-Glc PPases from all plants and green algae characterized so far are composed of α and β subunits, forming the heterotetrameric structure, $\alpha_2\beta_2$ (Frueauf et al. 2003; Preiss 1991; Sivak and Preiss 1998). These subunits are defined as "small" (α subunit,

50–54 kDa) and "large" (β subunit, 51–60 kDa). The difference in mass between the small and large subunits in potato tuber is not more than 1 kDa (Nakata et al. 1991, 1994). The small subunit of the higher-plant ADP-Glc PPase is highly conserved (85–95% identity), whereas the large subunit is less conserved (50–60% identity) (Smith-White and Preiss 1992). Nevertheless, both subunits are most likely derived from the same ancestor, on the basis of the high homology of the conserved regions in the two different subunits. Recent experiments have shown that replacement of just two amino acids in the large subunit that are equivalent to amino acids present in the small subunit can convert the large subunit to a catalytically active subunit (Ballicora et al. 2005). These results make it even more highly likely that both subunits are derived from the same ancestor.

5.1.2
Allosteric Properties of ADP-Glc PPase: Activator and Inhibitor Specificity

For almost all bacterial systems studied, glycolytic intermediates are the activators for ADP-Glc PPase activity. The glycolytic effectors at saturation provide maximal activity, and they may be markers or signals of energy excess. Therefore, they are utilized for synthesis of an energy reserve substituent.

The finding that regulation occurs at the ADP-Glc synthesis step is in agreement with the concept that regulation of a biosynthetic pathway occurs at the first novel step in the pathway.

The ADP-Glc PPases have been previously grouped into different classes (Ballicora et al. 2003, 2004; Preiss 1973, 1984, 1991, 1996) on the basis of specificity for activator and inhibitor. Those previous classifications can be updated and brought to nine distinctive classes of ADP-Glc PPases (Table 1) and enclose recent studies on the properties of gram-positive bacterial enzymes (Takata et al. 1997) and of endosperm tissue of higher plants (Gomez-Casati and Iglesias 2002).

Class I includes ADP-Glc PPases from bacteria having glycolysis as their main carbon disposition pathway. These microorganisms are typically enterobacteria, *E. coli*, *Salmonella typhimurium*, etc., and the enzymes are mainly regulated by fructose-1,6-bisphosphate (Fru-1,6-bisP), the activator, and AMP, the inhibitor (Preiss et al. 1966). The enzymes from class I are encoded by a single gene, giving rise to a native homotetrameric structure (α_4) of molecular mass about 200 kDa (Preiss 1991; Preiss and Romeo 1994). Other bacteria utilizing glycolysis as their major sugar utilization pathway contain ADP-Glc PPases activated not only by Fru-1,6-bisP but also by fructose-6-phosphate (Fru-6-P) and inhibited by AMP and ADP and are placed in class II. Those ADP-Glc PPases having no activator yet still inhibited by AMP are listed in class III (Table 1). The enzymes placed in class IV are from bacteria mainly utilizing the Entner–Doudoroff pathway, and the ADP-Glc PPases are distinctively activated by Fru-6-P and pyruvate, with ADP, AMP and P_i

Table 1 Relationship between carbon metabolism and regulatory properties of ADP–glucose (*ADP-Glc*) pyrophosphorylase (*PPase*) of different organisms

Organism	Main carbon utilization pathway	Class	ADP-Glc PPase Allosteric effectors Activator(s)	Inhibitor(s)
Prokaryotes				
Escherichia coli *Salmonella typhimurium* *Enterobacter aerogenes*	Glycolysis	I	Fru-1,6-bisP	AMP
Aeromonas formicans *Micrococcus luteus* *Mycobacterium smegmatis* *Rhodopseudomonas viridis*	Glycolysis (+ reductive carboxylic acid cycle)	II	Fru-1,6-bisP, Fru-6-P	AMP, ADP
Serratia marcescens *Enterobacter hafniae* *Clostridium pasteurianum*	Glycolysis	III	None	AMP
Agrobacterium tumefaciens *Arthrobacter viscosus* *Chromatium vinosum* *Rhodobacter capsulata* *Rhodomicrobium vaneilli*	Entner–Doudoroff pathway (+ reductive carboxylic acid cycle)	IV	Pyruvate, Fru-6-P	AMP, ADP
Rhodobacter gelatinosa *Rhodobacter globiformis* *Rhodobacter sphaeroides* *Rhodocyclus purpureus*	Glycolysis, Entner–Doudoroff pathway, and reductive carboxylic acid cycle	V	Pyruvate, Fru-6-P, and Fru-1,6-bisP	AMP, P_i
Rhodosprillum rubrum *Rhodosprillum tenue*	Reductive carboxylic acid cycle	VI		
Bacillus subtilis *Bacillus stearothermophillus*	TCA cycle during sporulation	VII	None	None
Cyanobacteria				
Synechococcus PCC 6301 *Synechocystis* PCC 6803 *Anabaena* PCC 7120	Oxygenic photosynthesis (Calvin cycle)	VIII	3PGA	P_i
Eukaryotes Green algae				
Chlorella fusca *Chlorella vulgaris* *Chlamydomonas reinhardtii*	Oxygenic photosynthesis (Calvin cycle)	VIII	3PGA	P_i

Table 1 (continued)

Organism	Main carbon utilization pathway	Class	ADP-Glc PPase Allosteric effectors Activator(s)	Inhibitor(s)
Higher plants				
Photosynthetic tissues				
Plant leaves; e.g., spinach wheat, pea, *Arabidopsis*, maize, rice	Oxygenic photosynthesis (Calvin or Hatch–Slack pathway)	VIII	3PGA	P_i
Nonphotosynthetic tissues				
Potato tuber, maize, endosperm	Sucrose catabolism and gluconeogenesis	VIII	3PGA	P_i
Barley and wheat endosperm	Sucrose catabolism and gluconeogenesis	IX	3PGA and Fru-6-P reverses P_i inhibition	P_i

ADP adenosine diphosphate, *AMP* adenosine monophosphate, *Fru-6-P* fructose-6-phosphate, *Fru-1,6-bisP* fructose-1,6-bisphosphate, P_i orthophosphate

behaving as inhibitors (Eidels et al. 1970; Uttaro et al. 1998). In class IV are photosynthetic organisms capable of utilizing the anaerobic photosynthetic reductive carboxylic acid pathway; *Chromatium vinosum*, *Rhodobacter capsulata*, and *Rhodomicrobium vaneilli*.

Of interest are those ADP-Glc PPases from organisms capable of having the Embden–Meyerhoff and Entner–Doudoroff pathways as well as the anaerobic photosynthetic reductive carboxylic acid pathway. Their ADP-Glc PPases are activated by three main effectors: Fru-1,6-bisP, Fru-6-P as well as pyruvate. This third activator places them in class V (Table 1) (Greenberg et al. 1983; Igarashi and Meyer 2000).

Class VI includes ADP-Glc PPases from anaerobic bacteria capable of growing under either heterotrophic conditions in the dark or autotrophic conditions in the light under anoxygenic photosynthesis (Table 1). These organisms cannot catabolize glucose but can grow very well on carbon sources such as pyruvate and tricarboxylic acid cycle (TCA) intermediates. Enzymes from class VI are specifically regulated by pyruvate (Table 1) (Furlong and Preiss 1969; Yung and Preiss 1981).

ADP-Glc PPases grouped as class VII are enzymes from sporulating *Bacilli* (Table 1). These microorganisms synthesize glycogen during sporulation, a process for survival in hostile environments (Takata et al. 1997). Under the latter conditions, the main pathway for carbon utilization is the TCA pathway that fully metabolizes by-products of glycolysis (Matsuno et al. 1999). The different recombinant enzymes from *B. stearothermophillus* were insensitive

to regulation by different metabolites typically affecting the activity of other bacterial ADP-Glc PPases (Takata et al. 1997). Thus, the enzymes grouped in class VII in Table 1 are very distinct from other ADP-Glc PPases, as they are apparently unregulated enzymes, being the only bacterial ADP-Glc PPases that exhibit a heterotetrameric structure of the type $\alpha_2\beta_2$.

The last group of bacterial ADP-Glc PPases are those from cyanobacteria, prokaryotes that fix CO_2 in an oxygenic photosynthetic process as seen in plants (class VIII, Table 1). These enzymes have 3-phosphoglycerate (3PGA) as the main activator and P_i as the inhibitor (Charng et al. 1992; Iglesias et al. 1991, 1992). Thus, the specificity for allosteric regulators of the cyanobacterial ADP-Glc PPase is identical to those of the eukaryotic photosynthesizers, the green algae and higher plants, also classified in class VIII (Table 1) (Iglesias et al. 1991). All these photosynthetic organisms utilize either the reductive pentose phosphate pathway (Calvin cycle) or the Hatch–Slack pathway utilizing atmospheric CO_2, to synthesize 3PGA as the first intermediate product. P_i, the inhibitor of the photosynthetic ADP-Glc PPases, decreases in concentration under light conditions as it is utilized to synthesize ATP through photophosphorylation (Iglesias and Podestá 1996). Therefore, class VIII ADP-Glc PPases are regulated by the 3PGA-to-P_i ratio under physiological conditions (Iglesias and Podestá 1996; Preiss 1991; Preiss and Sivak 1998; Sivak and Preiss 1998).

The ADP-Glc PPases from nonphotosynthetic tissues of higher plants can be distinguished as two different types (Table 1). The potato tuber ADP-Glc PPase from reserve tissues is typically activated by 3PGA and inhibited by P_i and is thus grouped as class VIII (Table 1) (Ballicora et al. 1995; Iglesias et al. 1993). The ADP-Glc PPases from reserve tissues of cereals have been shown to exhibit distinctive regulatory properties, such as a lower sensitivity to activators (Gomez-Casati and Iglesias 2002; Hylton and Smith 1992; Kleczkowski et al. 1993; Plaxton and Preiss 1987; Rudi et al. 1997; Weber et al. 1995). However, a complete characterization of the purified wheat endosperm ADP-Glc PPase (Gomez-Casati and Iglesias 2002) has shown that the enzyme is regulated in a different manner by metabolites. The wheat endosperm enzyme is allosterically inhibited by P_i, ADP and Fru-1,6-bisP. These inhibitions can be reversed by 3PGA and Fru-6-P. In the absence of the inhibitors however, 3PGA and Fru-6-P have no effect on enzyme activity. Thus, the wheat endosperm enzyme has distinctive regulatory properties accounting for a class IX group of ADP-Glc PPases (Table 1).

Of interest is that the cyanobacterial ADP-Glc PPase is homotetrameric in quaternary structure as seen for the ADP-Glc PPases from other bacteria in Table 1. However, it is regulated as, and is more related immunologically to, the plant enzymes (Charng et al. 1994; Iglesias et al. 1991). The main difference between the cyanobacterial and plant ADP-Glc PPases is the quaternary structure of α_4 for bacterial enzymes and $\alpha_2\beta_2$ for plants (Charng et al. 1994).

5.1.3 Allosteric Regulation of Bacterial ADP-Glc PPase is Functional, In Vivo

Much evidence has accumulated to indicate that the allosteric regulation observed *in vitro* is functional *in vivo* in bacteria as in plants. The evidence for the regulation being important in the modulating bacterial glycogen synthesis has been obtained from a study of *E. coli* B. (Govons et al. 1973; Preiss 1969, 1973, 1978, 1984, 1989, 1996a, b, 2000, 2002; Preiss et al. 1976b), *E. coli* K12 (Cattaneo et al. 1969; Creuzat-Sigal et al. 1972) and *Salmonella typhimurium* LT-2 (Steiner and Preiss 1977) mutants that are affected in their ability to accumulate glycogen and having ADP-Glc PPases modified in their allosteric properties. Mutants accumulating glycogen at a faster rate than their parent strain either had higher apparent affinity for the activator, Fru-1,6-bisP, or lower affinity for the inhibitor, AMP. *E. coli* deficient glycogen mutant SG14, which synthesized glycogen at 40% the rate of the wild type, had a lower affinity for the activator, Fru-1,6-bisP (Preiss et al. 1971). The mutant could no longer be activated by NADPH and could only be poorly activated by pyridoxal phosphate (pyridoxal-P), thus suggesting that Fru-1,6-bisP is the normal physiological activator. These data have been discussed in detail in several reviews (Ballicora et al. 2003, 2004; Preiss et al. 1983; Preiss 1996a, b; Preiss and Walsh 1981). Other data suggesting a causal relationship existing between the rate of glycogen accumulation and Fru-1,6-bisP concentration have been obtained by Dietzler et al. (1974, 1975).

It has also been established ADP-Glc PPase is also an important regulatory enzyme for plant starch biosynthesis. *Chlamydomonas reinhardtii* starch-deficient mutants have been isolated and a mutant was shown to have an ADP-Glc PPase that could not be activated by 3PGA (Ball et al. 1991). Support for physiological allosteric regulation by ADP-Glc PPase has also been obtained in *Arabidopsis thaliana* (Lin et al. 1988a, b). One mutant, TL25, lacked both subunits and accumulated only 2% of the starch seen in the normal plant (Lin et al. 1988a), indicating that starch synthesis is almost completely dependent on the synthesis of ADP-Glc. Another mutant, TL46, was starch-deficient and lacked the regulatory 54-kDa ("large") subunit (Lin et al. 1988b). The mutant had only 7% of the wild-type activity and subsequent studies (Neuhaus and Stitt 1990) showed that in high light (photosynthesis) the rate of starch synthesis of TL46 was only 9% and at low light only 26% of the rate of the wild type. This is supporting evidence that the regulation of ADP-Glc PPase is of *in vivo* importance. Moreover, a maize mutant has also been isolated where the ADP-Glc PPase was less sensitive to the inhibition by P_i than the normal enzyme. The mutant endosperm had 15% more dry weight and starch than the normal endosperm (Giroux et al. 1996). In both potato tuber (Stark et al. 1992) and wheat endosperm (Smidansky et al. 2002), genetic modification of ADP-Glc PPase activity led to higher amounts of starch. The allosteric mutant ADP-Glc PPases from maize endosperm and *Chlamydomonas reinhardtii* and

the resultant effects on starch synthesis provide strong evidence that even in plants the allosteric effects observed *in vitro* are operative in the *in vivo* situation (Ball et al. 1991; Giroux et al. 1996; Lin et al. 1988a, b; Preiss and Sivak 1998a, b; Sivak and Preiss 1998; Stark et al. 1992; Van den Koornhuyse et al. 1996).

5.1.4
Substrate and Allosteric Effector Sites. Identification of Important Amino Acid Residues for Regulation and Catalysis. Specificity of Activator

The structural requirements for the allosteric activator site of the *E. coli* ADP-Glc PPase can be satisfied by metabolites having two phosphate residues spatially situated apart as observed in Fru-1,6-bisP. Thus, sedoheptulose, 1,7-bisphosphate, 1,6-hexanediol bisphosphate and glycerate 1,3-bisphosphate have been shown to be potent activators of the enzyme (Preiss 1969, 1973, 1978, 1984, 1996a, b, 2002; Preiss and Walsh 1981). NADPH may be considered as an analog of Fru-1,6-bisP as a portion of its structure can be viewed as ribose 2,5-bisphosphate. However, some highly active effectors have only one phosphate plus an aldehyde or a carboxyl group (erythrose 4-phosphate, 4-pyridoxic acid-5-phosphate, 2-phosphate glycerate, phosphoenolpyruvate, pyridoxal-P and 4-pyridoxic acid 5-phosphate). Obviously basic amino acid residues in the effector binding site are important in the binding of the activators, and thus binding of the potential activators requires that they contain at least one phosphate residue and another anionic group, carboxyl or phosphate. If one of the protein binding residues is an ϵ-amino group of lysine, an aldehyde group may bind by Schiff base formation. Three-dimensional space-filling models indicate that the distance between the two phosphate groups of Fru-1,6-bisP either in furanose or in open-chain form is approximately equal to the distance from the aldehyde group to the phosphate group in pyridoxal-P. Evidence for all these diverse metabolites binding to the same site has been seen in kinetics (Gentner et al. 1969; Preiss et al. 1966) and binding data (Haugen and Preiss 1979).

5.1.5
Chemical Modification and Site-Directed Mutagenesis of Activator Sites

Chemical modification studies of the *E. coli* enzyme with [^{3}H]-pyridoxal-P (PLP) by reduction with $NaBH_4$ showed that it is covalently modified. PLP was bound to two different lysine residues. Fru-1,6-bisP protected binding to Lys39 and substrate ADP-Glc protected binding to Lys195 (Parsons and Preiss 1978a, b), suggesting that Lys39 was important for activator Fru-1,6-bisP binding and that Lys195 was important for substrate binding. The Lys39-PLP modified enzyme was highly active and the enzyme no longer required an allosteric activator for activity after incorporation of 0.5 mol of PLP

per mole of subunit (Parsons and Preiss 1978a). Allosteric activators, Fru-1,6-bisP and 1,6-hexanediol bisphosphate, as well as the inhibitor 5′-AMP protected against incorporation of pyridoxal-P, suggesting that the pyridoxal-P was bound at the activator site. Site-directed mutagenesis of the Lys39 with glutamate showed that that lysine is vital for the interaction of the activator Fru-1,6-bisP to the enzyme (Gardiol and Preiss 1990).

There is a predominance of lysine and arginine residues in the sequence especially in close proximity to the phosphopyridoxylated lysine. The activator site has six positively charged amino acid residues about the phosphopyridoxylated lysine: arginine at residues 28, 31 and 39; lysine at residues 33, 38 and 41 (Fig. 3). As indicated before, two basic residues of the protein have been suggested as being required for activator binding, and thus the sequence from residues 28 to 41 has the potential for containing residues to bind the phosphates, carboxyl or the aldehyde groups of the activator.

The various arginine residues at the allosteric site should be noted. Chemical modification with phenylglyoxal suggested the presence of an essential arginine at the allosteric activator site (Carlson and Preiss 1981, 1982). The modification resulted in a lessened ability of the activator Fru-1,6-bisP to stimulate activity. The apparent affinity for Fru-1,6-bisP and the V_{max} of the reaction at saturating concentrations of Fru-1,6-bisP were decreased by the arginine modification. Both 1,6-hexanediol bisphosphate and Fru-1,6-bisP were able to partially protect the enzyme from inactivation. Additional studies are required to determine the location of the modified arginine.

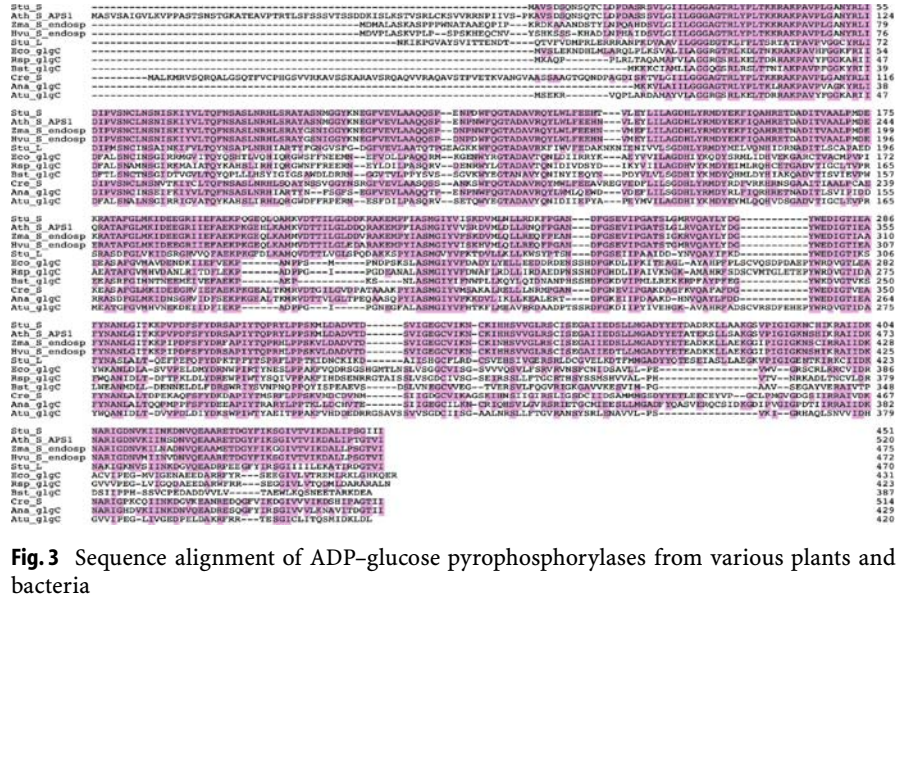

Fig. 3 Sequence alignment of ADP–glucose pyrophosphorylases from various plants and bacteria

Arginine residues were found in ADP-Glc PPases to be functionally important as shown by chemical modification with phenylglyoxal (Iglesias et al. 1992; Sheng and Preiss 1997). Alanine scanning mutagenesis of ADP-Glc PPase from *Anabaena* PCC 7120 indicated that Arg294 plays a role in inhibition by P_i. Recently, it was shown that replacement of this residue alanine or glutamine reversed the pattern of inhibitor specificity. The main inhibitor was NADPH rather than P_i (Frueauf et al. 2002). All of these results suggest that the positive charge of Arg294 may not be specifically involved in P_i binding but that it plays a role in determining inhibitor selectivity.

Alanine scanning mutagenesis of the arginine residues located in the *N*-terminal of the enzyme from *Agrobacterium tumefaciens* demonstrated the presence of separate subsites for the activators, Fru-6-P and pyruvate (Gomez-Casati et al. 2001). The R32A enzyme had reduced affinity for Fru-6-P (11.5-fold) and identical behavior to the wild type with respect to pyruvate activation. Both the R33A and R45A enzymes had higher activity than the wild-type enzyme in the absence of activators, no response to Fru-6-P, but could be partially activated by pyruvate. The mutant enzymes were also desensitized to phosphate inhibition (Gomez-Casati et al. 2001).

An analog of pyruvate, bromopyruvate, can covalently modify the *Rhodobacter sphaeroides* ADP-Glc PPase, and it appears that it is covalently linked to a cysteine residue in the protein at or near the activator site (Preiss et al. 1983). The position of that cysteine residue has not been determined.

5.1.6
Chemical Modification and Site-Directed Mutagenesis of Substrate Sites

In *E. coli* ADP-Glc PPase, site-directed mutagenesis of the Lys195 to glutamine, arginine, histidine, isoleucine or glutamate provided enzymes whose K_m for Glc-1-P was 100-fold to 10 000-fold greater than the that of the wild type (Hill et al. 1991). Kinetics constants however for ATP, Mg^{2+} and Fru-1,6-bisP were similar to those of wild-type enzyme, suggesting that this lysine is solely involved in the binding of the substrate, Glc-1-P. Moreover, K_{cat} for the glutamine mutant was similar to that of the wild type, ruling out the role of this residue in the catalytic reaction (Hill et al. 1991).

The equivalent amino acid in plant ADP-Glc PPase was found to be important also for Glc-1-P binding. Site-directed mutagenesis was done on the equivalent and conserved residue in the small (Lys198) and large (Lys213) subunits of potato tuber ADP-Glc PPase (Fu et al. 1998). Mutation of Lys198 of the small subunit with arginine, alanine or glutamic acid had little or no effect on the kinetics constants for ATP, Mg^{2+}, activator (3PGA) and inhibitor (P_i), but the apparent affinity for Glc-1-P decreased 135-fold to 550-fold. Similar mutations on Lys213 of the large subunit had a negligible effect on the affinity for Glc-1-P, indicating that Lys198 in the small subunit is directly involved in the binding of Glc-1-P and that the equivalent amino acid in the large sub-

unit is not (Fu et al. 1998). As indicated before this is consistent with the view that the large subunit does not have a catalytic role but only a modulatory one (Ballicora et al. 1995).

5.1.7
Chemical Modification and Site-Directed Mutagenesis of Catalytic Sites

An amino acid was identified as mainly involved in catalysis (Frueauf et al. 2001). Comparison with the three-dimensional structures of known PPase domains and prediction of the structure (Frueauf et al. 2001) led to the identification of highly conserved residues throughout the superfamily of PPases despite their low homology. Asp142 in the *E. coli* ADP-Glc PPase was predicted to be close to the substrate site. Site-directed mutagenesis of this residue to alanine and asparagine confirmed that the main role of Asp142 is catalytic (Frueauf et al. 2001). Kinetic analysis showed a decrease in specific activity of 4 orders of magnitude, whereas other kinetics parameters showed no significant changes.

In the PPase domain of the UDP-glucosamine PPase/*N*-acetyltransferase (GlmU) enzyme it was proposed that Arg18 could be a catalytic residue (Brown et al. 1999). Even though this residue seems to be important in ADP-Glc PPases, it is not very clear if it is directly involved in the catalytic reaction. Mutagenesis of the homologous Arg25 in the enzyme from *Agrobacterium tumefaciens* yields an enzyme with a reduced activity of 2 orders of magnitude (Gomez-Casati et al. 2001). Generally, it is expected that more dramatic effects would occur after mutation of catalytic residues. Most probably in *Agrobacterium tumefaciens* ADP-Glc PPase the catalytic residue equivalent to the *E. coli* enzyme Asp142 is Asp135.

5.1.8
Prediction of the Structure of ADP-Glc PPases

Information on the three-dimensional structure of an ADP-Glc PPase is currently available with respect to the plant enzyme potato tuber ADP-Glc PPase (Jin et al. 2005) but at present is not available for the bacterial ADP-Glc PPase. Previous to that finding, several methods were utilized to predict the ADP-Glc PPase enzyme structure (Frueauf et al. 2001; Rost and Sander 1993). A hydrophobic cluster analysis (HCA) (Lemesle-Varloot et al. 1990) was applied to several ADP-Glc PPases from different sources representing different classes according to the homology of subunits and tissue, i.e., *E. coli*, *Anabaena*, *Chlamydomonas*, potato (*Solanum tuberosum* L.) tuber "small" subunit and different "large" subunits from maize embryo, maize shrunken 2 and *Arabidopsis thaliana*. The HCA analysis suggested that ADP-Glc PPases are extremely similar in the distribution and pattern of the clusters, with both bacterial and plant enzymes. Thus, it is almost certain that the ADP-

Glc PPases share a common folding pattern, even though there is a different quaternary structure as seen for $\alpha_2\beta_2$ in plants and α_4 in bacteria including different specificities for activator in the eight groups.

If the ADP-Glc PPases from different sources have similar three-dimensional structures, their secondary structure predictions should also be similar. Thus, the sequences indicated before, as well as those from *Agrobacterium tumefaciens*, *B. stearothermophilus* and *Rhodobacter sphaeroides* were analyzed using the PHD program to predict their secondary structure (Rost and Sander 1993). The alignment helped to establish a structure for regions where the predictions may not be conclusive for one of the enzymes but may be very clear for the rest (Frueauf et al. 2001). An alignment of representative bacterial ADP-Glc PPases from each class is shown in Fig. 3. The small subunit sequences of the enzymes from *Chlamydomonas reinhardtii* and potato tuber were also included for comparative purposes with proteins from unicellular and multicellular eukaryotes (Fig. 3). From all these analyses, a general structure that fits all of these proteins can be postulated (Fig. 4). Biochemical data that support the model are also available (Frueauf et al. 2001).

Controlled proteolysis experiments are in good agreement with the model. The exposed loops are usually more sensitive to proteolytic cleavage, and other studies confirmed that proteases used cut-in sites predicted as loops

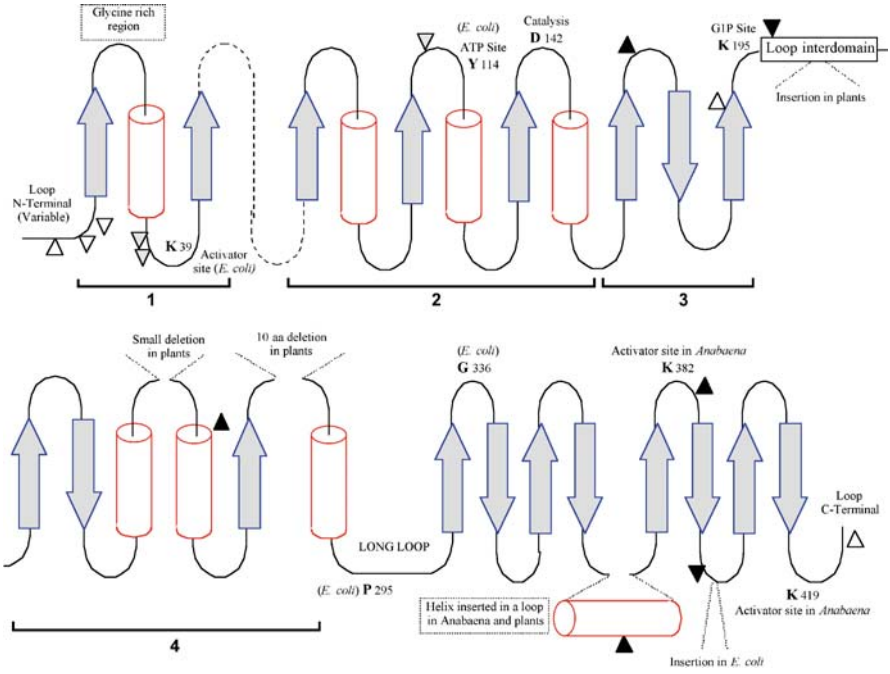

Fig. 4 Postulated secondary structure of bacterial ADP–glucose pyrophosphorylases

(Frueauf et al. 2001). The only exception is the α-helix predicted near the *C*-terminal on the *Anabaena* enzyme (Fig. 4). Since this is an insertion (20 amino acids) that is absent in *E. coli* enzyme, and is not predicted as "buried" by the PHD program, it is most likely that this helix is not part of the core but part of a loop in a domain of eight β-sheets (Fig. 4). Loops are prone to have insertions and deletions in homologous proteins, without altering the structure. In our model, all the insertions and deletions observed fall in loops (Fig. 4). The conserved amino acids known to have specific roles in the binding of substrates (*E. coli* Tyr114, Lys195) and activators (*E. coli* Lys39, *Anabaena* Lys382, Lys419) are located in loops. The residues Pro295 and Gly336 seem to be located in a region important for the regulation of the *E. coli* enzyme (Ghosh et al. 1992; Meyer et al. 1998) and are also in loops. The amino acid Asp142 in the *E. coli* enzyme was identified as a catalytic residue (Frueauf et al. 2001) and it is also situated in a loop.

A structure is observed in proteins that bind nucleotides and is also predicted in this model. Region 1 has a glycine- rich loop after a β-sheet, which is similar to a "P loop" in protein kinases or nucleotide binding sites (Saraste et al. 1990), and region 2 has three β-sheets and helices that are compatible with the Rossman fold (Rossman et al. 1974). Thus, regions 1 and 2 comprise a putative domain or subdomain that binds ATP. Moreover, Tyr114, which was shown to be reactive with the azido analog of ATP (Lee and Preiss 1986; Lee et al. 1986), is present in this region.

Structures α/β generally have a very particular topology regarding the loops. Some of them are "functional" because they carry important residues for binding and catalysis and some of the others are just "connectors" because they only connect one helix with the next sheet. It has been observed that functional loops are the ones that are located at the C-end of the β-sheets (Branden 1980). Supporting the model, those loops in regions 1, 2 and 3 are the ones that bear the most conserved amino acids. Hence, this is compatible with the idea that the ATP would be facing the "top" of the structure depicted in Fig. 4. Moreover, amino acid residues located at the loops that are at the *N*-terminal of β-sheets in regions 2 and 3 are not conserved at all. The exception is in region 1; however, there is evidence using chemical modification and site-directed mutagenesis that region 1 interacts with the activator Fru-1,6-bisP in the *E. coli* ADP-Glc PPase (Gardiol and Preiss 1990).

The first PPase domain crystallized and solved was present in a bifunctional enzyme that is the expression product of the gene *glmU* (Brown et al. 1999). One domain of the GlmU protein is an UDP-*N*-acetylglucosamine PPase and the other is an acetyltransferase. Later, other PPase domain structures were solved (Blankenfeldt et al. 2000; Kostrewa et al. 2001; Olsen and Roderick 2001; Sivaraman et al. 2002). All these structures verify the predicted secondary structure model of the ADP-Glc PPase (Frueauf et al. 2001). Regions 2, 3 and 4 are virtually identical. In region 4, the only difference is that two β-sheets were predicted rather than one because of the pres-

ence of a glycine (breaker). In the *N*-acetylglucosamine uridylyltransferase, there is only one sheet that is bent because of a glycine. Region 1 is very similar; there is a "P-loop"-like structure but our model predicted an extra β-sheet. It is possible that the prediction is wrong about this or that different sugar-nucleotide PPases vary in this region. When we predicted the secondary structure of GDPMan PPases, TDPGlc PPases, CDPGlc PPases and UDPGlc PPases this is the region with greatest variability. For this reason, the topology of the loop where Lys39 is present cannot be ascertained. The idea that the sugar-nucleotide PPases share a similar catalytic domain is supported by the observation that the homologous Glc-1-P site is present in the GDPMan PPase from *Pseudomonas aeruginosa* (May et al. 1995). The homology between ADP-Glc PPases and the *N*-acetylglucosamine uridylyltransferase is extremely low. However, an alignment has been done using the predicted structure to match helices and sheets. From the analysis several residues were found to be conserved in loops that face the substrate in the *N*-acetylglucosamine uridylyltransferase (Fig. 4). Lys195, the Glc-1-P binding site in ADP-Glc PPases, is present in the *N*-acetylglucosamine uridylyltransferase but is shifted by one position.

5.2 Bacterial Glycogen Synthase

The glycogen synthases in bacteria are specific for ADP-Glc. In systems where it has been studied the enzyme subunit size is about 50 kDa and the native form of the enzyme is either dimeric or homotetrameric.

An affinity analog of ADP-Glc, adenosine diphosphopyridoxal (ADP-pyridoxal), was used to identify the ADP-Glc binding site (Furukawa et al. 1990). Incubation of the enzyme with the analog plus sodium borohydride led to inactivation of activity. About one mole of analog per mole of enzyme subunit led to 100% inactivation. A labeled peptide was isolated after tryptic hydrolysis and the modified lysine residue was identified as Lys15. The sequence, Lys-X-Gly-Gly, where lysine is the amino acid modified by ADP-pyridoxal, has been found to be conserved in the mammalian glycogen synthase as well as in the plant starch synthases.

The structural gene for glycogen synthase, *glgA*, has been cloned from *E. coli*, *Salmonella typhimurium*, *Agrobacterium tumefaciens* and *B. stearothermophilus* (Preiss 1996a; Takata et al. 1997; Uttaro and Ugalde 1994) and their nucleotide sequences have been determined. The *E. coli* glycogen synthase sequence consists of 1431 base pairs specifying a protein of 477 amino acids with a molecular weight of 52 412. Replacement of other amino acids for lysine at residue 15 suggested that the lysine residue is mainly involved in binding the phosphate residue adjacent to the glycosidic linkage of the ADP-Glc and not in catalysis (Furukawa et al. 1990, 1993). The major effect on the kinetics of the mutants at residue 15 was the elevation of the K_m value

of ADP-Glc, of about 30-fold to 50-fold when either glutamine or glutamic acid was the substituted amino acid. Substitution of alanine for glycine at residue 17 decreased the catalytic rate constant, K_{cat}, about 3 orders of magnitude compared with the wild-type enzyme. Substitution of alanine for Gly18 only decreased the rate constant 3.2-fold. The K_m effect on the substrates, glycogen and ADP-Glc, was minimal. It was postulated that the two glycyl residues in the conserved Lys-X-Gly-Gly sequence participated in the catalysis by assisting in maintaining the correct conformational change of the active site or by stabilizing the transition state.

There is still appreciable catalytic activity of the Lys15Gln mutant. The ADP-pyridoxal modification was then repeated with the K15Q mutant and in this instance about 30-times higher concentration was needed for effective inactivation of the enzyme by ADP-pyridoxal. The enzyme was maximally inhibited by about 80% and tryptic analysis of the modified enzyme yielded one peptide containing the affinity analog and having a sequence Ala-Glu-Asn-modified Lys-Arg. The modified lysine was identified as Lys277 (Furukawa et al. 1994). Site-directed mutagenesis of Lys277 to form a glutamine mutant was done and the K_m for ADP-Glc was essentially unchanged but K_{cat} was decreased 140-fold. It is concluded that Lys277 was more involved in the catalytic reaction than in substrate binding.

A cysteine-specific reagent 5,5′-dithiobis(2-nitrobenzoic acid) was shown to inactivate the *E. coli* glycogen synthase (Holmes and Preiss 1982). To determine the residue responsible, all cysteines present in the enzyme, Cys7, Cys379 and Cys408, were substituted with serine. 5,5′-Dithiobis(2-nitrobenzoic acid) modified and inactivated the enzyme if and only if Cys379 was present (Yep et al. 2004a). The inactivation was prevented by the substrate ADP-Glc. Mutations C379S and C379A increased the $S_{0.5}$ for ADP-Glc 40-fold and 77-fold, whereas the specific activity was decreased 5.8-fold and 4.3-fold, respectively. Studies of inhibition by Glu 1-P and AMP indicated that Cys379 was involved in the interaction of the enzyme with the phosphoglucose moiety of ADP-Glc. Other mutations, C379T, C379D and C379L, indicated that this site is intolerant for bulkier side chains. Because Cys379 is in a conserved region, other residues were scanned by mutagenesis. Replacement of Glu377 by alanine and glutamine decreased V_{max} more than 10 000-fold without affecting the apparent affinity for ADP-Glc and glycogen binding. Mutation of Glu377 by aspartic acid decreased V_{max} only 57-fold, indicating that the negative charge of Glu377 is essential for catalysis. The activity of the mutation E377C, on an enzyme form without other cysteines, was chemically restored by carboxymethylation. Other conserved residues in the region, Ser374 and Gln383, were analyzed by mutagenesis but were found not to be essential. Comparison with the crystal structure of other glycosyltransferases suggests that this conserved region is a loop that is part of the active site. Thus, the results of this study indicate that this region is critical for catalysis and substrate binding with Glu377 being the catalytic residue (Yep et al. 2004a).

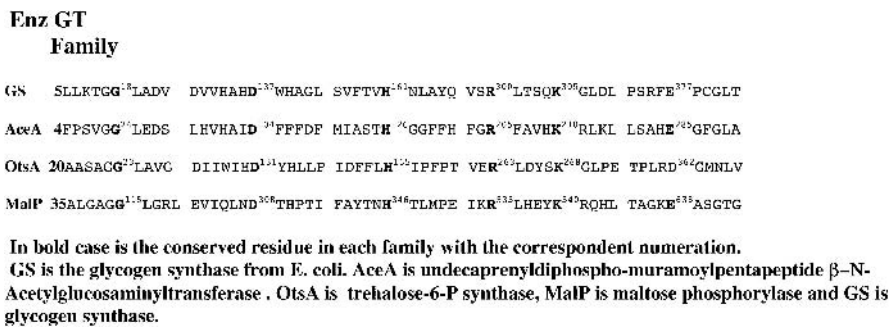

Fig. 5 Alignment of the regions surrounding the residues located in the active site of OtsA and MalP with glycogen synthase

Glycogen synthases are glucosyltransferases that retain the anomeric configuration of the glucosyl linkage of ADP-Glc to the nonreducing end of glycogen. The *E. coli* glycogen synthase was therefore modeled based on three other glycosyl-linkage-retaining glycosyltransferases having a GT-B fold (Yep et al. 2004b). Comparison between the model and the structure of the active site of crystallized retaining GT-B glycosyltransferases identified conserved residues in glycogen synthase with the same topology. These residues were studied in the *E. coli* glycogen synthase by site-directed mutagenesis to confirm the importance of these residues predicted by the model. Mutations D137A, R300A, K305A and H161A decreased the specific activity 8100-fold, 2600-fold, 1200-fold and 710-fold, respectively. None of these mutations increased K_m for glycogen and only H161A and R300A had a higher K_m for ADP-Glc of 11-fold and eightfold, respectively. These residues were essential, validating the model that shows a strong similarity between the active site of *E. coli* glycogen synthase and the other retaining GT-B glycosyltransferases *E. coli* glycogen synthase and the other retaining GT-B glycosyltransferases glycogen synthase and the other retaining GT-B glycosyltransferases k known to date (Yep et al. 2004b). Figure 5 shows the alignment of the amino acid residues essential for catalysis in the retaining glycosyl transferases, trehalose-P synthase, maltose phosphorylase and a muramoyl *N*-acetylglucosaminyltransferase, with glycogen synthase. The critical amino acids are in bold letters. Recently the glycogen crystal structure has been elucidated (Buschiazzo et al. 2004) and confirmed the correctness of our proposed model (Yep et al. 2004b).

5.3 Branching Enzyme

The structural gene of various branching enzymes has been cloned from many bacteria (Kiel et al. 1990, 1991, 1992, 1994; Rumbak et al. 1991; Takata

et al. 1994, 1996; Baecker et al. 1986). The nucleotide and deduced amino acid sequences of the *E. coli glg* B gene consisted of 2181 base pairs specifying a protein of 727 amino acids and with a molecular weight of 84 231 (Baecker et al. 1986).

The relationship in amino acid sequences between that of branching enzyme and amylolytic enzymes in the α-amylase family of enzymes has been compared (Jesperson et al. 1993; Svensson 1994). This family includes enzymes such as isoamylase, pullulanase and cyclodextrin glucanotransferase. There is a marked conservation in the amino acid sequence of the four putative catalytic regions of the amylolytic enzymes in the branching enzymes whether they are of bacterial, plant or mammalian source. Four regions that putatively constitute the catalytic regions of the amylolytic enzymes are conserved in the plant branching isoenzymes and the glycogen branching enzymes of *E. coli*. Analysis of this high conservation in the α-amylase family has been pointed out (Romeo et al. 1988) and greatly expanded by Jesperson et al. (1993) and by Svensson (1994) with respect to sequence homology but also in the prediction the (β/α)8-barrel structural domains with a highly symmetrical fold of eight inner, parallel β-strands, surrounded by eight helices, in the various groups of enzymes in the family. The (β/α)8-barrel structural domain was determined from the crystal structure of some α-amylases and cyclodextrin glucanotransferases.

Conservation of the putative catalytic sites of the α-amylase family in the bacterial and higher-plant branching enzymes is anticipated as the branching enzyme catalyzes two reactions in synthesizing α-1,6-glucosidic linkages by cleavage of an α-1,4-glucosidic linkage in an 1,4-α-D-glucan to form a nonreducing end oligosaccharide chain that is transferred to a C-6 hydroxyl group of the same or the other 1,4-α-D-glucan. The eight highly conserved amino acid residues of the α-amylase family are also functional in branching enzyme catalysis (Libessart and Preiss 1998). Recently the crystal structure of a truncated form of the *E. coli* branching enzyme was elucidated (Abad et al. 2002). Analysis of the three-dimensional structure of the branching enzyme is in process to determine the precise functions and nature of its catalytic residues and mechanism. The *C*-terminal and *N*-terminal portions of branching enzyme from various bacteria as well as in higher plants are dissimilar in sequence and in size. Studies by Kuriki et al. (1997) indicate that these amino acid sequence regions are functional with respect to branching enzyme specificity and to substrate preference (amylose or amylopectin) as well as to the size of the oligosaccharide chain transferred and the extent of branching. Indeed truncation of 113 amino acids of the *N*-terminal of the *E.coli* branching enzyme causes it to transfer longer branch chains than the wild-type enzyme (Binderup et al. 2000, 2002; Devillers et al. 2003), also indicating that the *N*-terminal region was involved in specifying the size of the chain transferred.

6
Genetic Regulation of Glycogen Synthesis in *E. coli*

The enzymes of the glycogen biosynthetic pathway are derepressed in the stationary phase when growth is limited for certain nutrients, for example, nitrogen. Similarly, the levels of the glycogen biosynthetic enzymes in *E. coli* increase as cultures enter the stationary phase (Krebs and Preiss 1975; Preiss 1969, 1984, 1996; Preiss and Romeo 1994). The rate of glycogen synthesis is inversely related to the growth rate.

In cells grown in an enriched medium containing 1% glucose, the specific activities of ADP-Glc PPase and glycogen synthase increase 11-fold to 12-fold, and the level of branching enzyme increases fivefold, as cells enter the stationary phase (Krebs and Preiss 1975; Preiss 1996a; Preiss and Romeo 1994). In minimal media, the ADP-Glc PPase and glycogen synthase activities are elevated in the exponential phase with only about a twofold increase in specific activity and branching enzyme is fully induced in the exponential phase. The same events are also observed with the glycogen biosynthetic enzyme levels in *Salmonella typhimurium* (Steiner and Preiss 1977).

The structural genes for glycogen biosynthesis are clustered in two adjacent operons, which also contain genes for glycogen catabolism. The structural genes for glycogen synthesis were shown to be located at approximately 75 min on the *E. coli* K-12 chromosome, and the gene order at this location was subsequently established by transduction to be *glg*P-*glgA*-*glgC*-*glg*X-*glgB*-*asd* (Latile-Damotte and Lares 1977; Romeo et al. 1988). *glg* A encodes glycogen synthase, *glg*C, ADP-Glc PPase and *glg*B encode glycogen branching enzyme, *glg*X encodes an isoamylase and glgP encodes glycogen phosphorylase. The genes are close to *asd*, the structural gene for the enzyme aspartate semialdehyde dehydrogenase (EC 1.2.1.11).

The arrangement of the glycogen biosynthetic genes encoded in plasmid pOP12 has also been determined by deletion-mapping experiments (Okita et al. 1981), and the nucleotide sequence of the entire *glg* gene cluster was determined (Preiss 1996a). The continuous nucleotide sequence of over 15 kb of this region of the genome has been determined and includes the sequences of the flanking genes *asd* and *glpD* (glycerol phosphate dehydrogenase; EC 1.1.99.5; EC 1.1.1.8) (Austin and Larson 1991).

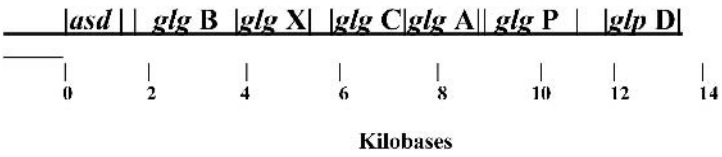

Fig. 6 Structure of the glycogen cluster in *Escherichia coli*. The restriction map is constructed from known contiguous sequences. All of the genes are transcribed from *left* to *right* (counterclockwise on the genome except for *glp*D)

Table 2 Genes affecting glycogen synthesis in *E. coli*

Regulatory gene	Gene product	Map site (min)	Comments
cya	Adenylate cyclase	85	Regulates catabolite repression
crp	Cyclic AMP receptor protein	74	Regulates catabolite repression
relA	(p)ppGpp synthase I	60	Mediates stringent response
spoT	(p)ppGpp3′-pyrophosphos-phohydrolase	60	response to carbon/energy
csrA	6.8-kDa polypeptide (CsrA)	58	Regulation of gluconeogenesis and *glgCA* and *glgB* transcription
csrB	A noncoding RNA molecule that binds to CsrA protein		Binds to CsrA protein
kat F	(rpoS) σs	59	Required for expression of *glgS*, not *glgCA*, pleiotropic
glgQ	Transacting factor (unidentified)	(?)	Transcriptional regulation of *glgCA* and *glgB*
glgR	*Cis*-acting site	75	Transcriptional regulation of *glgCA*
Structural gene	Gene product	Map site (min)	Comments
Biosynthetic			
glgC	ADP-glucose pyrophosphorylase	75	Synthesis of glucosyl donor
glgA	Glycogen synthase	75	α-1,4-Glucosyltransferase
glgB	Glycogen branching enzyme	75	α-1,6 branch synthesis
glgS	7.9-kDa polypeptide	67	Function?

The genetic regulation of the glycogen biosynthetic enzymes has been extensively reviewed elsewhere (Romeo and Preiss 1994; Preiss 1996a) and is summarized here.

1. Glycogen biosynthesis is under the direct control of at least three global regulatory systems, catabolite repression, (stimulation *glgABC* expression by cyclic AMP); stringent response (stimulation *glgABC* expression by 5-phosphoguanosine 3′-pyrophosphate) and repression of the *glgABC* expression by the *csrA* gene product, a 61 amino acid polypeptide (Liu et al. 1995, 1997; Romeo and Gong 1993; Romeo et al. 1993; Yang et al. 1996).

2. Although starvation for nitrogen as well as other nutrients leads to enhanced glycogen biosynthesis the *glg*ABC genes are not part of the nitrogen starvation regulon.
3. Transcription of *glgCA* expression appears to involve initiation events at least at four or more separate sites. All of the observed transcripts are in higher relative concentration in the stationary phase versus the exponential phase. Mutations leading to higher levels of the biosynthetic enzymes also result in enhanced accumulation of specific transcripts.
4. The *csrA* gene maps to 58 min on the *E. coli* chromosome (Romeo and Gong 1993). The *csrA* gene product negatively modulates posttranscriptionally by facilitating decay of *glgCA* messenger RNAs (Romeo et al. 1993). The *csrA* gene product is a specific messenger RNA binding protein binding to *csrB* RNA (Liu et al. 1995, 1997; Gudapaty et al. 2001). Binding of the *csrA* gene product to *csrB* RNA inhibits the repression of the of the *glgCA* operon and the *glgB* genes. Since *csrB* RNA increases in the stationary phase, it is believed that repression of the glycogen biosynthetic genes is relieved by the binding of *csrA* gene product by the *csrB* RNA.

Table 2 indicates the genes known presently to be involved in glycogen metabolism in *E. coli*. Figure 6 shows the *E. coli* glycogen gene cluster.

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Biogenesis and Structure of Polyhydroxyalkanoate Granules

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Abstract A large variety of prokaryotes are capable of accumulating polyhydroxyalkanoates (PHAs) as water-insoluble inclusions in the cytoplasm, and are referred to as PHA granules. Generally, PHAs represent storage compounds for carbon and energy, and they are synthesized under unbalanced growth conditions, i.e., when the carbon source is available in excess and when another nutrient is limited at the same time. In this case, further microbial growth is prevented, and PHAs are accumulated in the cytoplasm of the cells. These PHAs may possess molecular masses of up to several million daltons, and the polyester might represent the major cell constituent, contributing up to 90% or even more of the cellular dry weight. At the beginning of this chapter a brief overview about the PHA synthase and the different metabolic pathways occurring in prokaryotes will be given. The main topic focuses on the biogenesis of PHA granules and the chemical and physical properties of PHA granules produced by bacteria. The function of granule-associated proteins during the biogenesis and mobilization of PHA granules will also be discussed in detail. The chapter will be completed with an overview about applications of PHA granules as surface coatings and as nanoparticles.

1 Introduction

A large variety of prokaryotes are capable of accumulating polyhydroxyalkanoates (PHAs) as water-insoluble inclusions in the cytoplasm, and are referred to as PHA granules (Fig. 1). These polyoxoesters reveal some thermoplastic and/or elastomeric properties similar to those of synthetic polymers produced from petrochemicals such as polypropylene (Steinbüchel 1991; Müller and Seebach 1993). Owing to their biodegradability and origin from renewable resources, PHAs have attracted much interest, and many technical and medical applications have been developed in the last few decades (Asrar and Gruys 2002; Anderson and Dawes 1990; Hocking and Marchessault 1994; Williams and Martin 2002). Since the discovery of poly(3-hydroxybutyrate) (PHB) in *Bacillus megaterium* almost 80 years ago (Lemoigne 1926), many different hydroxyalkanoic acids have been identified as PHA constituents, and they comprise different carbon chain lengths of between three and 16 carbon atoms. The hydroxyalkanoic acids might also contain various substituents at different positions of the *R*-pendant groups of the polymer backbone. 3-Hydroxyalkanoic acid, 4-hydroxyalkanoic acid and 5-hydroxyalkanoic acid also occur as constituents (Steinbüchel and Valentin 1995) (Fig. 2). Today more than 150 different monomers have been identified. The composition of the PHA monomers is variable, and can be varied by means of the precursor used and of the growing conditions. Recently, it was shown that even polythioesters (PTEs) can be synthesized by PHA-accumulating bacteria, indicating an enormous unspecificity of the PHA-synthesizing enzymes (Lütke-Eversloh et al. 2001; Lütke-Eversloh et al. 2002).

Generally, PHAs represent storage compounds for carbon and energy, and they are synthesized under unbalanced growth conditions, i.e., when the car-

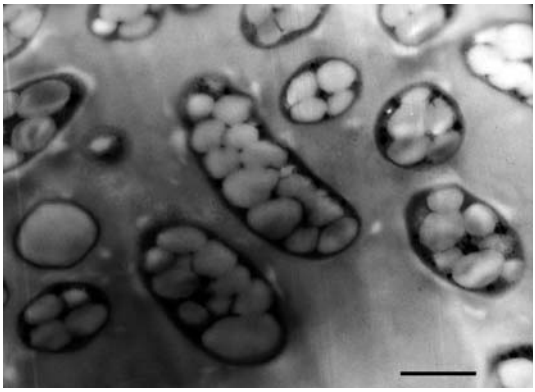

Fig. 1 Electron micrograph of an ultrathin section of *Ralstonia eutropha* H16 cells (*scale bar* 1 μm)

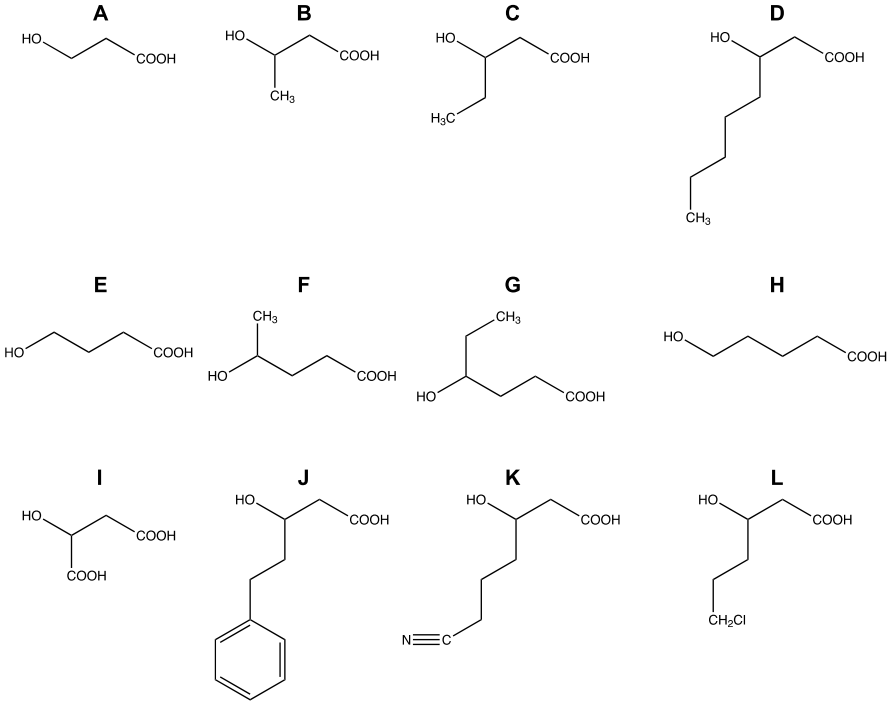

Fig. 2 Selected constituents of biosynthetic polyhydroxyalkanoates (PHAs). **a** 3-hydroxypropionic acid (Doi et al. 1990); **b** 3-hydroxybutyric acid (Lemoigne et al. 1926); **c** 3-hydroxyvaleric acid (Holmes et al. 1981); **d** 3-hydroxyoctanoic acid (De Smet et al. 1983); **e** 4-hydroxybutyric acid (Kunioka et al. 1988); **f** 4-hydroxyvaleric acid (Valentin et al. 1992); **g** hydroxyhexanoic acid (Valentin et al. 1995); **h** 5-hydroxyvaleric acid (Doi et al. 1987); **i** malic acid (Fischer et al. 1989); **j** 3-hydroxy-5-phenylvaleric acid (Kim et al. 1991); **k** 7-cyano-3-hydroxyheptanoic acid (Lenz et al. 1992); **l** 3-hydroxy-8-chlorooctanoic acid (Doi and Abe 1990)

bon source is available in excess and another nutrient is limited at the same time. In this case, further microbial growth is prevented, and PHAs are accumulated in the cytoplasm of the cells. These PHAs may possess molecular masses of up to several million daltons, and the polyester might represent the major cell constituent, contributing up to 90% or even more of the cellular dry weight (Anderson and Dawes 1990).

Interestingly, a completely different type of PHA with a yet unknown function occurs ubiquitously, i.e., in prokaryotes and eukaryotes. This PHA type contributes only 0.1% or less to the dry matter, and the molecules exhibit a much lower molecular mass of up to approximately 15 000 Da only. They are localized in the cytoplasmic membrane or are associated or even covalently bound to other cell constituents (Reusch 2002) and are therefore referred to as complexed PHAs (cPHA). However, enzymes and genes involved in

biosynthesis of cPHAs, which also occur in many bacteria like, for example, *Escherichia coli* and *Ralstonia eutropha*, have not been identified yet. This chapter summarizes the current knowledge of PHA inclusions. It focuses on the biogenesis and the structure of the surface of PHA granules.

2 Metabolism of PHA in Prokaryotes

In most bacteria PHA are accumulated if a carbon source is provided in excess and if at the same time growth is limited by another essential nutrient, for example, iron, magnesium, nitrogen, sulfur, phosphate or potassium (Steinbüchel and Schlegel 1989). In addition, accumulation of PHA is induced by oxygen limitation in many aerobic bacteria such as *Ralstonia eutropha* or *Azotobacter beijerinckii* (Senior and Dawes 1973).

Biosynthesis of the most frequently occurring type of PHA, PHB, proceeds in the facultative chemolithoautotrophic hydrogen-oxidizing bacterium *Ralstonia eutropha* (Schlegel et al. 1961) in three steps and starts from acetylcoenzyme A (acetyl-CoA) a central intermediate of metabolism. Two molecules of acetyl-CoA are condensed to acetoacetyl-CoA, catalyzed by a β-ketothiolase (PhaA), which is subsequently reduced by a stereospecific acetoacetyl-CoA reductase (PhaB) to *R*-(–)-3-hydroxybutyryl-CoA (Oeding and Schlegel 1973; Haywood et al. 1988a, b). The final step is the polymerization of 3-hydroxybutyryl-CoA to PHB with concomitant release of CoA, which is catalyzed by the PHA synthase (PhaC).

Ralstonia eutropha was the first organism from which the PHA biosynthesis genes were cloned and heterologously expressed in *E. coli* (Schubert et al. 1988; Slater et al. 1988; Peoples and Sinskey 1989). The PHA operon comprises three genes encoding a β-ketothiolase (*phaA*), an acetoacetyl-CoA reductase (*phaB*) and a PHA synthase (*phaC*). Today, many additional genes involved in PHA metabolism are known; however, the functions of several genes and proteins remain to be elucidated. Whereas PhaC is essential for storage PHA biosynthesis in *Ralstonia eutropha*, PhaA and PhaB can be replaced by isoenzymes. A well-known example is *bktB* coding for an additional β-ketothiolase, which is essential for the biosynthesis of 3-hydroxyvaleryl-CoA (Slater et al. 1998). As exhibited very recently by analysis of the genome sequence, *Ralstonia eutropha* possesses 15 *phaA* homologous genes (unpublished results).

One important goal of polymer research is the development of novel materials comprising special features. The impact of environmentally friendly materials, especially for disposable bulk products, is continuously increasing, and biopolymers originating from renewable resources will become more important in the near future. On the other hand, the demand for specific value-added polymers comprising unique physical properties is also very high. The

broad substrate range of PHA synthases and the comprehensive genetic and biochemical knowledge about PHA metabolism provide promising perspectives for the development of novel biopolymers. About 150 different hydroxyalkanoic acids are currently known as constituents of PHAs (Steinbüchel and Valentin 1995); some of these constituents occur as single constituent in homopolymers, but a large number of copolymers of different constituents are also known. Only a few PHAs can be synthesized from simple carbon sources such as glucose or fatty acids. The diversity of PHA constituents is at present predominantly achieved by the provision of suitable precursor carbon sources, which exhibit a chemical structure related to that of the respective constituent. Therefore, metabolic engineering of PHA-producing organisms will provide the opportunity to synthesize the respective substrates of the PHA synthases from central intermediates of the carbon metabolism.

2.1 Provision of Precursor

Pathways linking the central metabolism or the catabolism of various precursor carbon sources with PHA synthases and therefore providing the PHA synthases with their substrates have also been intensively studied, particularly in *Ralstonia eutropha*, *Pseudomonas putida* and recombinant strains of *E. coli*. Many investigations revealed connections betwween PHA biosynthesis and the amino acid metabolism, fatty acid β-oxidation, fatty acid *de novo* synthesis, as well as with the citric acid cycle (Madison and Huisman 1999; Steinbüchel 2001; Taguchi et al. 2002). Comprehensive knowledge of the relation between PHA biosynthesis and the central carbon metabolism provides the basis for the establishment of sustainable biotechnological processes to produce PHAs from renewable resources—preferentially carbon dioxide—in an economically feasible way (Steinbüchel and Füchtenbusch 1998).

The formation of PHAs can be divided into three essential steps. First, the carbon source has to be taken up by the bacterial cell by passive diffusion or by specific transporters. In a second step the carbon source is converted into a thioester of the respective hydroxyalkanoic acid. This CoA thioester can then used as a substrate by the key enzyme of PHA biosynthesis, the PHA synthase. The reactions comprising the second step of PHA biosynthesis are very complex and only partially understood. Three different strain specific pathways for biosynthesis of PHAs are known and these are compiled in Fig. 3.

2.1.1 *Ralstonia eutropha* Type

Biosynthesis of PHB in *Ralstonia eutropha* on carbohydrates, acetate or pyruvate starts from acetyl-CoA (Gottschalk 1964a, b). A β-ketothiolase catalyzes

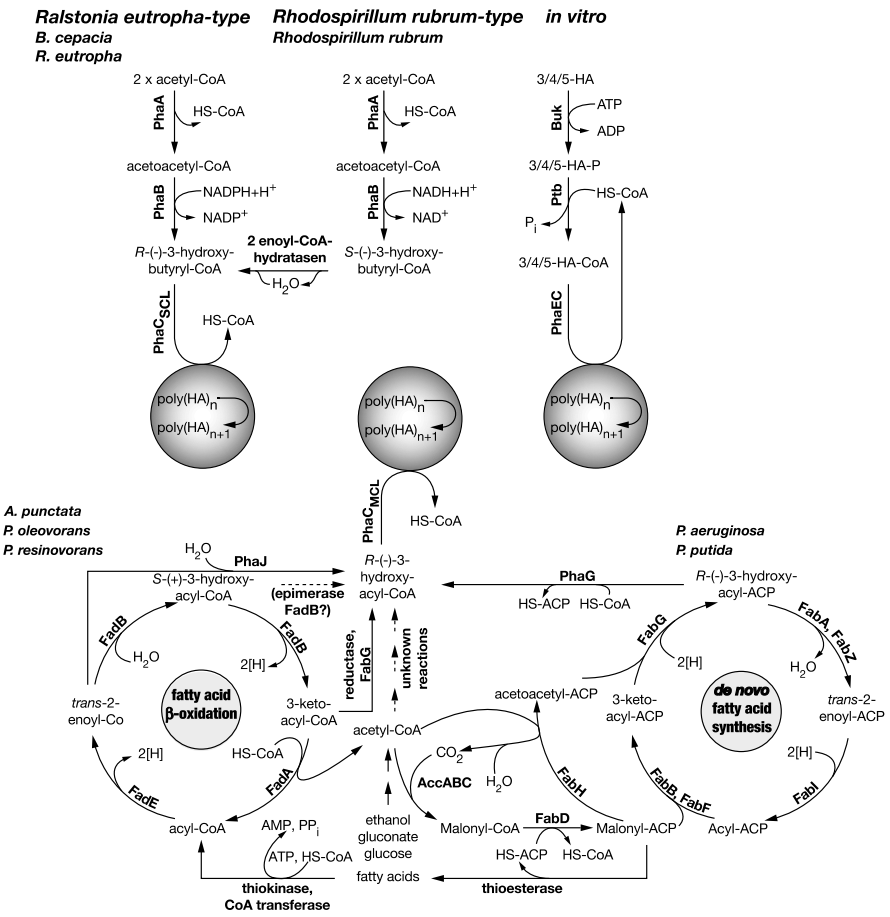

Fig. 3 Metabolic pathways of PHA biosynthesis in bacteria and in vitro (Steinbüchel 1991, 2001; Fukui and Doi 1997; Tsuge et al. 2000; Hoffmann et al. 2000; Liu et al. 2000; Rehm et al. 1998). Buk butyrate kinase, Ptb phosphotransbutyrase, CoA coenzyme A, Acc acetyl-CoA carboxylase, Fad enzymes of the β-oxidation of fatty acids, Fab enzymes of the de novo fatty acid biosynthesis. Other abbreviations are given in the text. The *dashed arrows* indicate unknown pathways

a Claison condensation of two molecules of acetyl-CoA and results in the formation of acetoacetyl-CoA and in the release of one CoA. The resulting acetoacetyl-CoA is than reduced to *R*-(–)-3-hydroxybutyryl-CoA by an NADPH-dependent stereospecific acetoacetyl-CoA reductase. This pathway is present in the majority of PHB-producing bacteria.

2.1.2
Rhodospirillum rubrum Type

In *Rhodospirillum rubrum*, two additional enzymes are involved in PHB biosynthesis, because the reduction of acetoacetyl-CoA yields *S*-(+)-3-hydroxybutyryl-CoA catalyzed by an NADH-dependent acetoacetyl-CoA reductase. Two enoyl CoA hydratases convert the *S*-(+)-3-hydroxybutyryl-CoA isomer to the *R* isomer (Moskowitz and Merrick 1969).

2.1.3
Pseudomonas sp. Type

Pseudomonads belonging to the ribosomal RNA homology group I are able to synthesize high amounts of PHA consisting of various 3-hydroxy fatty acids with carbon chain lengths ranging from six to 14 carbon atoms from aliphatic alkanes and their oxidation products, but also from sugars or other unrelated carbon sources (De Smet et al. 1983; Haywood et al. 1990; Steinbüchel et al. 1992; Timm and Steinbüchel 1990). Alkanes, such as octane, are oxidized in several steps to the corresponding fatty acids; the latter are then activated by thiokinases and degraded via the β-oxidation. Under cultivation conditions promoting PHA accumulation, intermediates of the β-oxidation cycle can be converted to *R*-(–)-3-hydroxyacyl-CoA by enoyl-CoA hydratases, epimerases or ketoacyl-CoA reductases, and are subsequently polymerized by the PHA synthase (Huisman et al. 1991). Most pseudomonads such as *Pseudomonas putida* are also capable of synthesizing PHA from unrelated carbon sources like glucose by the involvement of the fatty acid *de novo* synthesis pathway (Huijberts et al. 1992, 1994). The linking enzyme, a 3-hydroxyacyl-acyl carrier protein:CoA transferase (PhaG), transfers the *R*-(–)hydroxyacyl residue from the acyl carrier protein to CoA. This key enzyme was identified in *Pseudomonas putida* KT2440 and other pseudomonads (Rehm et al. 1998). In *Pseudomonas oleovorans* a cryptic *phaG* homologous gene was detected, explaining the inability of this strain to synthesize PHA from sugars (Hoffmann et al. 2000).

2.2
PHA Synthases

Many PHA synthases have been cloned and characterized at a biochemical and/or molecular level. Four different classes of PHA synthases are distinguished (Rehm and Steinbüchel 2002) (Table 1). Class I PHA synthases are represented by the enzyme of *Ralstonia eutropha*; they preferentially accept the CoA thioesters of short carbon chain length hydroxyalkanoic acids (HA_{SCL}, C3–C5 carbon atoms) as substrates and consist of only one type of subunit ($PhaC_{SCL}$). This type of PHA synthase occurs in most PHA_{SCL}-

Table 1 Polyester synthases can be divided into four classes (updated from Steinbüchel 2001)

Class	General structure of PHA operon	Subunits	Substrate	Representative species
I		~ 60–73 kDa	$3HA_{SCL}$-CoA $4HA_{SCL}$-CoA $5HA_{SCL}$-CoA $3MA_{SCL}$-CoA ~ C3–C5	*Ralstonia eutropha* *Sinorhizobium melioti* *Burkholderia* sp. many other bacteria
II		~ 60–65 kDa	$3HA_{MCL}$-CoA ~ C5–C14	*Pseudomonas aeruginosa* *P. putida* all pseudomonads belonging to ribosomal RNA homologous group I
III		PhaC ~ 40 kDa PhaE ~ 40 kDa	$3HA_{SCL}$-CoA ~ C3–C5 $3HA_{MCL}$-CoA ~ C5–C8 $4/5HA_{SCL}$-CoA	*Allochromatium vinosum* *Thiocapsa pfennigii* *Synechocystis* sp. PCC6803 purple sulfur bacteria all cyanobacteria
IV		PhaC ~–– 40 kDa PhaR ~ 22 kDa	$3HA_{SCL}$-CoA ~ C3–C5	*Bacillus megaterium* *Bacillus* sp. INT005 other strains are unknown

PHA polyhydroxyalkanoate, 3MA 3-Mercaptoalkanoate; further abbreviations are given in the text

accumulating bacteria. Most interesting, a second putative PHA synthase gene (*phaC2*) was detected in the genome of *Ralstonia eutropha*; this gene was clustered with a gene coding for an acetoacetyl-CoA reductase (*phaB*) homologue and a gene with unknown function (unpublished results). This was unexpected because *phaC1* insertion or deletion mutants were completely impaired in synthesis and accumulation of detectable PHAs (Reinecke and Steinbüchel, unpublished). Class II PHA synthases are represented by the enzyme of *Pseudomonas oleovorans*; they preferentially accept the CoA thioesters of medium carbon chain length hydroxyalkanoic acids (HA_{MCL}, C5–C14 carbon atoms) as substrates and consist also of only one type of subunit ($PhaC_{MCL}$). This type of PHA synthase occurs in all PHA_{MCL}-accumulating bacteria, particularly in pseudomonads *sensu strictu*. These bacteria generally posses two homologous PHA synthases. Class III PHA synthases are represented by the enzyme of *Allochromatium vinosum*; they consist of two different types of subunits (PhaC and PhaE) and accept the CoA thioesters of HA_{SCL} as substrates; some of them accept in addition also CoA thioesters of HA_{MCL}. Class IV PHA synthases are represented by the enzyme of *B. megaterium* and *Bacillus* sp. INT005 (McCool and Cannon 2001; Satoh et al. 2002); they also consist of two different types of subunits (PhaC and PhaR) and exhibit a similar substrate range as class III PHA synthases.

2.3 Biodegradation of PHAs

PHAs are degraded extracellularly and intracellularly by PHA depolymerases (PhaZ) through hydrolytic cleavage. The extracellular degradation of PHAs has been investigated in much detail and is well understood. It occurs under aerobic as well as under anaerobic conditions, and many different PHA-degrading bacteria and fungi from various habitats have been isolated and characterized. The rate of biodegradation depends on environmental factors such as moisture level, nutrient supply, temperature and pH. Furthermore, material parameters such as initial molecular weight, degree of crystallinity, formulation (e.g., presence of additives) and surface influence the degradation (Cox 1992). Generally, extracellular PHA depolymerases are synthesized and secreted, if the cells are cultivated in presence of PHAs (Jendrossek et al. 1996; Jendrossek 2002; Jendrossek and Handrick 2002). Previous studies showed that PHAs consisting of ω-hydroxy fatty acids are also accessible to hydrolytic cleavage by various other enzymes like lipases and esterases (Mukai et al. 1993; Jaeger et al. 1995).

In contrast, the intracellular degradation of PHAs is far less understood. It is initiated by PHA depolymerases, which exhibit properties different from those of extracellular PHA depolymerases. Only recently, six genes putatively encoding intracellular PHA depolymerases (*phaZ1*, *phaZ2*, *phaZ3*, *phaZ4*,

phaZ5 and *phaZ6*) were identified in *Ralstonia eutropha* H16 (Saegusa et al. 2001; York et al. 2003; Schwartz et al. 2003; Pötter et al. 2004; Abe et al. 2005). In addition, two hydroxybutyrate oligomer hydrolase genes were identified in *Ralstonia eutropha* (Saegusa et al. 2002; Kobayashi et al. 2005). The first analyses of the gene loci indicated that the translational products of at least three of these genes contribute to the intracellular degradation of PHB in this bacterium because only a triple PHA depolymerase mutant (defective in *phaZ1*, *phaZ2* and *phaZ3*) was impaired in mobilization of PHB as shown by York et al. (2003).

Since the PHB biosynthesis enzymes are constitutively expressed in *Ralstonia eutropha* and to avoid a futile cycle, a strict regulation of intracellular PHB degradation is required in the cells. It is completely unknown how this is achieved by the cells. Several years ago, two clustered genes (*phaH* and *phaI*) which are required for maximum PHB accumulation in *Ralstonia eutropha* were identified. Mutants harboring Tn5 insertions in either gene exhibited clearly a PHB-leaky phenotype mobilizing PHB much faster than the wild type after the cells had consumed their extracellular carbon source (Pries et al. 1991). According to this study and other data, a hypothesis was proposed (Fig. 4) by which either the PHB depolymerase enzyme activity is regulated by phosphorylation/dephosphorylation mediated by PhaH/PhaI or alternatively the transcription of *phaZ* is regulated by one of the PhaH forms (phosphorylated/nonphosphorylated) (Pries et al. 1991). A different situation occurs probably in *Rhodospirillum rubrum*. From biochemical studies on the PHB depolymerase of *Rhodospirillum rubrum* it is known that this enzyme requires

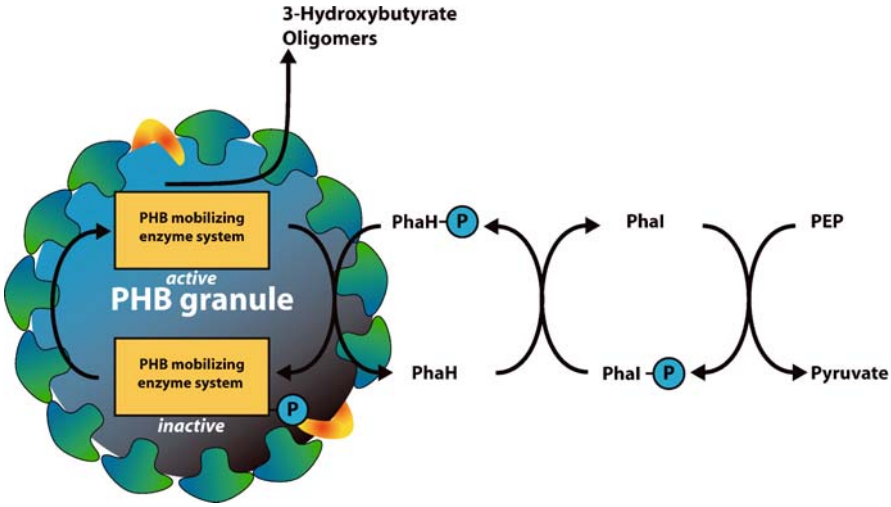

Fig. 4 Hypothetical functions of PhaH and PhaI in *R. eutropha* H16. (Adopted from Pries et al. 1991)

an extremely heat resistant and also otherwise stable activator (Handrick et al. 2004a), which exhibits homologies to phasins (Handrick et al. 2004b).

3 Structure of PHA Granules

PHA synthesis results in multiple cytoplasmic inclusions that are generally from 200 to 500 nm in size in the final accumulation phase (Anderson and Dawes 1990). The first investigations of PHA granules from bacteria were performed by Williamson and Wilkinson (1958) and also by Griebel et al. (1968). Isolated PHA granules contain approximately 97.5% PHA, 2% protein and 0.5% phospholipids (Griebel et al. 1968), although some estimates of the lipid contents were considerably higher (Steinbüchel et al. 1995). Lundgren et al. (1964) showed by electron microscopic studies that the surface of PHA granules from *B. megaterium* and *B. cereus* is covered by a membrane with a thickness of approximately 15–20 nm. The granule-associated proteins exhibited PHA polymerase and depolymerase activity (Griebel and Merrick 1971). A phospholipid monolayer surrounding the PHA granules was proposed by De Koning and Maxwell (1993) (Fig. 5). It was recognized that PHA granules contain proteins at their surface which are responsible for their structure, size and function (Steinbüchel et al. 1995). Whether the boundary layer consists of proteins or a membrane was analyzed by Mayer and Hoppert (1994) and Boatman (1964).

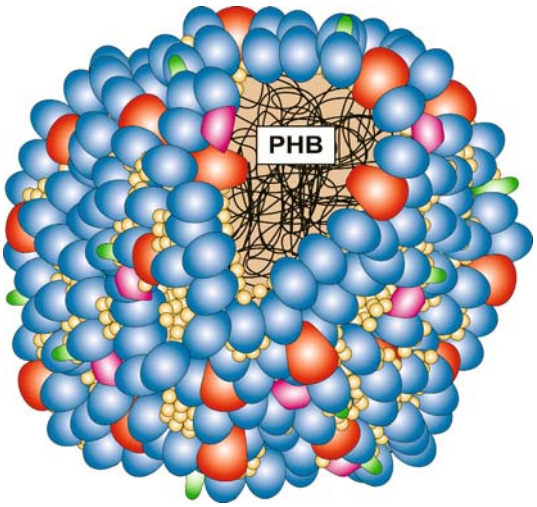

Fig. 5 Model of the PHA granule. *Red globes* PHA synthase, *blue globes* phasin, *magenta globes*, PHA depolymerases, *green globes* transcriptional repressor of phasin, *yellow globes* phospholipids

They determined the thickness of the layer as 4 nm. This result excludes a lipid bilayer, which has a thickness of approximately 8 nm.

Two physical states of the polyester occur in PHB granules: intracellular native, amorphous granules and partially crystalline granules (Jendrossek and Handrick 2002). The surface of the native granules is surrounded with a layer consisting of proteins and phospholipids. When the layer is damaged during the isolation of the granules, the crystallinity increases to 50–60%. Crystalline granules melt in the range between 170 and 180 °C (Scandola et al. 1998). The amorphous granule fraction has the same glass-transition temperature as native PHB granules ($T_g = 0$ °C).

Four different types of granule-associated proteins (GAPs) were found in PHA-producing bacteria. These are (1) the PHA synthases (PhaC), (2) the intracellular PHA depolymerases (PhaZi), (3) the phasins (PhaP) and (4) a regulator protein of the phasin expression (PhaR).

Phasins are structure proteins and are considered by far as the major proteins of the PHA granule surface layer. In *Ralstonia eutropha*, it was shown that PhaC is a soluble protein in the cytoplasm, which becomes only insoluble by granule binding after the onset of PHA accumulation under cultivation conditions permissive for PHA accumulation. This is due to the PHA chain, which remains covalently linked to the enzyme until synthesis of the polymer has finished (Gerngross et al. 1993; Liebergesell et al. 1992, 1994). In contrast, the first two enzymes of the pathway, PhaA and PhaB, are found in the cytoplasm during active PHA synthesis and do not bind to the granules (Haywood et al. 1988a, b). The phasin, PhaP1, is localized at the surface of the PHB granules, and it is synthesized in large quantities under storage conditions, and represents as much as 5% of the total protein (Wieczorek et al. 1995). PhaP1 adheres very tightly *in vivo* to native as well as *in vitro* to artificial PHB granules. By this, PhaP1 provides protection to the host cell by contributing to coverage of the hydrophobic surface of the polymer, preventing other proteins falsely binding to the hydrophobic granules (Bohmert et al. 2002). This hypothesis was confirmed by analysis of the granule proteom of *Ralstonia eutropha* wild type and phasin negative mutant, respectively, by two-dimensional gel electrophoresis. The granule proteom of the mutant exhibited huge amounts of the stress response proteins GroEL and DnaK which was not observable in the wild type (Pötter et al. 2004).

In *Rhodococcus ruber*, the anchoring region is located at the carboxy terminus; it was demonstrated that phasin molecules truncated at the carboxy terminal region lost their ability to bind to PHA granules (Pieper-Fürst et al. 1995). PHA granules can also be generated *in vitro* simply by incubating purified PHA synthase with a suitable substrate. The addition of phasins accelerates the rate of PHA synthesis. Furthermore, the size of these inclusions can be regulated by the addition of PhaP1, but electron microscopic studies revealed that the granules do not have the same surface structure as native inclusions (Jossek and Steinbüchel 1998).

The currently available genome sequence of *Ralstonia eutropha* H16 revealed three homologues (PhaP2, PhaP3, Pha4) to the phasin protein PhaP1 described peviously (Wieczorek et al. 1995). They exhibited 42, 49 and 45% identity or 61, 62 and 63% similarity to PhaP1, respectively (Schwartz et al. 2003; Pötter et al. 2004). The genes for the PhaP homologues are randomly distributed over the three replicons of the genome of *Ralstonia eutropha*.

Predictions of the secondary structure of the four phasin homologues from *Ralstonia eutropha* gave a ratio of approximately 90% helical structure of either protein. Genome analysis by the BLAST algorithm of *Sinorhizobium melioti* 1021 revealed no phasin homologue in this PHB-producing bacterium. But with the prerequisite to analyze the genom by the secondary database TIGRFAM, a collection of protein families based on hidden Markov models, a putative phasin with a prediction for a helical structure of *S. melioti* could be identified (unpublished).

In the case of PHA_{MCL} granules of *Pseudomonas putida* GPo1, it was suggested that the surface of the granule is covered by two distinct protein layers separated by a phospholipid layer (Fuller et al. 1992; Stuart et al. 1998). The granules contain two phasins of 15.4 kDa (PhaI) and 26.3 kDa (PhaF). It was shown that PhaF behaves not only as a structural protein but also as a transcriptional regulator of the biosynthetic *pha* cluster. Since both phasins are attached to the granules, it was postulated that the *N*-terminal region of PhaF might be responsible for attachment to PHA granules (Prieto et al. 1999).

3.1 Biogenesis of the PHA Granules

The formation of PHA granules is quite different from the formation of a prokaryotic neutral lipid inclusion like wax esters or triacylglycerols granules (Wältermann et al. 2005; Wältermann and Steinbüchel 2005). PhaC mediated the template-independent polymerization process from the respective HA-CoA. The PHA synthase is localized in the cytoplasm of exponentially grown cells which have not yet accumulated PHAs (Haywood et al. 1989). In contrast, in cells of *Ralstonia eutropha* and *Allochromatium vinosum* cultivated under conditions permissive for PHA synthesis, the PHA synthase is associated with the PHA granules (Haywood et al. 1989; Gerngross et al. 1993; Liebergesell et al. 1994), because the growing polyester molecule is covalently linked to the enzymes during polymerization, conferring amphiphilicity to the enzyme–polyester complex. Two models of *in vivo* PHA initiation, namely, micelle formation of PHB synthases and budding of PHB from the cytoplasm membrane have been proposed (Stubbe and Tian 2003) (Fig. 6).

The more accepted micelle formation model assumes that PHB synthase molecules without bound substrate molecules are randomly distributed in the cytoplasm and aggregate then to micellelike structures in the initial phase after the first polymerization reactions have occurred. PhaC represented the

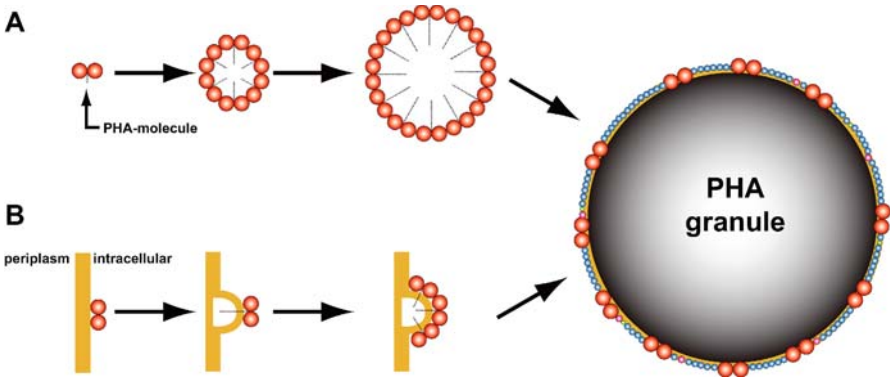

Fig. 6 Working models for the formation of PHA granules in bacteria. **a** micelle formation model; **b** budding model. For an explanation, see the text. *Red globes* PHA synthase as a dimer, *blue globes* phasin, *magenta globes* PHA depolymerases, *green globes* transcriptional repressor of phasin, *yellow line* phospholipids. (Modified from Stubbe and Tian 2003)

hydrophilic part and the prolonged polymer chain the lipophilic part of the amphiphilic molecule. With increasing length of the polymer chain and simultaneously increasing volume of the micelles, the PHA synthases are dispersed on the surface of the nascent granules. An increasing fraction of the growing PHA granules would be directly exposed to the cytoplasm, if these regions are not covered by other amphiphilic molecules, like phasins or phospholipids. However, there is so far no comprehensible explanation of how phospholipids could attach to the granule surface. Investigations to quantify phospholipids on PHA granules were done with granules extracted from crude extract (Griebel et al. 1968). Therefore, accurate data on the amounts of phospholipids are probably not available, because phospholipids could contaminate the granule during mechanical disruption of the cells. The non-specifically attached phospholipids could give higher amounts or could even be the only resason for phospholipids being on the granule surface.

The alternative budding model assumes that PHB synthase is released between the two layers of the phospholipid bilayer, resulting in the formation of a PHA granule that is surrounded by a phospholipid monolayer. The growing PHB granule is then released into the cytoplasm and specific proteins (e.g., phasins) are attached to the surface of the granules. This model resembles the model for the formation of eukaryotic neutral lipid bodies (Wältermann et al. 2005). However, this budding model neglects the fact that PhaC and phasins are never located at the cytoplasm membrane.

Tian et al. (2005a, b) suggested a third model for granule formation. When cells of *Ralstonia eutropha* are grown on PHB production medium, small, discrete granules appear to be localized close to a mediation element located in the center of the cells. During the accumulation period the granules in-

crease in size and stay close to the mediation element. After 24-h incubation time in the PHB production medium the mediation element is not observed anymore. The origin and scaffolding of this putative mediation element is obscure. It may be also an artifact due to sample preparation for transmission electron microscopy.

Very recently investigations on the early stages of PHB accumulation in *Ralstonia eutropha*, *Rhodospirillum rubrum* and in recombinant *E. coli* harboring the PHB biosynthesis genes of *Ralstonia eutropha* by fluorescence microscopy were reported by Jendrossek (2005). The PHB granules were stained with the fluorescent dye Nile red and by the phasin-enhanced yellow fluorescent protein fusion protein. PHB granules occurred near to the cell wall and to the cell pole in all three strains. These results emphasize that PHB is not synthesized randomly but in particular regions in the cell.

The first attempts to simulate PHB granule formation by a computer program were done by Marchessault and coworkers (Nobes et al. 2000; Jurasek et al. 2001). The program was extended to a more complex system, including phasins, to quantify their anticipated effect on the granule properties. This updated simulation program appears to be a useful tool for analyzing hypothetical options for the mechanism of the effects of phasins on properties of PHB granules *in vivo* (Jurasek and Marchessault 2002, 2004).

3.2
Effects of Phasins on the PHA Granule Size

PHB granule associated proteins have gained more significance because several laboratories have shown that defective or lacking phasins have substantial effects on PHA synthesis. PhaP1 from *Ralstonia eutropha* is the best studied phasin and several hypotheses for its function have been suggested. Further insights into the role of phasins were provided by genetics studies. Tn5-induced *phaP1* mutants with defective phasin biosynthesis are still able to synthesize PHB; however, they synthesize PHB at a significantly lower rate, and almost the entire PHB is present in only one single large granule in the cell (Wieczorek et al. 1995). Gene deletion experiments of *phaP1* demonstrated that the amount of PHB under a defined set of growth conditions was reduced by 50% compared with the situtaion for wild-type *Ralstonia eutropha* (York et al. 2001). Otherwise, overexpression of PhaP1 leads to an increase of the number of granules (Pötter et al. 2002).

3.3
Regulation of Phasin Expression

Further insights into the role of PhaP were provided by the detection of the transcriptional repressor PhaR in *Ralstonia eutropha* that regulates the expression of PhaP (Pötter et al. 2002; York et al. 2002). Using the *Ralstonia*

eutropha PhaR sequence and the BLAST algorithm, proteins homologous to PhaR were identified in various bacteria, for example, in *S. melioti*, *Paracoccus denitrificans*, *Burkholderia cepacia*, *Allochromatium vinosum*, and several other PHA_{SCL}-producing bacteria.

In the *phaR* deletion mutant strain of *Ralstonia eutropha*, PhaP is constitutively expressed at high levels. If *phaC* is deleted, no PhaP is synthesized (Wieczorek et al. 1995; York et al. 2001a, b). Importantly, deletion of both *phaR* and *phaC* results in high levels of PhaP expression, even though the cells cannot produce PHB. On the other hand, *in vitro* studies also revealed that *Paracoccus denitrificans* PhaR was able to cause a gel shift of a sequence of DNA to which it is proposed to bind and that this gel shift was reversed in the presence of PHB (Maehara et al. 2002).

To deduce a model for the regulation of phasin expression, additional experiments were performed with *Ralstonia eutropha*. Pötter et al. (2002) demonstrated that PhaR could bind to artificial PHB granules. Furthermore, Western immunoblotting and immunoelectron microscopic localization with antibodies raised against PhaR clearly showed that PhaR is bound to the PHB granules. Therefore, PhaR has the ability to bind at least to four different receptors in the cells of *Ralstonia eutropha*: the promoter regions of *phaP1* (1) *phaP3* (2) and *phaR* (3) and the surface of PHB granules (4). All these data support the following simple but elegant model for the regulation of PhaP expression in *Ralstonia eutropha* with PhaR functioning as a transcriptional repressor. Under cultivation conditions not permissive for PHA biosynthesis or in mutants defective in PHA biosynthesis, PhaR binds to the *phaP* promoter region and represses transcription of this gene. The cytoplasmic concentration of PhaR is sufficiently high to repress transcription of *phaP1* and *phaP3*. If physiological conditions are permissive for PHB biosynthesis, the constitutively expressed PHA synthase starts to synthesize PHB molecules, which remain covalently linked to this enzyme. At the beginning small micelles are formed which become larger and constitute the nascent PHB granules. Proteins with a binding capacity to the hydrophic surfaces like PhaR bind to the granules. From a certain point, the cytoplasmic concentration of PhaR becomes sufficiently low, and it no longer represses transcription of *phaP1* and *phaP3*. PhaP1 and also PhaP3 are therefore synthesized and subsequently bound to the surface of PHB granules. The granules become larger and reach their maximum size. Concomitantly, PhaP1 is continuously synthesized in sufficient amounts; in addition, small amounts of PhaP3 are also synthesized. When the PHB granules have reached the maximum possible size according to the physiological conditions, and when most of the PHB granule surface is covered by PhaP1, no more space will be available for binding of additional PhaR, or PhaR may even be displaced by PhaP1 (and PhaP3). Consequently, the cytoplasmic concentration of PhaR will increase and exceed the threshold concentration required to repress again transcription of *phaP1* and *phaP3*. As a consequence PhaP1 and PhaP3 proteins are as

no longer synthesized, and these phasins are therefore not overproduced, and thus do not exceed the amount required to cover the surface of PHB granules (Pötter et al. 2005).

To investigate the function of the phasin homologous deletion mutants various mutants were constructed and analyzed: from all single phasin deletion mutants of *Ralstonia eutropha* (Δ*phaP1*, Δ*phaP2*, Δ*phaP3* and Δ*phaP4*) only Δ*phaP1* exhibited a phenotype different from that of the wild-type strain, with respect to growth and PHB accumulation behavior as well as the size and number of granules formed. The single phasin deletion mutant Δ*phaP1* and the multiple phasin deletion mutants Δ*phaP12*, Δ*phaP123* and Δ*phaP1234* showed almost identical growth behavior; however, compared with the wild-type and the single deletions Δ*phaP2*, Δ*phaP3* and Δ*phaP4*, they accumulated PHB at a significantly lower level (Pötter et al. 2005).

The functions of PhaP2 and PhaP4 are not at all understood. Both proteins are expressed at much lower levels than PhaP1 and even than PhaP3, and transcription of both genes is not repressed by PhaR. In addition, PhaP2 seems *in vivo* not to be bound to the granules under the conditions investigated, although it is capable of binding to artificial PHB granules (Srinivasan et al. 2002; Pötter et al. 2004). PhaP2 and PhaP4 may therefore not be considered as phasins *sensu strictu* and may have different functions, for which only low concentrations of these proteins are required. Perhaps, the functions of these two proteins are related to mobilization of PHB and they may interact with one of the five PHA depolymerases of *Ralstonia eutropha*.

These new and unexpected findings should affect our current models of the structures of PHA granules and may also have severe impacts on the establishment of heterologous production systems for PHAs in other organisms.

Simultaneously, similar investigations were done in the laboratories of A.J. Sinskey (MIT, Cambridge, USA) on PhaR in *Ralstonia eutropha* (York et al. 2002) and of A. Maehara (RIKEN, Saitama, Japan) on a PhaR homologue in *Paracoccus denitrificans* (Maehara et al. 2002). These excellent studies were less complete but were fully consistent with our results and interpretation.

3.4 Physical Investigations on PHA Granules by Atomic Force Microscopy

In 2003 Dennis and coworkers carried out for the first time preliminary atomic force microscopy (AFM) analyses of the nanostructure of native PHB granules isolated from sonicated *Ralstonia eutropha* H16 cells in aqueous solution. They observed two different surface structures. The majority of the granules exhibited a rough and ovoid surface structure, whereas a smaller number of granules were smooth and spherical. These smooth granules exhibited linear strands in parallel arrays covering the surface at a distance

of approximately 7 nm. The individual strands did not cover the entire surface of the granule but ended after some distance at another array of strands traversing the granule surface in a different direction. The surface structures of the rough granules was much more complicated. They were covered with globular structures of 35 nm in diameter. The latter contained a central pore of about 12–15 nm in diameter and were connected by 4-nm-wide linear structures (filaments) which became wider in the regions where they were attached to the globular structures. These filaments were not restricted to the surface of an individual granule but were also bridged from one granule to another relatively frequently. When rough granules were washed with 0.5% (w/v) sodium dodecyl sulfate solution, these typical surface structures dissolved, and the remaining granules looked very much like the smooth granules.

Relying upon these results, Dennis and coworkers concluded that the rough granules exhibited the *in vivo* envelope structure, which had become lost in the smooth granules. The authors interpreted the arrays occurring at a distance of 7 nm as PHB and the space between the filaments and the globular structures of the rough granules as a phospholipid monolayer. This interpretation leads to the question of how substrates can enter the granule through the membrane and how the resulting monomers leave it. Dennis et al. (2003) regarded the globular structures as pores that have another size, allowing access to and egress from the granule, and that function as protein machinery that mediates structure, synthesis and depolymerization. It is possible that large parts of these structure are composed of phasins and allow the PHB synthase and/or PHB depolymerases to enter the granule. The granule-spinning filaments have a diameter of 4 nm, which is identical to the estimated diameter of PhaP1. This model and the hypothesis are rather speculative and preliminary and require much additional investigation for confirmation.

Simultaneously, the structure of PHB granules from *Comamonas acidovorans* was also investigated by AFM (Sudesh et al. 2004). Globular particles with diameters of 31 nm that were attached to large parts of the granule surface and that could be washed off with water to reveal a smooth granule structure were observed. The authors did not observe central pores in the globular structures. Furthermore, an *in vitro* model system simulating the formation of the surface envelope by adding purified PhaR and PhaP from *Paracoccus denitrificans* was used and analyzed by AFM. The incubation resulted in the formation of a surface membrane of globular particles with a thickness of approximately 4 nm. The proteins attached firmly and directly to the granule without a phospholipid monolayer.

Whatever structures the phasins may form at the surface of PHB granules, there is multiple evidence that they considerably affect PHB metabolism and the arrangement of PHB in the cells.

4
Biotechnological Production and Applications of Nanoparticles of PHAs

According to their beneficial properties, PHAs can be used for various technical applications, because they are thermoplastic and/or elastomeric, nontoxic, biodegradable and can be produced from renewable resources. The copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), which is sold under the trade name Biopol, as well as the homopolymer PHB are produced by fermentation of glucose-utilizing mutants of *Ralstonia eutropha* H16. These biomaterials originate from renewable resources and are applicable for biodegradable packaging materials such as bottles or foils (Asrar and Gruys 2002; Lee and Park 2002). Various commercial products fabricated from Biopol are presented in Fig. 7. Moreover, PHAs are biocompatible and can be

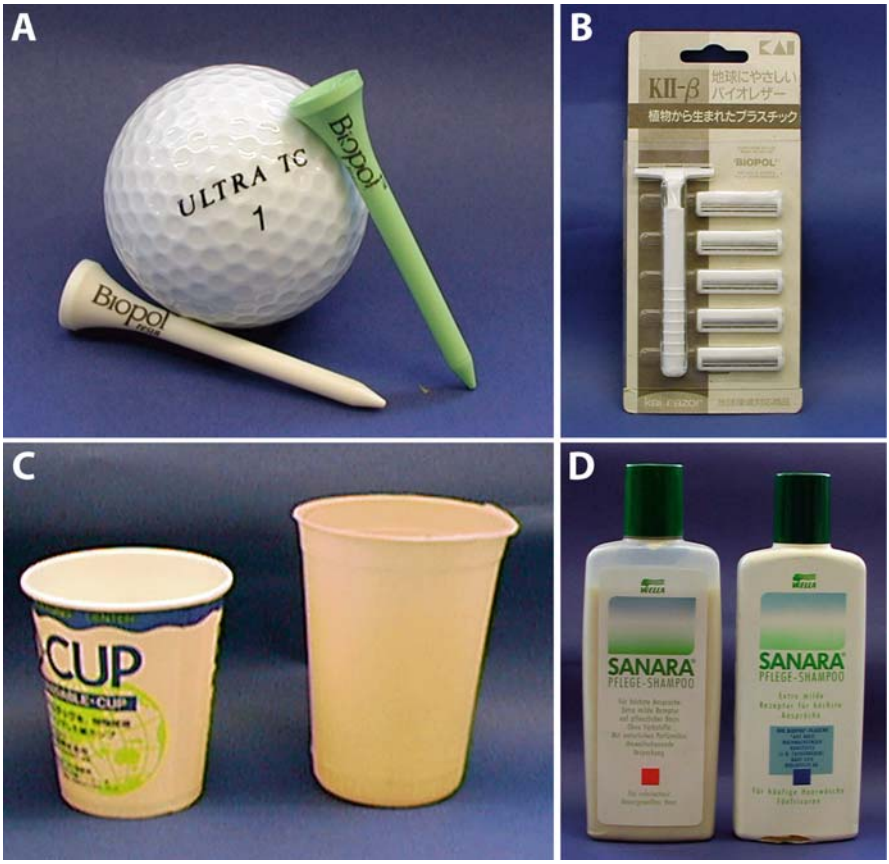

Fig. 7 Commercial products made from Biopol. **a** golf pins; **b** razor; **c** cups; **d** shampoo bottles

used for medical or pharmaceutical applications, for example, biodegradable sutures, retard materials or scaffolding material for cultivation of tissues (Williams and Martin 2002). PHA biosynthesis genes from *Ralstonia eutropha* H16 were also expressed in many other organisms, whereas transgenic plants, which produce Biopol directly from carbon dioxide, are of special interest (Poirier and Gruys 2002). Owing to the more elastic, partially rubberlike properties, PHA_{MCL}s are also considered for special applications such as hydrophobic coatings and latex; these polyesters can be produced at relatively low costs from plant oils by *Pseudomonas* species (Weusthuis et al. 2002).

At present, the production costs of bacterial PHAs from renewable resources are much higher than the costs of conventional plastics produced from fossil resources. Only the use of transgenic plants will probably provide economically feasible processes to obtain PHAs applicable for bulk products. In contrast, for specialized applications of PHAs, particularly in medicine and pharmacy, production costs are less relevant owing to the comparably small amounts of material required for these high-value products. In addition, these PHAs often comprise specific constituents conferring special physical and material properties required for the respective applications; such tailormade PHAs will more likely be available from bacterial fermentations. One well-studied example is poly(4-hydroxybutyrate), which is—in contrast to PHB—rapidly hydrolyzed by lipases and esterases, and is therefore the preferred PHA for use in tissue engineering owing to its fast resorption in the mammalian body (Williams and Martin 1999).

Excellent biocompatibility and biodegradability make PHAs also attractive for applications in nanotechnology. Most research on medical applications of PHAs described so far has been done with PHB. Toxicity tests according to ISO 10993 demonstrated that PHB is suitable for use as nanoparticles in animals. Early studies reported the use of PHB as a matrix in retard tablets (Korsatko et al. 1984). This and other studies confirmed that PHB degrades *in vivo*; however, *in vivo* degradation is slow, and PHB is completely resorbed in the body only after 24–30 months. In contrast, the homopolymer poly(4-hydroxybutyrate) behaves different and degrades more rapidly *in vivo*. This might be advantageous for those nanoparticle applications where faster disintegration of the matrixes is desirable as this is often required for pharmaceutical and medical applications (for a review see Pötter and Steinbüchel 2005). Also PTEs exhibit interesting material properties and are in contrast to PHAs obviously persistent with regard to biodegradation (Kim et al. 2005).

The production of fusion proteins, which are fused with effective targets, for industrial or pharmaceutical applications is very important. The generation of novel complex two-component biomaterials based on the PHA binding domains of phasins or other PHA granule associated proteins, which are suitable as nanoparticles and for coating of hydrophobic surfaces, is an

interesting and promising field. This will generate a very versatile and flexible system consisting of two components that can be used according to the modular-design principle for the generation of novel biomaterials.

In 1995 the C-terminally truncated variants of the GA14 protein of *Rhodococcus ruber* lacking the second domain or both hydrophobic domains was fused with the acetaldehyde dehydrogenase II of *Ralstonia eutropha* H16 (Pieper-Fürst et al. 1995). The fusion protein was able to bind to native and artificial PHB granules. This was the first example of modified PHA granule bioplastic consisting of a biodegradable matrix carrying an active enzyme.

Recently, a protein immobilization and purification system was developed based on the use of PHA_{MCL} (Moldes et al. 2004). The *N*-terminal domain of the PhaF phasin from *Pseudomonas putida* GPo1 was used as a polypeptide tag (BioF) to anchor fusion proteins to PHAs. The PHA granules carrying the BioF fusion proteins can be isolated by a simple centrifugation step and used directly for applications. Very recently, a method for economical purification of recombinant proteins was presented (Banki et al. 2005). Strains of *E. coli* producing PHB granules were used in combination with a self-cleaving intein tag to create a simple, economic alternative for conventional affinity-based protein purification. The intracellular granules are produced by *E. coli* to act as an affinity matrix for a coexpressed tagged protein.

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Wax Ester and Triacylglycerol Inclusions

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Abstract Neutral lipids such as wax esters (WEs) and triacylglycerols (TAGs) are frequently accumulated as energy and carbon stores in certain groups of bacteria. The biosynthesis of these lipids is promoted in cells grown during unbalanced growth, if an essential nutrient is limited and a surplus of a carbon source is available. They are again mobilized under conditions of carbon and energy deficiency. In general, neutral lipids are stored as insoluble inclusions with different shapes and sizes inside the cytoplasm, depending on the lipid, strain and culture conditions. The structure, morphology and biogenesis of these inclusions are the main topics of this chapter. A short overview of the metabolic pathways leading to the biosynthesis of TAGs and WEs and the enzymes involved therein is given. Parallels to and differences from the structure and formation of polyhydroxyalkanoate granules in bacteria and neutral lipid bodies in multicellular organisms such as plants and animals and unicellular eukaryotes are described.

1 Introduction

Accumulation of storage lipids is a widespread property among prokaryotes. All these lipids are constituted from esterified fatty or hydroxy fatty acids. These lipids serve as a depot for energy and carbon needed for maintenance of metabolic activity and synthesis of cellular metabolites during starvation. By far most prokaryotic species are able to synthesize and accumulate polymeric lipids like poly(3-hydroxybutyric acid) (PHB) (Fig. 1) or other polyhydroxyalkanoates (PHAs) such as poly(3-hydroxyoctanoate) (Fig. 1) (Steinbüchel 2001; Steinbüchel and Valentin 1995). A variety of species, mainly belonging to the actinomycetes group and some Gram-negative species, are able to synthesize and accumulate neutral lipids like triacylglycerols (TAGs) (Fig. 1) and wax esters (WEs) (Fig. 1) as intracellular storage lipids (Alvarez and Steinbüchel 2002; Bryn et al. 1977; Russel and Volkman 1980; Bredemeier et al. 2003). In contrast to their restricted occurrence in prokaryotes, TAGs are the primary energy store in eukaryotes (Zweytick et al. 2000; Murphy 2001), whereas intracellular accumulation of WEs occurs only in the seeds of the jojoba (*Simmondsia chinensis*) plant (Yermanos 1975). These lipids represent ideal storage compounds, since they exhibit a low biological toxicity compared with free fatty or hydroxy fatty acids and are water-insoluble and

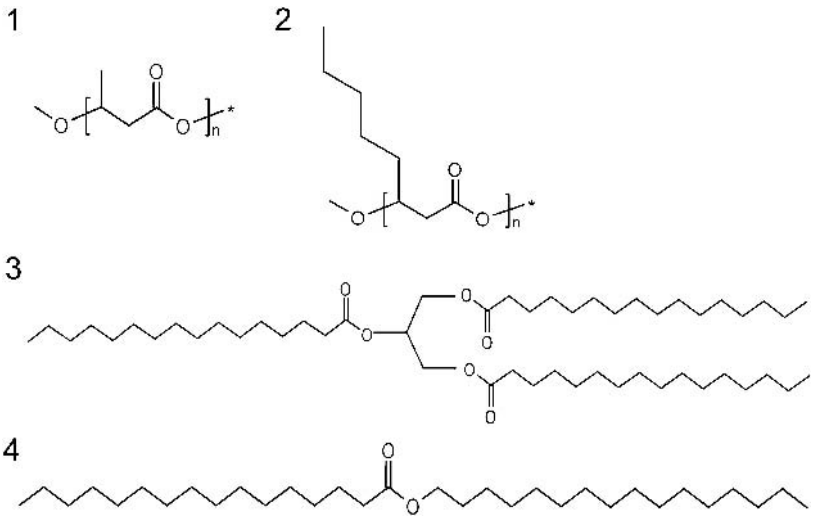

Fig. 1 Chemical structures of lipid storage compounds in bacteria. *1* Poly(3-hydroxybutyric acid) (PHB) as an example of a short-chain polyhydroxyalkanoate (PHA); *2* poly(3-hydroxyoctanoate) as an example of a medium-chain PHA; *3* tripalmitoylglycerol as an example of triacylglycerols (TAGs); *4* cetylpalmitate as an example of bacterial wax esters (WEs)

osmotically inert; therefore, they do not affect the water balance of the cells. Furthermore, accumulation of lipids is much more advantageous than that of proteins or carbohydrates, owing to their much higher caloric value and their relative compactness (Murphy 1993). Storage lipid biosynthesis in bacteria and unicellular eukaryotes is induced in response to stress imposed on the cells, for example, when the cells find themselves in a nutrient-limited environment and when a carbon source is available that can be readily used for storage lipid biosynthesis. Owing to their water insolubility, storage lipids are always deposited as intracellular inclusions.

In general, these inclusions are composed of a core of the respective storage lipid (TAGs, WEs, sterolesters or PHAs) which is surrounded by a layer of phospholipids and special classes of proteins (Wältermann and Steinbüchel 2005; Zweytick et al. 2000; Murphy 2001). However, although prokaryotic and eukaryotic lipid inclusions seem structurally related and relatively simple, they exhibit striking differences. The occurrence, composition, morphology and formation of prokaryotic TAG and WE inclusions and their relationships to PHA granules in bacteria will be the main topics of this treatise. In addition, a basic overview of the different situation of neutral lipid biosynthesis and lipid body structure and formation in plants, mammals and eukaryotic microorganisms will be given.

2
Occurrence of Storage Lipids in Prokaryotes and their Chemical Structure

Only a few prokaryotes lack the ability to accumulate lipids. These species exist mainly in nutrient-rich habitats, in which biosynthesis of lipophilic storage compounds seems meaningless (for example, lactobacilli, *Enterobacteriaceae*, and methanogens). Therefore, it is very likely that accumulation of lipophilic storage compounds is somehow advantageous for survival in natural habitats usually providing little carbon and during evolution.

2.1
TAGs in Bacteria

TAGs (trioxoesters of glycerol and long-chain fatty acids) as the main storage lipids in bacteria have mainly been described in *Actinomycetales* like *Actinomyces*, *Arthrobacter*, *Mycobacterium* sp., including *Mycobacterium tuberculosis*, *Nocardia* ssp., *Rhodococcus* sp., *Gordonia* sp. and *Dietzia* sp. (Koval'schuk et al. 1973; Wayman et al. 1984; Akao and Kusaka 1976; Alvarez et al. 1997; Alvarez and Steinbüchel 2002; Barksdale and Kim 1977) and in streptomycetes such as *Streptomyces coelicolor, Streptomyces lividans* and *Micromonospora echinospora* (Olukoshi and Packter 1994; Hoskisson et al. 2001). The amounts of TAGs in these species strongly varies from merely 10% up to an enor-

mous 80% of the cellular dry matter, for example, in *Rhodococcus opacus* PD630, which is one of the best studied model organisms with respect to its neutral lipid metabolism (Alvarez et al. 1996). It should be mentioned that a few actinomycetes are capable of accumulating significant amounts of PHAs simultaneously with TAGs. For example, *R. ruber* is capable of accumulating similar amounts of TAGs and the copolyester poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from glucose (Alvarez et al. 1997b; Kalscheuer et al. 2001). TAGs are also frequently accumulated in the Gram-negative genera *Acinetobacter* and *Alcanivorax* (Makula et al. 1975; Scott and Finnerty 1976; Singer et al. 1985; Bredemeier et al. 2003). These species accumulate minor amounts of TAGs besides major amounts of WEs, which serve as the predominant storage lipid. There was also a report of TAG biosynthesis in *Pseudomonas aeruginosa* strain 44T1 (De Andrés et al. 1991). However, the occurrence of TAGs in this strain could not be confirmed in our laboratory (Wältermann and Steinbüchel, unpublished results). So far, TAGs have never been reported to occur in Archaea.

Compositions and amounts of bacterial TAGs strongly depend on the cultivation conditions. In general, cells do not contain significant amounts of TAGs in their exponential growth phases or when cultivated in nutrient-rich complex media. The amount of TAGs in the cells increases when the cells enter the stationary growth phase, in particular when they are cultivated in unbalanced mineral salt medium with a high carbon-to-nitrogen ratio (Packter and Olukoshi 1995; Wältermann et al. 2005). The fatty acid composition of the accumulated TAGs depends strongly on the carbon source used for growth and fatty acid biosynthesis. In general, bacterial TAGs are composed mainly of long chain, even-numbered saturated and monoenic fatty acids such as palmitic and oleic acids when grown on structurally unrelated carbon sources like fructose, glucose or gluconate; however, significant variations also occur (Alvarez et al. 1997a, b). For example, TAGs from gluconate-grown cells of *R. opacus* PD630 contain considerable amounts of odd-numbered fatty acids such as margaric acid (11.4%) and heptadecenoic acid (10.6%) beside palmitic acid (36.4% of total fatty acid content) and oleic acid (19.4%). The fatty acid composition of TAGs in *R. opacus* varies strongly when the cells are cultivated on propionate or valerate. Under these conditions, the fraction of odd-numbered fatty acids strongly increases, whereas the fraction of even-numbered fatty acids decreases (Alvarez et al. 1997b). Biosynthesis of TAGs with polyunsaturated fatty acid residues from unrelated carbon sources has never been observed in bacteria. In addition, formation of TAGs does not only depend on fatty acid *de novo* synthesis, as was revealed by growth experiments using different hydrocarbons as carbon sources, which were directly converted into their respective acyl-Coenzyme A (acyl-CoA) and were incorporated into TAGs (Alvarez et al. 2001, 2002).

Similar to TAGs from plant or animal origin, the fatty acid distribution of the acyl residues at the glycerol backbone is not random as was re-

vealed from stereospecific chemical analyses. For example, in *Mycobacterium smegmatis* and *Mycobacterium bovis*, fatty acids with acyl chains of more than 20 carbon atoms were predominantly esterified to the *sn*-3 position of the glycerol, whereas C16 fatty acids occupied the *sn*-2 position and either stearyl, oleyl or tuberculostearyl residues were located at position *sn*-1 (Walker et al. 1970). In TAGs of gluconate-grown *R. opacus* PD630, shorter and saturated fatty acid residues tend to prefer position *sn*-2 against position *sn*-1 and *sn*-3. The unsaturated fatty acid residues are predominantly found at position *sn*-3, whereas unsaturated fatty acids and acyl chains with more than 17 carbon atoms were not found at position *sn*-2 (Wältermann et al. 2000).

2.2 WEs in Bacteria

For more than 30 years, biosynthesis and accumulation of WEs (oxoesters of primary long-chain fatty alcohols and long-chain fatty acids) has often been reported for species of the genus *Acinetobacter* (Fixter and Fewson 1974; Fixter and McCormack 1976; Gallagher 1971; Scott and Finnerty 1976; Kalscheuer et al. 1999) and less frequently also for *Moraxella*, *Micrococcus* and *Alcanivorax* (Bryn et al. 1977; Russel and Volkman 1980; Bredemeier et al. 2003). Also in actinomycetes, WEs have been described, for example, in *Corynebacterium*, *Mycobacterium tuberculosis* and *Nocardia* (Bacchin et al. 1974; Raymond et al. 1960; Wang et al. 1972). Similar to the accumulation of PHAs and TAGs, WE accumulation occurs during growth limitation owing to a lack of a suitable nitrogen source. Under this condition, *Acinetobacter* sp. also synthesize WEs from carbon sources like acetate, sugars and sugar acids; however, the total amount of WEs is low in contrast to that of cells grown on alkanes, in which the total WE content can reach about 25% of the cellular dry matter (Fixter et al. 1986). The chemical structures of WEs are similar to those produced by jojoba and the sperm whale (*Physeter macrocephalus*). They consist mainly of 32–36 carbon atoms, with saturated and monoenic C16 and C18 fatty acid and fatty alcohol residues, respectively (Ervin et al. 1984; Ishige et al. 2002). *Acinetobacter* sp. also synthesize WEs from long-chain alkanes, with fatty acid and fatty alcohol residues according to the chain length of the assimilated alkanes. Furthermore, WEs with chain lengths of two and four carbon atoms less were also synthesized (Dewitt et al. 1982). The degree of saturation of the accumulated WEs depends on the culture temperature, with higher ratios of monoenic and dienic WEs occurring at lower temperatures (Ervin et al. 1984). Interestingly, *Acinetobacter* sp. strain ADP1 and *Alcanivorax jadensis* are also capable of synthesizing unusual wax diesters in the presence of diols or even from hexadecane (Kalscheuer et al. 2003; Reers, Wältermann, Luftmann and Steinbüchel, unpublished results).

WEs do not exclusively occur as intracellular storage compounds, since some strains of *Acinetobacter* sp. and *Alcanivorax jadensis* were reported to also synthesize extracellular WEs from hydrocarbons, but the origin and possible function of these extracellular WEs remain to be elucidated (Dewitt et al. 1982; Makula et al. 1975; Singer et al. 1985; Bredemeier et al. 2003).

3
Metabolism of Storage Lipids in Bacteria

As the interest of academia and industry in bacterial storage lipids is continuously rising, much research has been focused on the biochemical and molecular basics of storage lipid biosynthesis during recent years.

3.1
TAG Metabolism

In general, TAG biosynthesis in bacteria is carried out via the Kennedy pathway, involving three sequential acylation reactions (Fig. 2) (Lehner and Kuk-

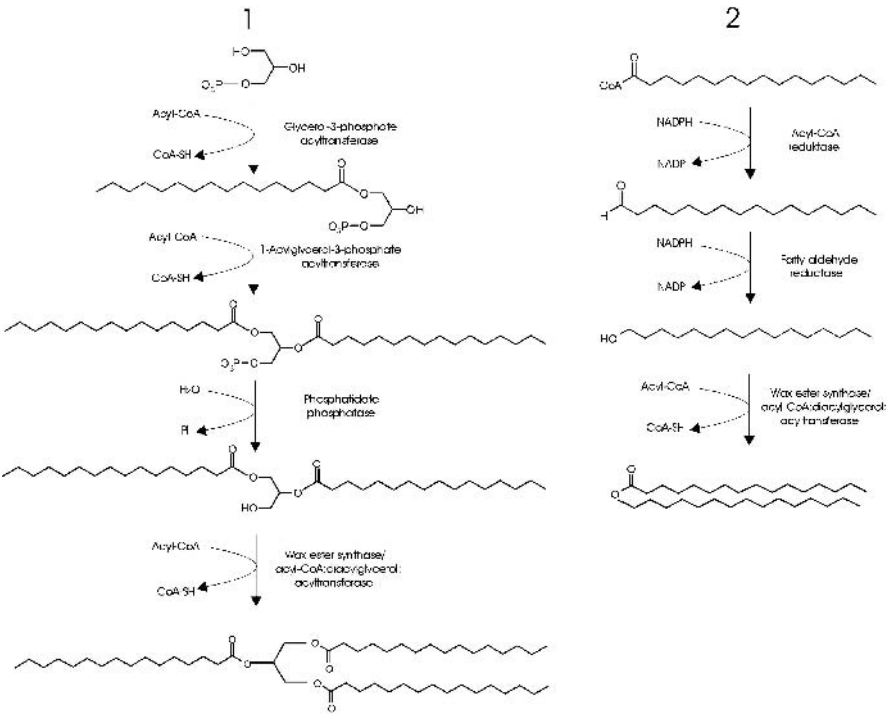

Fig. 2 Metabolic pathways for the biosynthesis of (*1*) TAGs and (*2*) WEs

sis 1996). This pathway combines the formation of TAGs and phospholipids, since biosynthesis of phosphatidate and diacylglycerol (DAG) from glycerol-3-phosphate share the same enzymes. Biosynthesis of DAG starts in the formation of lysophosphatidate (1-acylglycerol-3-phosphate) from glycerol-3-phosphate through catalysis of glycerol-3-phosphate acyltransferase. In *Escherichia coli*, this enzyme is able to use acyl-CoAs and also fatty acids bound to acyl carrier protein (ACP) as substrates, and it is localized at the cytoplasm membrane (Wilkinson and Bell 1997). Subsequently, lysophosphatidate is converted to phosphatidate by lysophosphatidate acyltransferase, which is also localized at the cytoplasm membrane (Coleman 1990). Finally, DAG is synthesized from phosphatidate through dephosphorylation catalyzed by phosphatidate phosphatase (Icho and Raetz 1983). In *Acinetobacter* sp. strain ADP1, the last step in biosynthesis of TAGs is mediated by a promiscuous WE synthase/acyl-CoA:DAG acyltransferase (WS/DGAT).

This WS/DGAT is responsible for biosynthesis of TAGs and WEs with acyl-CoA as an acyl donor and DAG or long-chain fatty alcohol as acyl acceptors, respectively (Kalscheuer and Steinbüchel 2003). The activity of WS/DGAT using DAG as an acyl acceptor instead of fatty alcohols is one order of magnitude lower, with a high specificity using the *sn*-1 and *sn*-3 positions of glycerol as acyl acceptors, rather than the *sn*-2 position (Stöveken et al. 2005). The substrate specificities regarding fatty alcohols and DAG as acyl acceptors resemble the distribution of both storage lipids in *Acinetobacter* sp. strain ADP1 (Kalscheuer and Steinbüchel 2003). Furthermore, this enzyme exhibits also monoacylglycerol acyltransferase activity (Stöveken et al. 2005). Although this WS/DGAT (also Atf1) is responsible for WE and TAG biosynthesis in *Acinetobacter* sp. strain ADP1, this type of long-chain acyl-CoA acyltransferase is not related to the known WS from jojoba, or the DAG:acyltransferase 1 (DGAT1) and DAG:acyltransferase 2 (DGAT2) families present in yeasts, plants and animals, and the phospholipid:DGAT catalyzing TAG formation in yeasts and plants (Cases et al. 1998, 2001; Hobbs and Hills 1999; Routaboul et al. 1999; Zou et al. 1999; Bouvier-Navé et al. 2000; Sandager et al. 2002; Dahlquist et al. 2000). Although there is only one *atf1* gene present in *Acinetobacter* sp. strain ADP1, genes for Atf1 homologues seem to be widespread among the *Actinomycetales*, since an extensive group of related genes occurs in streptomycetes and mycobacteria, with, for example, 15 homologous genes in *Mycobacterium tuberculosis* H37Rv (Kalscheuer and Steinbüchel 2003; Daniel et al. 2004).

Similar to the degradation of WEs or PHAs, TAGs are degraded under conditions of carbon and energy deficiency. In this context, Alvarez et al. (2000) demonstrated mobilization of more than 90% of the accumulated TAGs in *R. opacus* and *R. ruber* within 120 h, when the cells were cultivated in the absence of a suitable carbon source and in the presence of ammonium. However, knowledge of the degradation of intracellular TAGs in bacteria is very scarce, and the enzymes involved therein and their regulation are still unknown.

3.2
WE Metabolism

The metabolic pathway for biosynthesis of WEs in *Acinetobacter* sp. involves three enzymatic reactions (Fig. 2) (Ishige et al. 2003). First, long-chain acyl-CoAs, which are derived from fatty acid *de novo* synthesis or alkane oxidation, are reduced to the corresponding fatty aldehydes by an NADPH-dependent acyl-CoA reductase (Acr1). The corresponding gene (*acr1*) encodes a membrane-localized enzyme of 32.5 kDa and accepts acyl-CoAs with chain lengths from 14 to 22 carbon atoms (Reiser and Somerville 1997). The next reduction step is mediated by an NADPH-dependent fatty aldehyde reductase, yielding the corresponding fatty alcohols; however, the gene encoding this enzyme has not been identified yet. The crucial metabolic step, which condenses long-chain acyl-CoAs and long-chain fatty alcohols forming the respective WEs, is mediated by WS/DGAT encoded by *atfA* (Kalscheuer and Steinbüchel 2003). This 51.8-kDa enzyme comprises a putative membrane-spanning region, and was also demonstrated to be mainly membrane-localized, but seems also to be distributed throughout the cytoplasm and on the surface of intracelluar lipid bodies (Wältermann et al. 2005; Stöveken et al. 2005; Kalscheuer et al. 2006). WS/DGAT from *Acinetobacter* sp. strain ADP1 shows a broad ability to utilize fatty alcohols and also to some extent DAGs as acyl acceptors *in vitro*. Actually, this enzyme is also responsible for biosynthesis of the minor amounts of storage TAGs occurring in *Acinetobacter* sp. strain ADP1 (Kalscheuer et al. 2003; Stöveken et al. 2005).

Since WEs act as true storage lipids, degradation of the waxes has to occur in growth-accommodating environments and under carbon limitation. The initial step in the degradation of intracellular WEs is surely not the reverse reaction mediated by the WS/DGAT enzyme. Most probably, degradation of storage WEs is catalyzed by an intracellular esterase or lipase. Attempts to identify such an enzyme have not been successful so far.

4
Structure and Morphology of Prokaryotic Lipid Inclusions

All storage lipids in bacteria are stored in discrete, intracellular lipid inclusions. When observed by light microscopic techniques, these inclusions appear as intensively light scattering particles inside the cytoplasm. In transmission electron microscopic studies, lipophilic inclusions appear as electron-transparent structures of different sizes and shapes and without a visible internal structure but with a single boundary layer. Although their structure seems relatively simple, interesting differences between the different types of inclusions occur.

4.1 TAG Inclusions

TAG inclusions in actinomycetes, whose formation has been completed, occur as spherical and electron-transparent storage droplets inside the cytoplasm, with diameters ranging from 50 to 400 nm (Fig. 3). The number and size of these inclusions vary depending on the respective strain, growth phase and cultivation conditions. Similar to their plant, animal and yeast analogues, TAG inclusions in bacteria were frequently reported to possess a thin boundary layer comprising half the width of the plasma membrane, which most probably shields the lipid core from the cytoplasm (Packter and Olukoshi 1995; Alvarez et al. 1996). However, in cells harboring high amounts of TAGs, inclusions show some indication of coalescence, most probably caused by tight direct interactions of the inclusions (Packter and Olukoshi 1995; Alvarez et al. 1996). Similar to lipid bodies in cultured mammalian cells, bacterial TAG inclusions split into numerous internal fracture planes when freeze-fractured, leading to a lamellar internal view of the hydrophobic core (Fig. 3) (Packter and Olukoshi 1995; Robenek et al. 2004; Wältermann et al. 2005). These fracture planes are very similar to that of membrane fracture faces. It is not known whether these fracture planes are caused by physical artifacts during the fracturing process or are a true series of tightly compressed lipid layers making up the TAG core (Wältermann et al. 2005). It should be mentioned that PHA inclusions inside the bacterial cytoplasm can hardly be distinguished from WE and TAG inclusions by transmission electron microscopy (Fig. 3). However, they behave quite differently in freeze-fracture experiments. Depending on the chemical composition of PHA and the temperature at which the fracturing is performed, PHA granules exhibit needle-like or mushroomlike artifacts, which occur owing to a plastic deformation process. Fractured granules are often observed to be composed of a central, stretchable inner core and an outer core which withstands deformation while fracturing (Fig. 3) (Preusting et al. 1993).

A particularly interesting situation occurs in actinomycetes, which are capable of simultaneously accumulating TAGs and PHAs in similar amounts like in *R. ruber*. It remains to be elucidated whether TAGs and PHAs occur in discrete, separated inclusions, or if both types of storage lipids are deposited in one and the same type of inclusion. However, it could be demonstrated that the *R. ruber* phasin, a class of structural proteins thought to be specific for PHA granules, coated all intracellular inclusions (Pieper-Fürst et al. 1994). TAG inclusions from gluconate-grown cells of *R. opacus* PD630 were first isolated from cellular lysates by density-gradient ultracentrifugation, and the chemical composition was investigated by Alvarez et al. (1996). These inclusions were mainly composed of TAGs (87%), DAGs (approximately 5%), phospholipids (1.2%) and proteins (0.8%). TAG inclusions exhibited a very complex protein pattern strongly resembling that of total cell

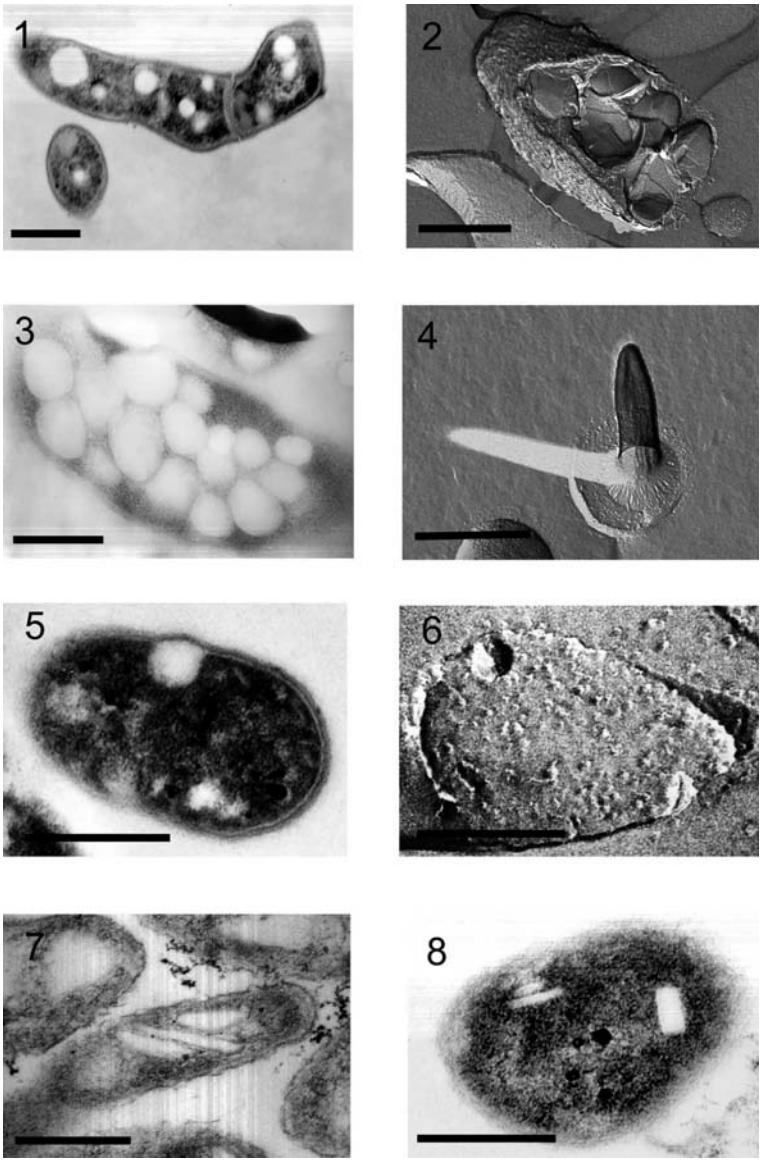

Fig. 3 Morphology of bacterial lipid inclusions. *1* Lipid inclusions and lipid prebodies in a cell of *Rhodococcus opacus* PD630; *2* freeze-fracture preparation of a cell of *R. opacus* PD630 exhibiting lamellar fracture planes on intracellular TAG inclusions; *3* cell of *R. eutropha* H16 with PHB granules; *4* needlelike deformation of a PHB granule in *R. eutropha* H16 in a freeze-fracture preparation; *5* cell of *Acinetobacter* sp. strain ADP1 with a nascent, spherical WE inclusion; *6* freeze-fractured cell of *Acinetobacter* sp. strain ADP1 accumulating WE inclusions; *7* cells of *Alcanivorax jadensis* exhibiting half-moon-like and disclike WE inclusions; *8* rectangular WE inclusions in *Acinetobacter* sp. strain ADP1. *Bars* 1 μm

lysates when resolved by polyacrylamide gel electrophoresis. We were unable to identify structural proteins specifically bound to the surface of these inclusions like proteins accomplishing similar functions as the oleosins on the surface of oil droplets in plants or phasins on the surface of PHA granules (Kalscheuer et al. 1999; Wältermann and Steinbüchel 2005). All proteins found to be strongly associated with the surface of isolated TAG inclusions could not be related to lipid metabolism or lipid body structure, indicating that these proteins became bound unspecifically only during cell disruption. Thus, the surface of bacterial TAG inclusions is most probablely comparable to that of WE inclusions in *Acinetobacter* sp. and lipid bodies in yeasts or the mesocarb tissue from olive or avocado, which also lack specific structural proteins (Kalscheuer et al. 2006; Murphy and Vance 1999; Murphy 2001).

4.2 WE Inclusions

Since the amounts of WEs in the investigated bacteria are relatively low in comparison to those of some TAG-accumulating bacteria, only a limited number of WE inclusions occur in the cytoplasm (approximately three to five). WE inclusions in bacteria show a wide variety of different morphologies in electron microscopic images, ranging from (1) spherical and ellipsoid to (2) disclike structures and to (3) even rectangular shapes:

1. Spherical inclusions in hexadecane-grown cells of *Acinetobacter* sp. strain HO1-N exhibited average diameters of 200 nm. These inclusions were first misinterpreted as depots of the unmodified carbon source. However, hexadecane was only a minor component, whereas WEs constituted the dominant part of these inclusions. On the basis of freeze-etching and transmission electron micrographs, the authors suggested that these inclusions were separated by a phospholipid monolayer membrane. Interestingly, also intracellular bilayer membranes were observed in these preparations (Scott and Finnerty 1976). Similarly, *Acinetobacter* sp. strain ADP1 accumulates spherical, electron-transparent WE inclusions when cultivated on carbohydrates or hexadecane (Fig. 3). These inclusions were also demonstrated to be surrounded by a phospholipid monolayer membrane (Wältermann et al. 2005). Freeze-fracturing of spherical WE inclusions leads to irregular internal fracture faces, with different fracture planes and sharply delineated edges, confirming a surrounding membrane (Fig. 3) (Wältermann et al. 2005; Scott and Finnerty 1976).
2. In *Acinetobacter* sp. strain M-1, grown on hexadecane, WE inclusions occur as electron-transparent, smooth and disclike structures. On the basis of electron micrographs, these structures were reported to lack a surrounding membrane; however, the authors did not perform further

attempts to identify a possible membrane structure surrounding these inclusions. These WE inclusions grew to about the same diameter as the cell. The time course of their formation was also investigated. The authors described the sequential formation of WE discs, with one disc completion resulting in the formation of the following one (Ishige et al. 2002). However, the formation of disclike WE inclusions in this strain seems not to be a curiosity or an artifact, since similar structures were also observed in *Alcanivorax* sp. accumulating WEs from hexadecane and to some extent also in *Acinetobacter* sp. strain ADP1 in parallel with spherical inclusions (Fig. 3) (Manilla, Wältermann and Steinbüchel, unpublished results).

3. Singer et al. (1985) reported on electron-transparent, rectangular inclusions with a typical bilayer structure at their surface in strain *Acinetobacter* sp. strain HO1-N, when cultivated on hexadecanol (Fig. 3). These rectangular structures were 100–200 nm in length, exhibited a width of approximately 30 nm and consisted mainly of hexadecylpalmitate (85.6%) and minor amounts of hexadecanol (4.8%) and phospholipids (9.6%). The inclusions also occurred in parallel with cytoplasmic membrane structures. Whether the rectangular WE inclusions reported by Singer et al. (1985) and the disclike WE inclusions reported by Ishige et al. (2002) and our laboratory are just morphological variations of the more frequently observed spherical inclusions or whether their formation relies on mechanisms different from that described and discussed later remains to be elucidated.

Isolated WE inclusions from different *Acinetobacter* strains exhibited a complex pattern of copurified proteins, and no protein identified among them has been structurally related to these inclusions so far, reminding us of the situation of isolated TAG inclusions from *R. opacus* and *R. ruber* (Scott and Finnerty 1976; Alvarez et al. 1997a; Kalscheuer et al. 2001; Wältermann and Steinbüchel 2005). A very recent report on recombinant *Escherichia coli*, using an artificial pathway for WE production employing a bifunctional acyl-CoA reductase from jojoba and *Acinetobacter* sp. strain ADP1 WS/DGAT describes the formation of intracellular WE inclusions in this strain. The inclusions had the same size and spherical shape as those described for *Acinetobacter* sp. strain ADP1, which proves that structural proteins are not needed for the formation and structural integrity of WE inclusions (Kalscheuer et al. 2006). Recently, it was demonstrated that WS/DGAT is partly localized on the surface of WE inclusions of *Acinetobacter* sp. strain ADP1 and also in recombinant *E. coli*; however, it remains questionable if the enzyme exhibits a significant biosynthetic activity at this location *in vivo* (Wältermann et al. 2005; Kalscheuer et al. 2006).

5
Formation of Prokaryotic TAG and WE Inclusions and PHA Granules

In eukaryotes, storage lipid synthesis and formation of lipid bodies are thought to arise most probably from the endoplasmic reticulum (ER) by a unique mechanism. Since prokaryotes do not possess an ER, the mechanism of the formation of neutral lipid inclusions must be quite different.

5.1
TAG Inclusions

Under appropriate experimental conditions (see before), strains like *R. opacus* PD630 or *Mycobacterium smegmatis* reach their maximum TAG content within a period of 72 h. During this time, nearly TAG and lipid inclusion free cells transform to cells in which the cytoplasm is substantially filled with lipid inclusions. Application of fluorescent dyes as lipid markers on TAG inclusion biogenesis indicated that neutral lipid accumulation was initiated at peripheral lipid domains of the cells, and that large lipid inclusions localized in the cytoplasm occur only at a later stage of lipid accumulation. When observed by phase-contrast microscopy, these early lipid domains appear as small, intensively light scattering granules, whereas large lipid inclusions observed at later stages occur as clearly visible droplets filling almost the whole cytoplasm (Christensen et al. 1999; Wältermann et al. 2005). It was assumed that the formation of TAG inclusions in actinomycetes underlies the same mechanism as the formation of spherical WE inclusions in *Acinetobacter* sp. strain ADP1, which was investigated in more detail using purified WS/DGAT in *in vitro* experiments (Fig. 4; Sect. 5.2) (Wältermann et al. 2005). However, significant differences also occur.

Specimens of ultrathin sections of both *Mycobacterium smegmatis* and *R. opacus* exhibited a strong morphological diversification from low TAG containing cells to cells harboring a maximum lipid content in the final stage. In early stages of lipid accumulation, flat-to-spherical structures of a higher electron density compared with matured TAG inclusions occurred close to the cytoplasm membrane (Fig. 3). These structures correspond to the peripheral lipid domains visible in fluorescence microscopy, and were referred to as lipid prebodies. Lipid prebodies appear to be in conjunction with a distinct layer of varying thickness facing the cytoplasmic site of the cytoplasm membrane, which was referred to as an oleogenous layer. Lipid prebodies were observed to enlarge until they reached an average diameter of about 300 nm, before they left the oleogenous layer at the membrane and migrated into more central parts of the cytoplasm. Lipid prebodies bound to the cytoplasm membrane and cytoplasm-localized prebodies exhibit a relatively diffuse shape compared with that of the electron-transparent lipid inclusions found in later stages of lipid accumulation, and are not surrounded

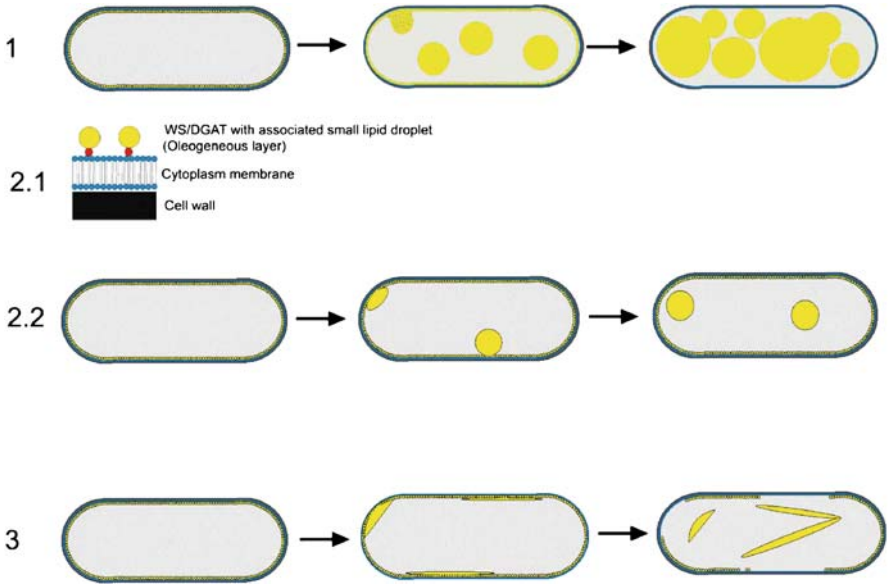

Fig. 4 Formation of intracellular neutral lipid inclusions in bacteria. *1* Formation of TAG inclusions; *2.1*, formation of small lipid droplets at the cytoplasmic face of the cytoplasm membrane; *2.2* formation of spherical WE inclusions; *3* hypothetical model for the formation of nonspherical WE inclusions. *WS* WE synthase, *DGAT* diacylglycerol acyltransferase (Updated from Wältermann and Steinbüchel 2005)

by a clearly visible outer layer in electron micrographs. It was assumed that lipid prebodies do not exhibit a homogeneous lipid core, but are composed of an emulsion-like aggregation of very small lipid droplets (SLDs) in conjunction with phospholipids and proteins. How lipid prebodies convert to matured lipid inclusion is unknown. It was speculated that the emulsion-like aggregation of SLDs aggregate to structures with a homogeneous lipid core by proceeding, thermodynamically driven coalescence (Wältermann et al. 2005; Wältermann and Steinbüchel 2005).

5.2 WE Inclusions

Similar to the formation of TAG inclusions in the actinomycetes mentioned earlier, formation of spherical WE inclusions in *Acinetobacter* sp. strain ADP1 occurs at peripheral sites of the cells, as can be easily observed using lipophilic dyes and fluorescence microscopy techniques. According to this, nascent WE inclusions can be observed in proximity to the cytoplasm membrane in transmission electron microscopy preparations. They are often in conjunction with a thin layer facing the cytoplasmic site of the membrane (Fig. 3). The situation that WS/DGAT is mainly localized at the cytoplasm

membrane indicates that this compartment is the natural site of WE biosynthesis and the origin of the formation of WE inclusions. As suggested by Wältermann et al. (2005), biosynthesis of WEs by membrane-bound WS/DGAT in *Acinetobacter* sp. strain ADP1 leads to the formation of very small WE droplets with diameters of only some nanometers at the cytoplasmic face of the cytoplasm membrane (Fig. 4). These SLDs remain associated with the enzyme and form a small, emulsion-like layer of WE biosynthesis. This layer is permeable for hydrophilic solutes and metabolites, thus enabling normal metabolic processes at the membrane. By proceeding WE biosynthesis, the WS/DGAT:SLD complexes are released, and SLDs conglomerate or coalesce owing to hydrophobic interactions to larger structures at distinct sites of the membrane, which can be observed as nascent WE inclusions in transmission electron microscopy. This mechanism was confirmed by scanning force electron microscopy and quartz microbalance experiments on immobilized model phospholipid membranes using purified WS/DGAT for *in vitro* formation of WEs and TAGs. By these methods, the *in vitro* formation of small membrane-bound WE and TAG droplets and their conglomeration/coalescence to lipid prebodies were observed. Although not investigated in detail, it was assumed that phospholipids coat the nascent lipid inclusions owing to their proximity to the cytoplasmic membrane and phospholipid biosynthesis. However, in contrast to the situation of TAG inclusions in actinomycetes, nascent WE inclusions do not exhibit a more electron dense inner structure in comparison to cytoplasmic WE inclusions. The cause of this phenomenon is unknown, but might be due to an earlier coalescence of WEs inside the nascent inclusions in comparison to TAG prebodies (Fig. 4).

Although no detailed experiments on the formation of nonspherical WE inclusions, for example, in *Alcanivorax* sp. or *Acinetobacter* sp. strain M-1, have been performed so far, it may be speculated that their formation underlies principally the same mechanism. Electron micrographs of *Acinetobacter* sp. strain M-1, *Alcanivorax borkumensis* and *Alcanivorax jadensis* revealed the occurrence of disc-, ellipsoid-, half-moon- or rectangular-like WE inclusions of different sizes, of which a considerable amount was in direct conjunction with the cytoplasm membrane (Ishige et al. 2002; Reers, Wältermann and Steinbüchel, unpublished results). Since the first steps in the formation of WE inclusions in *Acinetobacter* sp. strain ADP1 are membrane-associated, and thus a nearly two-dimensional process, it is likely that nascent WE inclusions formed by this mechanism are not forced to become spherical structures (Wältermann et al. 2005). It might be speculated that WEs form inclusions, the shapes of which are molded from respective sites of the cytoplasm membrane. During proceeding biosynthesis of small WE droplets at the membrane and without their conglomeration to spherical WE prebodies, a growing WE layer could be formed at the membrane; the WEs could occur as ellipsoid, disclike or otherwise irregular inclusions in transmission

electron microscopy observations after they have left the membrane and migrated into the cytoplasm. However, although this assumption would not explain the occurrence of such large disclike structures observed by Ishige et al. (2002), it would indicate, that WEs in nonspherical inclusions occur in a nonliquid or crystalline state (Fig. 4.3).

Although significant progress in the formation of lipid inclusions and neutral lipid biosynthesis has been made in the last few years, the general mechanism of the formation of intracellular lipid inclusions is still a matter of debate. Although WS/DGAT is localized mainly at the cytoplasm membrane in *Acinetobacter* sp. strain ADP1, the enzyme exhibits also a distribution throughout the cytoplasm and at the surface of WE inclusions (Wältermann et al. 2005; Stöveken et al. 2005). This distribution was also reported to occur in bioengineered, WE-accumulating *E. coli* by Kalscheuer et al. (2006). This strain is capable of synthesizing and accumulating small amounts of fatty acid butyl esters (FABE) and WE in media supplied with butanol. This strain was able to synthesize a small number of intracellular lipid inclusions of approximately 200 nm in diameter. Owing to its low lipid content, the authors suggested that this strain was virtually arrested in an early state of lipid accumulation, and that all lipids synthesized would be localized as a WS/DGAT:lipid complex at the cytoplasm membrane, and that no intracellular lipid inclusions could be formed according to the model suggested by Wältermann et al. (2005). This assumption is in contrast to the situation in cells of *Acinetobacter* sp. strain ADP1 in early stages of WE accumulation, in which the WE content is also very low and WE inclusions can also be observed. On the basis of these findings, the authors suggested that the main location of FABE biosynthesis and also long-chain WE biosynthesis in this highly artificial system is associated with the surface of intracellular lipid inclusions, because both substrates for their formation are water-soluble. Although the possibility of substantial lipid biosynthesis at the surface of lipid inclusions cannot be excluded, experimental evidence for the latter mechanism is missing. In wild-type bacteria accumulating neutral lipids, TAGs and WEs are synthesized from acyl-CoAs and hydrophobic acyl acceptors, whose biosynthesis is associated with the cytoplasm membrane, and thus the putative situation of FABE synthesis from soluble butanol and acyl-CoA in the case of this recombinant *E. coli* strain does not reflect the natural situation in a lipid-accumulating strain and is not satisfactory to refute the current model for the formation of neutral lipid inclusions in bacteria at all. A significant biosynthetic activity at the surface of lipid inclusions would require that hydrophobic intermediates have to shuttle between the cytoplasm membrane and the surface of lipid inclusions. Furthermore, this would suggest that neutral lipid inclusions could arise *de novo* in the cytoplasm from soluble WS/DGAT starting from a small, hypothetical soluble enzyme:lipid complex continuously growing to a lipid inclusion. This would be similar to the formation of PHA inclusions from soluble substrates in PHA-accumulating bacteria

(Jurasek and Marchessault 2004). However, such a situation would lead to many small lipid inclusions inside the cytoplasm, and with proceeding coalescence larger inclusions would be formed. However, such a situation has never been observed, not even in this recombinant strain. Furthermore, this model does not explain the incorporation of phospholipids into the surface of nascent lipid inclusions. Finally, although soluble WS/DGAT is capable of synthesizing lipids from soluble acyl-CoA and fatty alcohol emulsions, an *in vitro* formation of neutral lipid inclusions, similar to the *in vitro* formation of PHB granules reported several years ago (Jossek et al. 1998), has never been reported.

5.3 PHA Granules

Since PHAs represent a polymeric form of carbon and an energy store, the formation of PHA granules is quite different from the formation of prokaryotic neutral lipid inclusions. Biosynthesis of PHB is a template-independent homopolymerization process which forms an insoluble polymer from soluble substrates, mediated by the PHA synthase (PhaC) (Stubbe and Tian 2003). PhaC is a soluble enzyme and is found in the cytoplasm in cells harboring no PHB granules. In contrast, under conditions of PHA accumulation, PhaC becomes strictly bound to the surface of PHB granules. The biogenesis of PHB granules is not understood in detail. According to the most accepted model, cytoplasm-localized PHB-linked PhaC proteins generate micellelike structures, with the growing PHB chain representing the hydrophobic core and PhaC itself representing the hydrophilic head group. During proceeding PHB synthesis, PhaC remains covalently bound to the elongated PHB chain while the resulting surface area of the nascent granule increases, thus dispersing PhaC on the surface of the granule. At this state of granule formation, the formation of phasin protein is induced, and the amphiphilic proteins invade into the empty space on the hydrophobic granule surface, thus preventing the granules from coalescing. In addition, formation of a large hydrophobic surface in the cells is prevented, which could lead to unspecific binding of other proteins. This function is similar to the situation of oleosins in plant seeds. In addition to phasins, which make up the major protein on the surface of PHB granules, PHB granules are assumed to be surrounded also by a monolayer of phospholipids. However, there is no plausible explanation for how phospholipids could reach the surface of growing PHB inclusions from their site of synthesis at the cytoplasm membrane. It may also be speculated whether they are required at all for maintenance of the structural integrity of the granules (Fig. 5) (Stubbe and Tian 2003; Jurasek and Marchessault 2004).

An alternative model for the formation of PHB granules has been proposed, and shares some similarities with the formation of eukaryotic neutral lipid bodies. In this model, PhaC becomes membrane-bound and could syn-

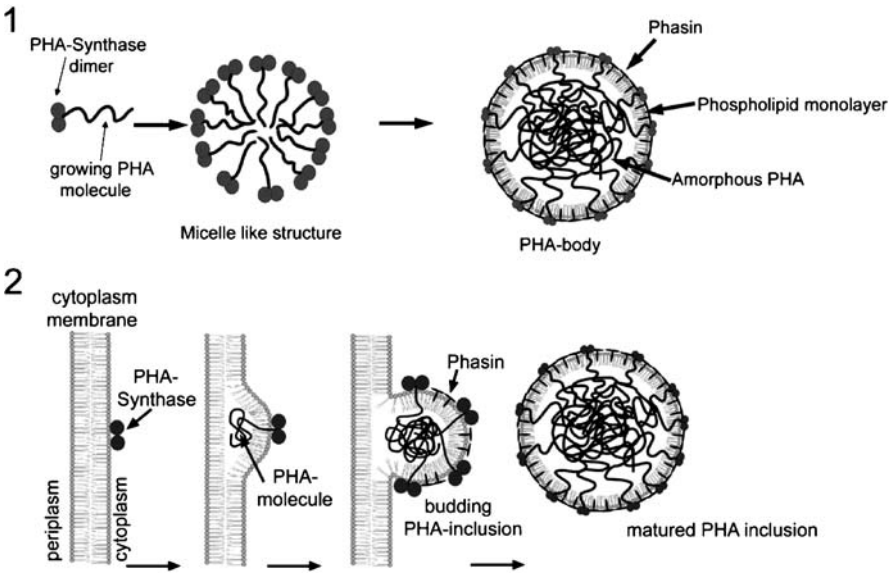

Fig. 5 Models for the formation of PHA granules. *1* The micelle model; *2* the budding membrane model (Wältermann and Steinbüchel 2005)

thesize a growing PHB molecule in the space between both leaflets of the plasma membrane, thus leading to a swelling of the membrane and a subsequent budding of a PHB granule which is coated by a monolayer membrane derived from the cytoplasmic face of the plasma membrane. Similar to the incorporation of oleosins into budding plant seed oil bodies along the cytoplasmic site of the ER membrane, incorporation of phasins could be facilitated by insertion of phasins into the plasma membrane (Jendrossek 2005; Stubbe and Tian 2003). Although this model provides a comprehensible explanation for the incorporation of phospholipids, it is contrary to the strict cytoplasmic localization of PhaC. Furthermore, phasins have never been reported to be membrane-associated in *R. eutropha* (Fig. 5).

For a detailed overview of the basics of PHA metabolism the reader should refer to the review of Pötter and Steinbüchel in this volume.

6
Structure, Formation and Protein Equipment of Eukaryotic Lipid Inclusions

Prokaryotic lipid inclusions and PHA granules are considered to be structurally related to eukaryotic lipid bodies, which are referred to as lipid particles, lipid globules, lipid bodies or especially in plants oil bodies, spherosomes and oleosomes. Although the basic structure of eukaryotic lipid bodies is very similar to those of prokaryotes, these compartments are discussed

whether they constitute a true organelle or not (Zwytick et al. 2000). The structure of eukaryotic lipid droplets and oil bodies is generally rather simple; a hydrophobic core consisting of the respective storage lipid is surrounded by a phospholipid monolayer with special proteins embedded therein. The main function of these inclusions is storage of neutral lipids as an energy source and as a source of components needed for membrane biogenesis or formation of other lipophilic components, like steroides. In higher eukaryotes, lipid storage occurs mainly in specialized tissues, for example, white and brown adipose tissue, steroidogenic cells or the seeds of oleogenic plants (Zweytick et al. 2000). However, the formation and protein equipment of lipid bodies in eukaryotic cells are quite different from those in prokaryotes.

6.1 Plants

In some plant seeds, for example, rapeseed or sunflower, storage lipids account for more than half of the total weight. TAGs represent the dominant storage lipid of plants, whereas steryl esters generally constitute only a minor component. Although WEs are abundant in plants as a component of hydrophobic barriers, pathogen protection and for light reflection, jojoba, a desert shrub native to southwest USA and Mexico, is the only exception among angiosperms known to accumulate WEs as energy stores (Yermanos 1975; Post-Beitenmiller 1996). In plants, TAGs are formed via the Kennedy pathway from glycerol-3-phosphate and acyl-ACP or acyl-CoA in the ER, plastids and mitochondria. First, phosphatidic acid is formed from glycerol-3-phosphate through acylation at the *sn*-1 and *sn*-2 positions by glycerol-3-phosphate acyltransferase and 1-glycerol-3-phosphate acyltransferase, respectively. Subsequent dephosphorylation of phosphatidic acid is mediated by phosphatidate phosphatase (Frentzen 1998). Further acylation of the resulting DAG is catalyzed by either an enzyme of the DGAT1 or DGAT2 families and yields TAG. DGAT1 and DGAT2 are encoded by two unrelated genes in plants, from which one is closely related to acyl-CoA:cholesterol acyltransferase, whereas the other form does not resemble any other known genes (Zou et al. 1999; Lardizabal et al. 2000). As an acyl-CoA independent pathway, Dahlquist et al. (2000) described the formation of TAGs from DAG and phospholipid as an acyl donor in different plants and yeasts.

In electron microscopy plant oil bodies appear as electron-transparent structures with an electron-dense phospholipid/protein layer. The average diameter of oil bodies in dessication-tolerant seeds varies from 0.6 to 2.5 μm, depending on the species (Tzen et al. 1993). This size is assumed to be optimal for TAG mobilization during germination of seeds, since it provides an ideal volume-to-surface ratio (Murphy 1993; Zweytick et al. 2000). In most plants, the main proteins embedded in the surrounding monolayer of seed oil bodies are oleosins; but they were also found in flowers, pollen and tape-

tum. Oleosins are structural proteins of low molecular masses (15–26 kDa) (Murphy 1993; Roberts et al. 1991; Robert et al. 1994). It is assumed that these proteins form a meshworklike organization on the surface of lipid bodies which protects oil bodies from unspecific attack of lipases and coalescence. This is particularly important in plant seeds, in which an optimal surface-to-volume ratio is essential for rapid mobilization of TAGs. Oleosins are composed of three structural domains, an expanded conserved α-helical region (approximately 70 hydrophobic amino acids) flanked by two amphiphilic regions of varying sizes. The central hydrophobic domain directly interacts with the lipid core of the oil body, and a proline knot motif in the central region of this domain was identified as a putative targeting signal to the lipid core (Chen et al. 1997; Lacey et al. 1998; Abell et al. 1997). However, although oleosins represent the majority of proteins on the surface of lipid bodies, it is unlikely that they play an essential role in their formation. This is confirmed by the situation of oil bodies in the seeds of olive or avocado, which do not possess oleosins (Murphy and Vance 1999; Ross et al. 1993). However, the lipid bodies in these species exhibit diameters of about 20 μm, and are seemingly not degraded at all.

The budding model is the most accepted model for the assembling of plant oil bodies. In this model, TAG formation occurs between both phospholipid leaflets at special subdomains of the ER. This causes a swelling of the bilayer, and TAG droplets with a surface derived from the outer ER layer bud-off. This model is supported by ultrastructural observations and the fact that the plant ER is equipped with enzymes and structural proteins involved in biosynthesis of TAGs and oil bodies (Frey-Wyssling et al. 1963; Wanner et al. 1981; Wanner and Theimer 1978; Tzen et al. 1993; Galili et al. 1998). The coating of the nascent oil bodies with oleosins is mediated by a cotranslational insertion of the oleosins into the ER membrane. Owing to their hydrophobic character mediated by their central hydrophobic domain, oloesins invade regions of TAG accumulation between the phospholipid leaflets and accumulate at the surface of nascent oil bodies (Hills et al. 1993; Loer and Herman 1993). The other hypothesis for plant oil body formation is the postencasement model. In this model, storage TAGs arise naked from the ER and form droplets inside the cytoplasm and associate subsequently with oleosins and phospholipids. However, although this model is supported by the observation that in some species oleosin formation lags temporarily TAG accumulation, it does not explain the formation of a phospholipid surface layer and insertion of oleosins (Murphy 1993; Ichihara 1982).

6.2 Mammals

Rather than being mere storage droplets for TAGs and sterol esters, which provide cells with fatty acids as an energy source, for membrane remodeling

or with precursors for hormone synthesis, lipid bodies in mammals are recognized as indispensable and metabolically active organelles that participate in cell signaling, vesicle trafficking and several important diseases, like obesity and arteriosclerosis (Umlauf et al. 2004; Liu et al. 2004; Wang et al. 1999). In mammals, TAGs, the primary bodily energy stores, are mainly synthesized by action of DGAT1 and DGAT2, which are associated with the microsomal fraction (Polokoff and Bell 1980; Anderson et al. 1994). However, DGAT-deficient mice were reported to synthesize TAGs, indicating that an additional pathway for TAG biosynthesis must exist (Smith et al. 2000). Steryl esters, which constitute the second major lipid in mammalian lipid bodies, are synthesized by esterification of cholesterol with long-chain acyl-CoAs mediated by acyl-CoA:cholesterol acyltransferase. This enzyme is also located at the ER (Chang et al. 1997; Suckling and Stange 1985).

Lipid bodies occur in almost all mammalian cell types. However, the main location for TAGs is white adipose tissue. White adipose cells contain one or a few enormously large lipid bodies (10–100 μm in diameter), whereas steroidogenic cells and brown adipose cells, which occur in some hibernating animals and fetuses/neonates of many mammals, contain higher amounts of lipid droplets of much smaller size (2–10 μm) (Murphy and Vance 1999; Hammerson 1985; Weiss 1983; Weather et al. 1987). When mammalian lipid bodies are inspected by electron microscopic methods, they appear as distinct, electron-transparent structures surrounded by an electron-dense layer, often observed in a close association to the ER or mitochondria (Schlunk and Lombardi 1967). Blanchette-Mackie (1995) described a junction between the ER membrane and the lipid body surface on the basis of freeze-fracture observation, indicating that the formation of mammalian lipid bodies probably underlies a similar mechanism to that of oil bodies in plants, in which the lipids accumulate between the leaflets of the ER membrane. Distension of the membrane and budding of the membrane leaflet facing the cytoplasm are thought to leave a droplet surrounded by a monolayer consisting of the former cytoplasmic leaflet of the ER membrane.

The main proteins associated with mammalian lipid bodies are proteins of the PAT family, namely, perilipins, adipose differentiation related protein (ADRP; orthologously termed adipophilin in humans) and tail-interacting protein of 47 kDa (TIP47) (Londos et al. 1999; Wolins et al. 2001; Wang et al. 2003). PAT family proteins are similar in their entire sequences, with the exception of an N-terminal extension of approximately 20 amino acids in TIP47 and a C-terminal extension of varying length in the different perilipins. Recently, caveolin, lipotransin, a 200-kDa capsular protein and the hepatitis C virus core protein were reported as lipid body associated proteins (Robenek et al. 2004; Syu and Saltiel 1999; Wang et al. 1997; Hope et al. 1998). Perilipins are localized at the surface of lipid bodies in adipocytes and steroidogenic cells, but are virtually absent in all other types of cells. Perilipins are encoded by a single copy gene yielding three isoforms, A, B

and C, by alternative splicing (Lu et al. 2001). Perilipin A is predominantly found in differentiated adipocytes, but can also be found in steroidogenic cells. Isoform B can be found in lower amounts in adipocytes and steroidogenic cells, whereas isoform C coats exclusively the cholesteryl ester droplets in steroidogenic cells. Perilipins can be highly phosphorylated and seem to be involved in TAG lipolysis and lipid body stabilization (Londos et al. 1995). Both adipocytes and steroidogenic cells use a cyclic AMP (cAMP) stimulated process to activate a hormone-sensitive lipase for lipolysis of TAGs and cholesterol, respectively. After phosphorylation by protein kinase A , which is stimulated by hormonally controlled cAMP, perilipins are released from the lipid body surface, thus enabling the lipase to bind to the lipid core and hydrolyze the embedded lipids, wheras nonphosphorylated perilipin suppresses lipolysis (Sztalryd et al. 2003). ADRP and TIP47 are ubiquitously expressed in all other cells and early-differentiated adipocytes. The functions of ADRP and TIP47 in lipid metabolism are not well understood. Since both proteins exhibit 50% sequence identity and owing to their similarities to perilipin, both proteins might therefore be expected to have similar structural or metabolic roles. Similar to perilipins, ADRP has been found only associated with lipid droplets and not in any other subcellular compartment, whereas TIP47 is also abundant in the cytoplasm (Barbero et al. 2001; Miura et al. 2002). In later stages of adipocyte differentiation, ADRP is replaced by perilipins. Therefore, these enzymes are assumed to play an essential role in lipid droplet formation and during conversion of adipocytes from preadipocytes. In contrast to the oleosins in plants, which are assumed to be transferred to the lipid body surface along with the cytoplasmic leaflet of the ER, PAT family proteins are thought to be directed from cytosolic sites of synthesis to the surface of nascent lipid droplets (Londos et al. 1999).

6.3 Eukaryotic Microorganisms

A wide variety of unicellular eukaryotes are capable of accumulating TAGs (Christiansen 1978; Leman 1997; Stahmann et al. 1994). The biochemistry of TAG and steryl ester biosynthesis and lipid body formation has been mostly investigated in oleogenous yeasts and filamentous fungi, particularly for biotechnological production of single-cell oils which are rich in diverse polyunsaturated fatty acids (Clausen et al. 1974; Ratledge 2002). Oleogenous microorganisms are defined as organisms able to accumulate more than 20% lipids of their cellular dry weight (Ratledge 1989). Similar to the situation in prokaryotes, lipid accumulation in an eukaryotic microorganism starts upon deletion of a nutrient from the medium, for example, nitrogen, but when an excess of carbon still remains. The carbon source continues to be assimilated and is used for lipid synthesis. In eukaryotic microorganisms, accumulation of lipids parallels the presence of ATP:citrate lyase, which is localized in the

cytoplasm and converts citrate to oxalacetate under hydrolysis of ATP and transfers the acetyl residue to CoA (Boulton and Ratledge 1981; Ratledge 1989). Acetyl-CoA is formed by oxidative decarboxylation of α-keto acids and β-oxidation of fatty acids in the mitochondrion. Because the inner membrane of the mitochondrion is impermeable for acetyl-CoA, it is condensed with oxalacetate in the mitochondrial matrix and converted to citrate, which is then transported into the cytoplasm where it is finally released by ATP:citrate lyase. The cytoplasm-localized acetyl-CoA enters fatty acid *de novo* synthesis and is incorporated into lipids (Leman 1997; Harwood 1994; Ratledge 1989). Because prokaryotes are not compartmented, this "acetyl-CoA shuttle" can only be a criterion for oleogenity in eukaryotic microorganisms.

Similar to plants and animals, lipids are stored in lipid bodies in yeasts. These lipid bodies exhibit a spherical shape and diameters between 0.3 and 2 μm, and have a hydrophobic core constituted of similar amounts of TAGs and steryl esters (Schaffner and Matile 1981), which is surrounded by a phospholipid monolayer and proteins. (Leber et al. 1994). Biosynthesis of TAGs in *Saccharomyces cerevisiae* is mediated mainly by two proteins, an acyl-CoA:DGAT (Dga1p; belonging to the DGAT2 family) and a phospholipid:DGAT (Lro1p), which utilize acyl-CoA and phosphatidylcholin as acyl donors, respectively. Lro1p activity was reported to occur exclusively in the microsomal fraction, whereas Dga1p activity was mainly localized in the lipid body fraction and to some extent in the microsomal fraction (Sorger and Daum 2003). In yeasts, steryl esters are formed by two acyl-CoA:sterol ester acyltransferases (ARE1 and ARE2), which are localized at the ER (Yang et al. 1996; Yu et al. 1996). Unlike higher eukaryotes, structural proteins comprising functions similar to that of perilipins or oleosins could not be identified on yeast lipid bodies, but most proteins identified so far are involved in sterol, fatty acid and TAG metabolism (Athenstaedt and Daum 1997, 2003; Leber et al. 1994, 1998; Milla et al. 2002; Smid et al. 1995; Tsitsigiannis et al. 2004). Yeast lipid particles are assumed to originate from the ER, as was similarly described for plant and mammalian lipid bodies, because the ER and lipid droplets share certain proteins and are frequently observed in proximity to each other (Zweytick et al. 2000).

7
Concluding Remarks and Future Perspectives

Research in the last few years on biosynthesis of neutral lipids and the structure and formation of their deposits has revealed a tremendous amount of knowledge. Much research has still to be done to address unresolved problems, for example, to identify enzymes involved in the intracellular degradation of accumulated lipids, to identify the mechanism involved in excretion of neutral lipids in *Alcanivorax* sp., and the overall regulation of neutral lipid

metabolism in bacteria. Furthermore, a biotechnological production of lipids, for example, the production of WEs as a cheaper substitute for jojoba oil, might be achievable in the near future. Furthermore, WE and TAG inclusions as well as PHA granules could be modified and used as a basis for the production of biodegradable, self-assembling nanoparticles, which could be useful for a wide range of sophisticated technical applications and in medicine.

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Cyanophycin—an Ideal Bacterial Nitrogen Storage Material with Unique Chemical Properties

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Abstract Cyanophycin (CGP) is a nitrogen-rich, polypeptide-like storage material of cyanobacteria and heterotrophic bacteria. CGP inclusions were discovered more than 100 years ago and from that time on have attracted the interest of many researchers with both basic and applied research interests. The discovery of CGP goes along with the development of the light microscope and the electron microscope, respectively, as well as application of differential staining techniques and refined analysis methods that were employed to determine its chemical structure. CGP has different physiological functions depending on the cell type and organism in which it occurs, but can be considered as

a widespread intracellular nitrogen reserve. Its biosynthesis is mediated by the activity of a single enzyme, the cyanophycin synthetase, that possesses two putative active sites responsible for the alternating incorporation of the amino acids arginine and aspartic acid. Heterologous expression of CGP synthetase genes (*cphA*) and the activity of the enzyme during in vitro studies led to the formation of CGP with altered monomer composition. By employment of recombinant bacterial strains that accumulate large amounts of CGP, strategies were developed to produce CGP in semitechnical amounts in comparably cost effective and time saving biotechnological processes. CGP inclusion body formation in transgenic plant lines demonstrated the potential of eukaryotic organisms to serve as hosts for heterologous expression of *cphA* and as potential future CGP production organisms.

1
Cyanophycin—an Ideal Bacterial Nitrogen Storage Material with Unique Chemical Properties

Among the various inclusion bodies or subcellular structures regularly occurring in cyanobacteria (blue-green algae) which are the phycobilisomes, polyphosphate granules, the carboxysomes (polyhedral bodies), polyglucose granules (glycogen granules), gas vacuoles, poly(3-hydroxybutyric acid) granules, lipid bodies (reviewed by Allen 1984) and cyanophycin (cyanophycin granule polypeptide, CGP) granule (Fig. 1a); the last ones are the only inclusions composed of a nitrogen-rich homopolymeric material with a unique comblike chemical structure.

1.1
The Structure of CGP—a Reflection of its Reserve Function

Regarding its classification, CGP is a poly(amino acid) with a polymer backbone consisting of α-linked aspartic acid residues. Arginine residues are attached to the β-carboxylic groups of the poly(α-aspartic acid) backbone of naturally occurring CGP forming isopeptide bonds via their α-amino groups (Simon 1973a; Simon and Weathers 1976). Because of its mode of biosynthesis (see later) presumably all aspartic acid side chains are initially linked to arginine residues in cyanobacterial CGP. Therefore, CGP can be alternatively described as poly(β-Asp-Arg). This structure has been confirmed recently also by enzymatic degradation studies employing CGP-hydrolyzing enzymes (CGPases) which led to the release of β-Asp-Arg dipeptides from CGP (Richter et al. 1999; Obst et al. 2002, 2004; Ziegler et al. 2002). The released dipeptides can be further cleaved to aspartic acid and arginine by the hydrolytic activity of "plant-type" asparaginase of cyanobacteria with isoaspartyl dipeptidase activity (Hejazi et al. 2002), thus demonstrating the presence of a complete metabolic cycle of CGP synthesis and degradation in cyanobacteria (Fig. 2).

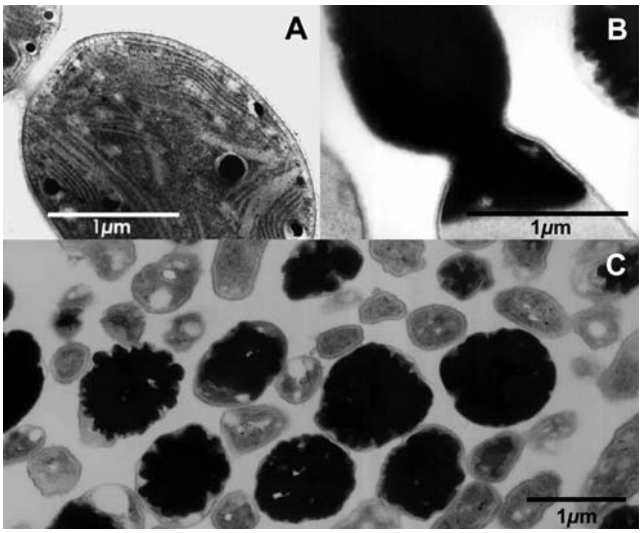

Fig. 1 Cyanophycin granule polypeptide (*CGP*) inclusion body formation (*black inclusions*) in the CGP synthesizing bacterium *Synechocystis* sp. strain PCC6308 (**a**) and in cells of *Acinetobacter* sp. strain ADP1 (**b**, **c**) cultivated under storage conditions (phosphate limitation). *Synechocystis* sp. strain PCC6308 was cultivated in BG11 mineral medium in the presence of chloramphenicol for enhanced CGP accumulation and *Acinetobacter* sp. strain ADP1 (**b**; Krehenbrink 2002; Elbahloul et al. 2005) was cultivated in arginine-supplemented mineral salt medium. In cells of *Synechocystis* sp. strain PCC6308 the granules are located in the same area as the thylakoid membranes in the peripheral cytoplasm (**a**), whereas in the heterotrophic *Acinetobacter* sp. strain ADP1 one granulum per cell is present, and growth proceeds radially leading to the formation of "star-shaped" structured granules (**c**). During cell division CGP is distributed unequally between the dividing cells (**b**, **c**)

CGP is like all other homopolyamide of natural origin produced independently of the ribosomal protein synthesis apparatus and independently of an RNA template. Therefore, cyanophycin synthesis is resistant to a large variety of antibiotics which effect protein biosynthesis like chloramphenicol, erythromycin, streptomycin, tetracycline and rifampicin (Simon 1976). As a product of a template-independent biosynthesis process (reviewed by Stubbe et al. 2005), cyanobacterial CGP exhibits a high degree of polydispersity (n = 90–400; Fig. 3b, lane 2; Berg et al. 2000) corresponding to a molecular mass distribution which ranges from 25 to 100 kDa. In contrast, CGP synthesized by cells of *Acinetobacter* sp. strain ADP1 (Krehenbrink and Steinbüchel 2004) as well as CGP produced in recombinant bacteria (Fig. 3b, lanes 3–5) showed a markedly lower dispersity (22–43 kDa). In recent studies, Allen et al. (2005) found low molecular mass CGP (30–60 kDa) also in cells of *Synechocystis* sp. strain PCC 6308. The CGP was extended rather than de novo synthesized during 24 h of incubation yielding CGP molecules of appar-

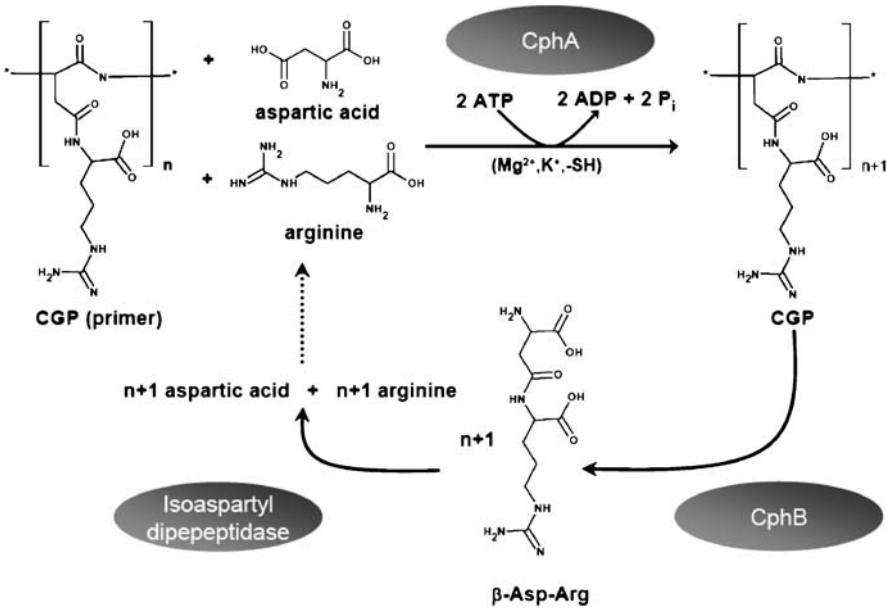

Fig. 2 CGP metabolism of cyanobacteria. The CGP synthetase (*CphA*) catalyzes the ATP-dependent formation of CGP from aspartic acid and arginine. Cyanophycin molecules consisting of at least three β-Asp-Arg monomers serve as the most efficient primers in the CphA reaction in vitro (Berg et al. 2000; Aboulmagd et al. 2001a). The reaction is dependent on the presence of Mg^{2+}, K^+ and a sulfhydryl reagent (– SH). Accumulated CGP can be mobilized by the activity of the intracellular CGPase (*CphB*), releasing β-Asp-Arg isodipeptides (Richter et al. 1999). By the activity of an isoaspartyl dipeptidase (Hejazi et al. 2002), the isodipeptides are hydrolyzed to arginine and aspartic acid, which are reutilized by the bacterial cells

ent masses of up to approximately 100 kDa. The observed mass differences may be due to (1) the absence of a further catalytic or regulatory factor in recombinant bacteria as well as in *Acinetobacter* or (2) differences in the enzyme-to-substrate ratio in these cells (Aboulmagd et al. 2000).

Although isopeptide bonds are typically found also in other bacterial poly(amino acids) like, for example, in poly(γ-glutamic acid) or in poly(ε-lysine), CGP exhibits some chemical peculiarities which make it, in contrast to the other extracellular poly(amino acids), an ideal intracellular storage material with high nitrogen content (Allen 1984; Simon 1987): (1) CGP contains five nitrogen atoms in each polymer building block; (2) CGP is insoluble and uncharged in the cytoplasm and does not have adverse effects on other polymeric molecules like DNA; (3) CGP does not change the osmolarity of the cytoplasm like, for example, poly(glutamic acid) that builds up the high osmotic pressure in the nematocysts (stinging capsules) of the eukaryotic organism *Hydra* (Weber 1989, 1990). CGP can accumulate in the cytoplasm of

Fig. 3 Structure and properties of CGPs. **a** The chemical structure of CGP of cyanobacterial origin which is poly(β-Asp-Arg) (n = 90–400, Berg et al. 2000). The sodium dodecyl sulfate polyacrylamide gel electrophoresis (**b**) shows the comparative analysis of the spectra of apparent molecular masses of different CGPs synthesized by cyanobacteria (*Synechocystis* sp. strain PCC6308; *lane 2*) and recombinant bacteria [*Escherichia coli* DH1 (pMa/c5-914::*cpha*$_{PCC6803}$), *lane 3*; *Ralstonia eutropha* HF39 (pBBR1::*cpha*$_{PCC6308}$), *lane 4*; *Pseudomonas putida* KT2440 (pBBR1::*cpha*$_{PCC6308}$), *lane 5*] harboring *cphA* of *Synechocystis* spp. *Lane 1* molecular mass protein standard. The distinct bands in *lane 4* are protein contaminations which are due to incomplete purification. Purified and dried CGP is a slightly yellowish, fine-grained powder and is shown in **c**

vegetative cells of cyanobacteria from 8 to about 18% of the cellular dry matter during transition from the exponential to the stationary growth phase (Simon 1973a; Weathers and Allen 1978; Allen et al. 1980; Lawry and Simon 1982; Mackerras et al. 1990) and can be found also in significant amounts in akinetes, the "spores" of cyanobacteria as a storage compound.

1.2 Solubility of CGP

The insolubility of CGP or its granular appearance under physiological conditions and at typical ionic strength of the cyanobacterial cytoplasm may be explained by its amphoteric nature, which is the result of the presence of free carboxylic and free guanidino groups in the arginine side chains and the formation of a relatively stable β-sheet secondary structure at neutral pH and under slightly acidic conditions which was proposed on the basis of analysis of CGP samples with laser Raman spectroscopy and circular dichroism spectroscopy (Simon et al. 1980). However; purified CGP (Fig. 3c) or CGP granules contained in biomass can easily be solubilized in dilute acids (pH < 2) and under alkaline conditions (pH > 9), respectively (reviewed by Simon 1971 and Oppermann-Sanio and Steinbüchel 2003).

Only recently, Ziegler et al. (2002) isolated a water-soluble polymer from recombinant *Escherichia coli* harboring a plasmid containing the CGP synthetase gene (*cphA*) from *Desulfitobacterium hafniense*. The polymer exhibited in several experiments chemical properties identical to those of CGP, indicating that under certain physiological conditions CGP can be solubilized without significant alteration of the polymer properties. In this regard, Füser and Steinbüchel (2005) analyzed the solubility behavior of CGP in simple inorganic salt solutions and found that water-insoluble CGP became water-soluble if alkali metal or alkaline-earth metal salts were applied to purified CGP preparations at significantly high concentrations. Accordingly, it can be assumed that CGP inclusions behave similarly as α-linked polypeptide (proteins) which are water-soluble only at specific ionic strength (salinity) of their environment and can be likewise dissolved or "salted in". In this regard the observations of Füser and Steinbüchel (2005) could serve as a plausible explanation for the solubility behavior of the CGP-like polymer isolated by Ziegler et al. (2002). Another explanation would be the occasional occurrence of other linkages or branches in the CGP molecules which was discussed by Ziegler et al. (2002) on the basis of the difference in mass spectra obtained for both polymer types in enzymatic degradation experiments. However, up to now such branching has never been demonstrated.

A population of CGP molecules in a given granulum can be separated into two subpopulations by employing isoelectric focusing. Simon and Weathers (1976) found that the two subpopulations occurred at pH 4.75 (90%) and 6.1 (10%), respectively, in a 6 M urea gel. In 2004 Krehenbrink and Steinbüchel (2004) also observed the occurrence of two molecule subpopulations which were, on the basis of two-dimensional gel electrophoresis analysis and theoretical consideration, identified as CGP molecules with either *C*-terminal aspartic acid residues [poly(β-Asp-Arg)-Asp] or *C*-terminal arginine residues that appear in the polymer side chain [poly(β-Asp-Arg)], thus also indicating the alternating incorporation of these two amino acids during CGP biosynthesis and confirming the structure of CGP that was proposed by Simon and Weathers in 1976 (see later).

1.3 Other Properties of CGP and CGP Granules

Another characteristic feature that CGP inclusion share with typical protein inclusions (see Neubauer et al., this volume), is the absence of a confining membrane (Allen and Weathers 1980) usually found in connection with other, mostly more hydrophobic cellular inclusions like polyhydroxyalkanoates or lipid inclusions (Wältermann et al. 2005; Wältermann and Steinbüchel 2005; Pötter and Steinbüchel 2005; Preiss, this volume; Pötter and Steinbüchel, this volume). Furthermore, CGP inclusions also lack surface proteins which are typically associated with these inclusions and play a role in regulation of their

formation, degradation, number and size. It remains to be determined how the number and the size of CGP inclusions or the size of CGP molecules is controlled by the (cyano)bacterial cells. It can only be stated here that these parameters vary greatly among the different CGP-synthesizing microorganisms and with changes of cultivation conditions (see later).

2
Cyanophycin Inclusions in Cyanobacteria and Chemotrophic Bacteria—Early Microscopic Observations and Analyses of CGP Granules

Many experiments were performed to elucidate the structure and chemical composition of the CGP granules. The first experiments, before the chemical composition of CGP was determined by Simon and coworkers in the 1970s, were mostly based on light microscopic observations, on analysis of the solubility behavior of CGP inclusions and on the application of different staining techniques.

Since the first light microscopic observation of highly refractive inclusions in filamentous cyanobacteria for which Borzi (1887) created the name *cianoficina*, CGP granules have been discovered in the cytoplasm of most cyanobacteria from all taxonomic groups (Allen et al. 1980; Lawry and Simon 1982; Allen 1984, 1988; Simon 1971, 1976, 1987; Golecki and Heinrich 1991; Liotenberg et al. 1996; Mackerras et al. 1990; Hai et al. 1999; Wingard et al. 2002) as bodies with diameters of up to 0.5 μm (Pankratz and Bowen 1963) or even more than 1 μm (Dembinska and Allen 1988) especially in cells of older cultures (Lang 1968). Dembinska and Allen (1988) also observed that the number of granules remained constant during the entire cultivation period, indicating that CGP accumulation at least in *Synechocystis* sp. strain PCC 6308 leads to enlargement of already existing granules and not to de novo synthesis of additional granules.

2.1
Electron Microscopic Studies

Drews and Niklowitz (1957) introduced the name "structured granules" for electron-dense inclusions which they detected in cyanobacteria. Other commonly used names for these structured granules were *Ektoplasten* (Baumgärtel 1920; Drawert 1949), *fermentaktiv Granula* (Drews and Niklowitz 1957), *geschichtete Granula* (Fuhs 1958) or type I granules (Maugini 1966). These many names were mainly used before the unambiguous identification of the granules as CGP inclusions. A general problem was that the substructural patterns of CGP inclusions were visible only in some of the samples analyzed and that they were dependent on the fixatives and poststains used during inclusion body analysis (Lang 1968). However, the oc-

currence of the inclusions as, one the one hand, regular refractive inclusion bodies under the light microscope, and on the other hand as a "mass of tightly packed undulating, flattened sacs" (Ris and Singh 1961) on electron micrographs has led the to creation of the great variety of names for CGP inclusions and to the controversy about the nature of these inclusions that lasted until the 1970s.

Systematic analysis of the simultaneous occurrence of "structured granules" in electron micrographs and the refractive CGP inclusions in light micrographs of identical samples was started by Simon (1971), who isolated native CGP granules from *Anabaena cylindrica* cells by differential centrifugation, determined the molecular apparent mass distribution of CGP and determined its amino acid composition by sodium dodecyl sulfate polyacrylamide gel electrophoresis, gel filtration and automated amino acid analysis, respectively, and by Lang et al. (1972), who studied structure and staining behavior of isolated granules and of the material accumulating at the poles of heterocysts. Lang et al. (1972) identified both inclusions as CGP granules and demonstrated that CGP can be solubilized in dilute HCl, a characteristic of the formerly microscopically observed refractive CGP granules (Fritsch 1945), or in NaOH solutions. Furthermore, application of denaturating agents like sodium dodecyl sulfate or urea and solutions of cesium chloride had a similar solubilizing effect (Lang et al. 1972). The latter observation was later confirmed and can be explained on the basis of the studies of Füser and Steinbüchel (2005), who systematically analyzed how simple inorganic salt solutions can be employed to dissolve CGP (see before). In contrast, the isolated granules remained insoluble in organic solvents like methanol, ethylene glycol, dimethyl sulfoxide and formamide, 2% (v/v) Triton X-100, and 1% (w/v) sodium deoxycholate (Lang et al. 1972).

2.2 Staining of CGP Granules and Chemical Analysis

Moreover, isolated CGP granules were shown to have similar staining properties and showed a similar staining reaction with the dyes acetocarmine and neutral red (Lang et al. 1972) as granules of CGP-accumulating cyanobacterial cells that were stained in situ with the same reagents (Fritsch 1945; Fogg 1951). The dye Amido Black 10B stained the isolated CGP granules, and methylene blue showed a weak staining reaction despite its inability to stain the granules in the living cells (Lang et al. 1972; Baumgärtel 1920). Application of Millon's reagent to cyanobacterial cells or isolated CGP granules showed the absence of aromatic amino acids both in the refractive inclusions and in CGP (Lang et al. 1972; Hegler 1901).

It was observed by several researchers that some, but not all, protein-specific reagents could stain CGP granules in microscopic preparation. For example, the Sakaguchi reagent, which reacts with free arginine but in add-

ition also with protein-bound arginine residues, forming naphtol–arginine complexes (Messineo 1966), was later successfully employed to stain CGP (Fogg 1951), thus indicating its high arginine content. On the basis of the Sakaguchi reaction, Simon (1973a) developed an assay for the quantitation of CGP granules in cyanobacteria. Only much later more sensitive and versatile quantitative methods were developed. For example, high-performance liquid chromatography (HPLC) analysis for quantitative measurement of amino acid constituents of CGP was successfully employed during CGP synthetase characterization studies (Aboulmagd et al. 2000). Suarez et al. (1999) applied ^{1}H, ^{13}C and ^{15}N nuclear magnetic resonance (NMR) spectroscopy to characterize CGP. Erickson et al. (2001) used ^{1}H-NMR for rapid quantitative analysis of CGP containing samples and Obst et al. (2004) used a simple, modified Bradford assay to follow the kinetics of CGP degradation by extracellular CGPases (CphE).

Simon and Weathers (1976) were the first researchers to take advantage of the solubility of CGP in dilute acid and base (Fogg 1951; Simon 1971) and developed a CGP isolation protocol based mainly on repeated solubilization at pH 1, centrifugation, detergent washing steps and precipitation of CGP at neutral pH. This method was simplified later by Frey et al. (2002) to allow direct large-scale CGP isolation from CGP containing biomass obtained by fermentation of recombinant *E. coli* harboring CGP synthetase genes of cyanobacteria (see later).

Detailed chemical and physiological studies on CGP and CGP biosynthesis followed in the 1970s when Simon and coworkers (Simon 1971, 1973a, b, 1976; Simon and Weathers 1976) did not only determine the chemical structure of CGP by analysis of the monomer composition and the type of linkages occurring between the amino acids arginine and aspartic acid (Fig. 1a) but provided in addition a first insight into the biosynthesis mechanism of the CGP synthetase (see later). The CGP structure proposed by Simon and Weathers (1976) soon became widely accepted and was confirmed later by many researchers who applied a brought spectrum of different qualitative and quantitative analysis methods to CGP samples, like HPLC, NMR, electrospray ionization tandem mass spectrometry or enzymes specifically acting on α-amide or β-amide bonds of CGP or its degradation products.

3 Metabolic Functions of GGP

Accumulation of CGP in different cell types of cyanobacteria and in heterotrophic bacteria as well as regulation of CGP biosynthesis by a variety of different abiotic factors reflect its various functions in bacterial metabolism.

3.1 Occurrence of CGP

Besides their occurrence in the cytoplasm where CGP inclusions are often located close to thylakoid membranes in the peripheral cytoplasma (Fig. 1a), CGP granules were found to accumulate in akinetes of cyanobacteria to a large extent (Fritsch 1945; Miller and Lang 1968; Lang and Fisher 1969) and accumulation usually started at the beginning of sporulation (Sarma and Khattar 1986). Other experiments showed that the CGP granules disappeared gradually or completely during akinete germination (Lang 1968; Miller and Lang 1968; Simon 1987) and that their presence was a prerequisite for germination (Lang 1968; Leganés et al. 1998), indicating that they are "reserve sites for arginine-containing proteinaceous material" (Fogg 1951) and possibly have a similar function as seed storage proteins in higher plants.

For more than 100 years after its discovery, CGP was thought to be exclusively synthesized by cyanobacteria (Lawry and Simon 1982; Allen 1984, 1988; Simon 1987) until Krehenbrink et al. (2002) and Ziegler et al. (2002) detected genes in obligate heterotrophic bacteria with up to 40% similarity to *cphA* and *cphB* of cyanobacteria. Although CGP inclusions had been studied most intensively in cyanobacteria, the occurrence of similar inclusions and genes of CGP metabolism in microorganisms of largely different taxonomic affiliation like *Acinetobacter calcoaceticus* or *D. hafniense* showed that CGP accumulation is not necessarily linked to nitrogen fixation or photoautotrophic growth although such a linkage was postulated and has been demonstrated recently for *Anabaena* sp. strain PCC7120 (Laurent et al. 2004).

The discovery of similarities to *cphA*, *cphB* and *cphE* for genes of physiologically highly diverse bacteria (e.g., *Bordetella* sp., *Clostridium botulinum*, *D. hafniense* and *Nitrosomonas europaea*) in those studies and detection of an at least fivefold higher number of genes with similarity to genes of CGP metabolism in more recent data base searches (data not shown) indicates the function of CGP as an almost ubiquitous storage compound. Thus, CGP may have a higher ecological importance as a transiently accumulated nitrogen storage material in the global nitrogen cycle than hitherto thought. Furthermore, CGP possibly plays an important role in many of the well-known cyanobacterial symbioses that are ecologically and in many cases also economically important and that have existed in some cases for several hundred million years. Cyobacteria can be symbiotically associated with higher (eukaryotic) organisms, for example, with plants (Chiu et al. 2005; Nilsson et al. 2005; Qiu and Yu 2003; Gorelova and Kleimenov 2003), animals (Schmidt et al. 2005; Steindler et al. 2005) or fungi (Yuan et al. 2005), and often provide nitrogen-containing compounds to their symbiotic partner. One example of a symbiontic cyanobacterium that produces CGP granules is a strain with high similarity to *Nostoc punctiforme* that lives in association with coralloid roots of the cycad *Encephalartos altensteinii* (Caiola 1975).

3.2 Regulation of CGP Biosynthesis

Although the general role of CGP in the metabolism of many bacteria under various environmental conditions and in particular its role in cyanobacterial symbioses is not yet fully understood, throughout the literature CGP synthesis in bacteria is described as being correlated with a limitation of bacterial cultures mostly owing to the lack of the macroelements phosphorous or sulfur (Lawry and Simon 1982; Stephan et al. 2000; Allen et al. 1980; Ariño et al. 1995), thus indicating that low concentrations of these nutrients trigger CGP accumulation. Furthermore, formation of CGP granules occurs under limiting conditions in all groups of CGP-producing bacteria analyzed so far, including the chemoorganoheterotrophically growing *Acinetobacter* sp. strain ADP1 that formed CGP inclusions during phosphate limitation (Fig. 1b, c). In addition, it was observed that in cyanobacteria accumulation of CGP as large granules usually occurred during transition from the late exponential to the stationary growth phase where nutrients, including nitrogen, become limiting (Hegler 1901; Tischer 1957; Stephan et al. 2000; Mackerras et al. 1990; Liotenberg et al. 1996).

In natural habitats of cyanobacteria, nitrogen limitation or depletion occurs quite regularly (Howarth and Cole 1985) and a strong competition between members of the bacterial communities for exogeneous nitrogen is the consequence. Carr (1988) developed the hypothesis that CGP is accumulated under such conditions as an intracellular buffer against the change of extracellular nutrient concentrations allowing CGP accumulating cyanobacteria a kind of "forward planning" and giving them a competitive advantage over nonaccumulating bacteria (Mackerras 1990), which have to endure a stressful period of starvation. Therefore, it was not surprising that Liotenberg (1996) detected the highest CGP synthesis rates when the extracellular ammonia level reached a minimum but was not yet fully exhausted. When growth is resumed owing to a change in culture conditions, CGP is reutilized by the CGP-accumulating cells (Mackerras et al. 1990).

To meet the enhanced amino acid demand during CGP accumulation, isoenzymes of the arginine biosynthesis pathways are present in cyanobacteria and allow a regulatory differentiation between arginine supply for either protein biosynthesis or CGP production (Leganés et al. 1998). Furthermore, an enhanced CO_2 fixation rate was observed by Weathers and Allen (1978) in cyanobacterial cultures during transition from the exponential to the stationary growth phase when CGP is accumulated.

Other abiotic factors, like high light (Hegler 1901; Allen et al. 2005), low light, low temperature (van Eykelenburg 1980), low aeration of the culture medium (Sarma et al. 2004) or the provision of (excess) nitrogen from various sources (arginine, aspartic acid, asparagine, glycine, ammonia, nitrate urea or others (Rippka and Stanier 1978; Allen et al. 1980; Lawry and Simon

1982; Sarma and Khattar 1986; Liotenberg 1996), play a role as triggers of CGP accumulation in many cyanobacteria. However, the inductive effect of these parameters cannot be generally stated for all cyanobacteria as the influence of the individual factors varies greatly from species to species or even among strains of the same species. In some cyanobacteria salt stress seems to promote CGP accumulation (Page-Sharp et al. 1998), and salt-sensitive mutants of *Synechocystis* sp. strain PCC 6803 showed increased CGP accumulation (Zuther et al. 1998).

Inhibition of protein biosynthesis by application of transcriptional or translation inhibitors led in various studies to enhancement of CGP biosynthesis (e.g., rifampicin, Rodriguez-Lopez et al. 1971, or chloramphenicol, Simon 1973b; Pandey and Talpasayi 1982; Allen and Hawley 1983; Hai et al. 2000). This may be due to a decreased concentration of the intracellular CGPase (CphB) and/or an improved availability of free amino acids like arginine and aspartic acid that are no longer directed to the ribosomes.

Although many species-dependent or even strain-dependent differences exist regarding the prerequisites for CGP biosynthesis, its role as a nitrogen and possibly also an energy and carbon storage compound in the different types of cells is widely accepted (Simon 1973a; Mackerras et al. 1990; Liotenberg et al. 1996; Krehenbrink et al. 2002; Picossi et al. 2004). However, the importance of CGP as a nitrogen storage material has been repeatedly controversially discussed, and it has been shown that CGP accumulation is neither a prerequisite for growth of vegetative cells under laboratory conditions nor is its presence of crucial importance for nitrogen fixation (Ziegler et al. 2001).

Another role of CGP that was postulated was that of a "metabolic sink" in the transient accumulation of newly fixed nitrogen in heterocyst -forming cyanobacteria where it occurs as polar "plugs" at the junctions to adjoining vegetative cells (Fogg 1951; Lang et al. 1972; Stewart et al. 1969; Rippka and Stanier 1978; Wolk and Wojciuch 1971a, b; Sherman et al. 2000; Li et al. 2001; Ziegler et al. 2001). Fritsch (1945) suggested as long ago as 1945 that the polar granules are a prominent feature of heterocysts of any cyanobacteria (blue-green algae) and that they consist of CGP. Fogg (1951) concluded from the positive Sakaguchi reaction that the "plugs" occurring at the cell poles consist of "protein, of which arginine appears to be a universal constituent".

Besides CGP, the phycobilines (or phycobilisomes) and soluble cellular proteins represent other potent "metabolic sinks" for nitrogen and possibly also important reserve sites in cyanobacteria (Simon 1973b; Tandeau de Marsac and Houmard 1993). Simon et al. (1973a, b) found that under nitrogen-limitation conditions the phycobilines (phycocyanin) were degraded similarly to CGP granules when nutrients other than nitrogen were provided in excess. Other studies revealed evidence that CGP is synthesized from cellular proteins (Allen and Hawley 1983).

4
CGP Synthetase and CGPase—the Key Enzymes of CGP Metabolism

The partial solubility of different CGPs in the cytoplasm (Ziegler et al. 2002; Füser and Steinbüchel 2005; see before) indicates a high degree of polymer chain mobility in the CGP molecules despite their occurrence in solid granules. Therefore, one can assume that the polymer chain remains accessible to the three known intracellular enzymes of cyanobacterial CGP metabolism, which are the cyanophycin synthetase (CphA, Ziegler et al. 1998), the intracellular CGP-degrading cyanophycinase (CphB, Richter et al. 1999) and the isodipeptide (β-Asp-Arg) degrading "plant-type" asparaginase (isopeptidase, Hejazi et al. 2002) (Fig. 2). These enzymes do not require other additional proteins for their activity neither in vivo nor in vitro. Furthermore, CGP synthetases possess similar high affinity for CGP as two extracellular cyanophycinase (CphE) of heterotrophic bacteria which strongly bound to the polymer during enzyme purification studies (Obst et al. 2002, 2004; Krehenbrink and Steinbüchel 2004). It remains to be demonstrated under which conditions the enzymes of CGP metabolism are attached to the surface of CGP granules in the living cells and when they occur in a dissolved state in the cytoplasm.

4.1
CGP Synthetases

The CGP synthetase was enriched (92-fold) and characterized for the first time by Simon (1976) and was later purified from several bacteria (*Anabaena cylindrica*, Ziegler et al. 1998; *Synechococcus* sp. strain MA19, Hai et al. 1999; *Synechocystis* sp. strain PCC 6308; Aboulmagd et al. 2001a). CphA is a peptide ligase consisting of identical subunits of 90–130 kDa which most probably form active dimers. CphA of *Anabaena cylindrica* possesses a pH optimum of 8.2 and requires besides the aspartic acid and arginine building blocks ATP, Mg^{2+}, K^{+} and a sulfhydryl reagent like 2-mercaptoethanol or dithiotreitol for its activity (Fig. 2; Simon 1976). Knockout of *cphA* in *Anabaena variabilis* led to complete loss of CGP production in vegetative cells and to the loss of the ability to form polar nodules in heterocysts (Ziegler et al. 2001), thus demonstrating the function of CphA in both cell types.

For detection of CphA activity a CGP primer as a starting point for polymerization must be present. Improvement of a radioactive CGP synthetase assay made it possible to measure the primer specificity of CphA (Aboulmagd et al. 2000, 2001a). According to Berg et al. (2000), who employed matrix-assisted laser desorption/ionization time-of-flight analysis to identify in vitro synthesis products of the CphA reaction using synthetic primers, the primer must at least be a trimer of β-Asp-Arg [(β-Asp-Arg)$_3$]. Hai et al. (2002) found evidence that different fractions (e.g., cell walls or other components) of cells can serve as alternative priming substances in the CphA reaction. This might

be the reason why CGP synthesis can also occur in recombinant bacteria that do not posses a CGP molecule or β-Asp-Arg oligomers per se.

The mechanism of chain elongation results in alternating incorporation of aspartic acid and arginine and was suggested first by Simon (1976), who used radioactive arginine and aspartic acid isotopes, respectively. This was confirmed by Berg et al. (2000), who measured the stepwise amino acid incorporation at the *C*-terminus of the growing polymer chain. Each reaction cycle, in which two amide bonds are formed, consumes two molecules of ATP which are employed for activation (phosphorylation) of the α-carboxylic and β-carboxylic groups, respectively, of the *C*-terminal aspartic acid residue of the growing CGP molecule. Since many CGP synthetases are not strictly specific for arginine, other amino acids with basic functional groups like lysine, ornithine, citrulline and canavanine are also incorporated in in vitro experiments (Berg et al. 2000; Aboulmagd et al. 2001a). Furthermore, also in vivo lysine incorporation into the CGP molecule was observed in recombinant *E. coli* strains (Ziegler et al. 1998, 2002; Frey et al. 2002). Merrit et al. (1994) found glutamic acid to be incorporated instead of arginine in CGP isolated from nitrogen-limited cultures of *Synechocystis* sp. PCC 6308, and Wingard et al. (2002) detected comparably large amounts of glutamic acid (15 mol %) in CGP from a marine halotolerant *Synechococcus* strain. However, purified CphA did not accept glutamic acid as a substitute for arginine, and the acidic nature of glutamic acid makes it seem rather unlikely that glutamic is incorporated as a side-chain amino acid (Aboulmagd et al. 2001a).

On the basis of similarities of its primary structure, a close relatedness of the enzyme to other known ligases was expected. The *N*-terminal half of the enzyme matched the B-loop sequence of the ATP-grasp fold of members of the superfamily of enzymes with carboxylate–amine/thiol ligase activity, whereas the *C*-terminal region showed similarities to proteins of the superfamily of three substrate ligases. Remarkable sequence homologies were also detected with the *capB*-translational product of *Bacillus anthracis*, which is involved in extracellular poly(glutamic acid) biosynthesis (reviewed by Oppermann and Steinbüchel 2003). Therefore, it was concluded that two different domains with different specificity and peptide ligase activity are responsible for the alternating incorporation of aspartic acid and arginine.

4.2 CGPases

During studies on CGP biosynthesis and CGP material properties it was soon discovered that CGP is remarkably resistant against the enzymatic attack of a large variety of proteases and other enzymes that act on structural motifs occurring in CGP (Simon and Weathers 1976; Simon 1987 and unpublished data). This resistance is most probably due to the branched polypeptide structure and the β-linkages occurring in the CGP side chains which make it

sterically difficult for polypeptide-hydrolyzing enzymes to bind to CGP or to get access to the peptide and isopeptide bonds of CGP, respectively. It was therefore not surprising that a highly specialized enzyme is employed by the cyanobacterial cell for CGP mobilization.

Although numerous studies on CGP biosynthesis have been performed, only a few reports are available on the intracellular CGP-degrading enzyme CphB of cyanobacteria (Simon et al. 1980; Richter et al. 1999). Gupta and Carr (1981) as well as Allen et al. (1984) reported on the occurrence of CGP degradation in crude extracts prepared from cells of cyanobacteria. Later, Richter et al. (1999) identified β-Asp-Arg dipeptides as the main products of cyanophycin hydrolysis by gel filtration. The first and only intracellular CGPase (CphB protein) that was characterized in detail is the enzyme of *Synechocystis* sp. strain PCC 6803 (Richter et al. 1999). By employment of inhibitors as well as nucleotide sequence analysis, it was shown that CphB is most probably a serine-type peptidase with an active center containing a catalytic triad with a histidine, a glutamic acid and a serine residue (nucleophile) within a lipase box (GxSxG) motif. In vitro experiments showed that CphB was highly specific for CGP as a substrate and degraded it to β-Asp-Arg dipeptides. Later, Hejazi et al. (2002) demonstrated that asparaginase, like the heterologously expressed plant-type asparaginase of *Synechocystis* sp. PCC6803, can hydrolyze isodipeptides like β-Asp-Arg to the amino acid monomers. It was obvious that these enzymes often even prefer β-Asp-Xaa dipeptides (Xaa is Ala, Arg, Leu, Lys or Phe in the case of the *Synechocystis* sp. PCC6803 enzyme) to their putative natural substrate asparagine, indicating their potential role in CGP catabolism where β-linked dipeptides (isodipeptides) are formed as primary CGP degradation products.

Employment of these highly specialized hydrolytic enzymes and of purified extracellular CGPases (cphE) of several Gram-positive and Gram-negative heterotrophic bacteria for in vitro CGP degradation confirmed also the proposed structure and chemical composition of CGP by specific cleavage of peptide and isopeptide bonds, respectively, of CGP or β-Asp-Arg (Gupta and Carr 1981; Allen et al. 1984; Richter et al. 1999; Obst et al. 2002, 2004; Hejazi et al. 2002). Furthermore, also during anaerobic cultivation of a CGP-degrading bacterial consortium with CGP as the sole carbon source β-Asp-Arg dipeptides were formed, thus indicating that cleavage of the CGP polymer backbone at the α-amide bonds and subsequent degradation of the released isodipeptides is a common principle in nature (Obst et al. 2005).

5
Cyanophycin Synthesis in Recombinant Bacteria and Transgenic Plants

The production of CGP in recombinant bacteria started when several *cphA* genes were cloned by Ziegler et al. (1998), Oppermann-Sanio et al. (1999),

Aboulmagd et al. (2000), Berg et al. (2000) Hai et al. (2002), Krehenbrink et al. (2002) and Ziegler et al. (2002) and were heterologously expressed in *E. coli*. These studies were done to purify CphA and characterize the enzyme biochemically and to learn more about its catalytic mechanism (see before) but provided also the first genetic constructs for heterologous CGP production. Later strategies were developed to optimize genetic constructs for high-level expression of *cphA* to establish economically feasible CGP production processes based on recombinant strains and to obtain sufficient amounts of CGP to test on a large scale its material properties (Aboulmagd et al. 2001b; Frey et al. 2002; Voss et al. 2004). Insertion of *cphA* into a vector where transcription can be controlled via a thermosensitive lambda repressor (cI857) significantly increased CGP production (Frey et al. 2002) which was dependent on the application of rather expensive chemical inducers like isopropyl-β-D-thiogalactopyranosid to plasmid containing recombinant strains. However, CGP production was still relatively cost intensive because, presumably by depletion of the host cells of the amino acids arginine and aspartic acid during growth in a fermentor, complex nutrient components like casamino acids had to be employed to produce CGP efficiently on a large scale (Frey et al. 2002). A similar high demand for arginine (75 mM) for optimum CGP synthesis rates [41% of cellular dry weight (CDW)] was recently observed for *Acinetobacter* sp. strain ADP1 cells which were cultivated in arginine-supplemented mineral medium (Elbahloul et al. 2005a), again indicating that high CphA activity efficiently depletes the cellular pool of free arginine.

CphA of *Synechocystis* sp. strain PCC 6308 was also expressed in several other widely used industrially relevant bacteria such as *Corynebacterium glutamicum*, *Ralstonia eutropha* and *Pseudomonas* sp. (Byrom 1992; Eggerling and Sahm 1999; Füchtenbusch et al. 2000) using different types of shuttle vectors to test the capability of these organisms to produce CGP in large amounts in mineral salt media. The recombinant strains showed comparably high CphA activities and accumulated CGP up to 11% of CDW as inclusion bodies. Figure 4 shows as examples cells of recombinant *E. coli* (Fig. 1a) at an early stage of CGP production and of *R. eutopha* with large CGP inclusions (Fig. 1b, C). It should be noted that in recombinant cells of production strains usually only one granulum per cell occurred. In some cases structured surfaces were formed (Fig. 4c) similarly as in *Acinetobacter* sp. strain ADP1 where only one "radiating" granulum was found (Fig. 1b, C). Similar surface patterns are known also from cyanobacterial "structured granules" (Lang et al. 1972). Stephan et al. (2000) detected the formation of similar star-shaped "structured" granules during cultivation of *Synechocystis* sp. strain PCC 6308 in the presence of asparagine. Geitler (1932) mentioned refractive granules in the cytoplasm of cyanobacteria with an irregular round-to-crystalloid shape consisting mainly of protein and indicating that variations in the structure of CGP granules occur quite regularly. Cyanobacteria usually synthesize a higher number of granules than recombinant bacteria, and

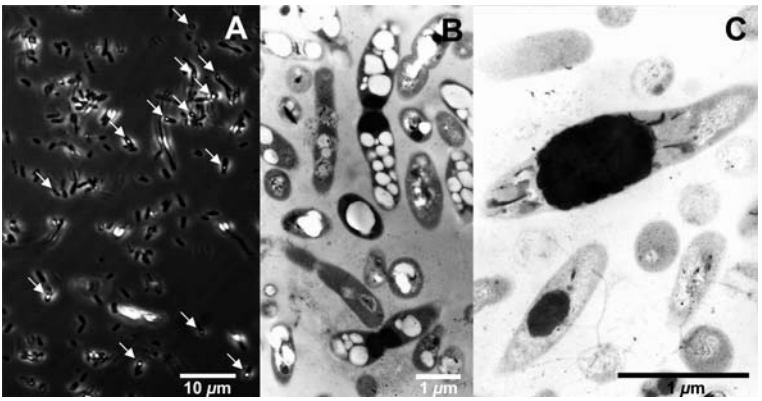

Fig. 4 CGP inclusions in recombinant bacteria. **a** A phase-contrast light micrograph of *E. coli* DH1 (pMa/c5-914::$cpha_{PCC6803}$) cells grown on solid Luria–Bertani complex medium in which light refractive inclusions of CGP are visible (marked by *arrows*). **b** Transmission electron micrographs of cells of *R. eutropha* strain H16 (pBBR1::$cpha_{PCC6308}$) harboring the CGP synthetase of *Synechocystis* sp. PCC6308 (Aboulmagd et al. 2001b). CGP granules appear as *black inclusion bodies*, beside *white inclusions* representing polyhydroxybutyric acid (*PHB*) granules. **c** Cells of the *R. eutropha* H16-$PHB^{-}4$ (pBBR1::$cpha_{PCC6308}$) which lack PHB granules but exhibit large CGP granules

the granules are often located at specific sites, i.e., close to the thylakoid membranes or cross walls in filaments and at the junctions between heterocysts and neighboring vegetative cells but usually not in the centroplasma (cf. Fig. 1; Geitler 1932; Fritsch 1945). Whether this location of CGP granules and their presence in a higher number are due to the presence of CGP primer molecules in the peripheral regions of cyanobacterial cells or whether thylakoid membranes play a role in separating the individual granules of a given cell that otherwise would merge and form large granules as observed in *Acinetobacter* and recombinant bacteria, respectively, is unknown.

Only recently *cphA* of *Thermosynechococcus elongatus* strain BP-1 was expressed constitutively in plants (Neumann et al. 2005). The transgenic tobacco and potato plants, which exhibited alterations of leaf morphology and showed decelerated growth, produced up to 1.1% dry weight of a polymer with cyanophycin-like properties that formed inclusions or aggregates of up to 4 μm in diameter in the plant cells (Fig. 5). However, although heterologous CGP biosynthesis was successfully established, incorrectly processed messenger RNA and an adverse effect of cytoplasmatically produced CGP on plant growth showed that the expression of *cphA* in the plant hosts is not yet optimized and needs to be improved if one day plants should serve as cost-effective CGP production organisms. The studies also indicated that the direction of CphA to the chloroplast helps to increase the polymer content significantly and reduces the negative side effects on growth of the respective plant lines (Neumann et al. 2005).

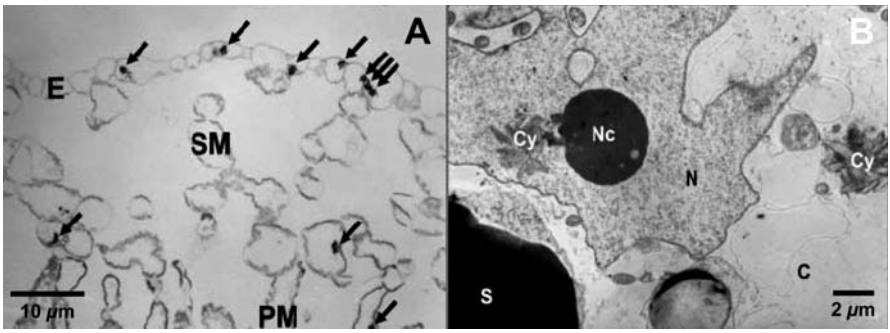

Fig. 5 CGP synthesis in transgenic plants (Neumann et al. 2005). Expression of *cphA* of *Thermosynechococcus elongatus* strain BP-1 led to accumulation of CGP in tobacco leaf cells (**a** light micrograph of a cross section stained with Naphtol Blue Black; CGP granules are marked by *arrows*) and in tubers of trangenic potato plants (**b** electron micrograph of a tuber cell). *C* cytoplasm, *Cy* cyanophycin, *N* nucleus, *Nc* nucleolus, *PM* palisade mesophyll, *s* starch, *SM* spongy mesophyll. (Reproduced from Neumann et al. 2005; with permission of Blackwell Publishing Ltd.)

These results demonstrate that production of CGP can be established in non-cyanobacterial production strains and in principle also in higher (eukaryotic) organisms.

6 Biotechnological Production of CGP and Potential Technical Applications

Many CGP synthetase have been characterized so far and exhibit different substrate specificities regarding the incorporation of amino acids other than arginine in the polymer side chain or discrimination of amino acids other than arginine as it is the case for CphA from *Acinetobacter* (Ziegler et al. 1998, 2002; Berg et al. 2000; Aboulmagd et al. 2001a; Krehenbrink et al. 2002; compare above). Furthermore, many other *cphA* homologous genes were found in the genomes of a variety of bacteria with largely different biochemical capabilities (Krehenbrink et al. 2002; Ziegler et al. 2002 and unpublished data). This finding probably opens up new perspectives for the production of different CGPs and possibly also other polyamides with various different monomer compositions.

6.1 Production of CGP and CGP Derivatives

At least for lysine-containing CGP and for lysine-free CGP the production in a biotechnological process has been demonstrated (Frey et al. 2002; El-

bahloul et al. 2005a) and synthesis of CGPs that contained basic amino acid monomers other than lysine (canavanine, citrulline or ornithine) has been shown in in vitro studies with purified CphAs (Berg et al. 2000; Aboulmagd et al. 2002a).

An important characteristic of technical enzymes is their stability under various conditions. In this regard, CphA of the thermophilic cyanobacterium *Synechococcus* sp. strain MA19 is a good candidate as it shows increased thermostability and remains active even after 2-h incubation at 50 °C (Hai et al. 2002). This enzyme may therefore be used also in in vitro CGP synthesis systems (Oppermann-Sanio and Steinbüchel 2002).

Furthermore, employment of production strains that synthesize a water-soluble CGP-like polymer (Ziegler et al. 2002) or solubilization of CGP by salt treatment (Füser and Steinbüchel 2004) could lead to development of production processes yielding CGP that can be easily modified in the dissolved state by chemical treatment of aqueous CGP solutions.

The production of large amounts of CGP is rather time-consuming (about 21 days) if cyanobacterial cell are employed and requires special fermentors and elaborate fermentation techniques (Hai et al. 2000). Furthermore, the CGP content of cyanobacterial cells even under optimum growth conditions is often comparably low and requires supplementation of arginine and application of chloramphenicol (Hai et al. 2000). Therefore, production of CGP in recombinant bacterial strains or heterotrophic CGP-producing bacteria like *Acinetobacter* sp. strain ADP1 with CGP contents of up to 24% CDW for *E. coli* (Frey et al. 2002) or 41% CDW for *Acinetobacter* (Elbahloul et al. 2005a) and much shorter fermentation periods of approximately only 1 day seems advantageous and will certainly be preferred by industry in terms of cost effectiveness.

Limitation of CGP production by insufficient arginine biosynthesis in various CGP-producing strains, including cyanobacteria (Hai et al. 2002; Frey et al. 2002; Elbahloul et al. 2005a), as also indicated by the low K_M values (and high affinities) of CGP synthetases for arginine (usually about 50 μM; Aboulmagd et al. 2001a; Krehenbrink and Steinbüchel 2004), demonstrates that depletion of the cellular arginine pool occurs quite regularly during cultivation of various different bacteria. One of the main challenges in the future for large-scale CGP production will therefore be the improvement of the arginine supply for CphA, presumably by metabolic engineering and selection of arginine-overproducing bacterial strains suitable for large-scale cultivation in fermentors.

It became obvious during studies on large-scale CGP production (Hai et al. 2002; Frey et al. 2002; Voss et al. 2004; Elbahloul et al. (2005b)) that *E. coli* is presumably the most suitable bacterium for CGP production in complex media and media with relatively high salt and high nutrient content, whereas *R. eutropha*, *Pseudomonas putida* and *Corynebacterium glutamicum* may be preferentially cultivated in mineral salt media in which they showed comparably good productivity.

6.2 Potential Applications and Commercialization of CGP

For CGP, no definite applications have been developed yet. In the future, CGP and CGP derivatives may possibly serve as a raw material for the production of bio-based, biodegradable and biocompatible polymeric products as happened before when the other known bacterial poly(amino acids) poly(glutamic acid) and poly(ε-lysine) were commercialized in Japan and are still used in technical as well as in food applications (reviewed by Oppermann-Sanio and Steinbüchel 2002; Obst and Steinbüchel 2004).

Because the production and efficient isolation of CGP in semitechnical amounts was established only during the last few years (Aboulmagd et al. 2001; Frey et al. 2002; Elbahloul et al. 2005a), considerations to commercialize CGP were only recently made. In contrast, economically important applications have been established already for poly(aspartic acid) (PAA), which is a structural element (polymer backbone) of CGP and which is used as a substitute for nonbiodegradable polyacrylates (Schwamborn 1998). PAA is a bulk chemical and can be employed as a water softener or a scale inhibitor in many industrial applications as well as being an ingredient of many daily-life commodity products, including paints, suntan lotions and washing detergents, and can be used as an additive in the paper, building or oil industries (reviewed by Joentgen 2003). By hydrolysis in dilute acid, CGP can be converted to a derivative with reduced arginine content (Joentgen et al. 1998).

Because a thermochemical synthesis process is employed for chemical synthesis of PAA, it contains aspartic acid residues that are either linked via their α-carboxylic or β-carboxylic groups and consists of a racemic mixture of the levo and dextro isomers of aspartic acid. In addition, branching of the polymer chain was observed. Therefore, chemosynthetic PAAs are poly(α,β-D/L-aspartic acids) in contrast to the polymer backbone of CGP that solely consists of poly(α-L-aspartic acid). Although chemically synthesized PAA is partially biodegradable (Tabata et al. 2001; Hiraishi et al. 2003a, b), it seems reasonable to assume that linear poly(α-L-aspartic acid), which is structurally more closely related to proteins, but exhibits similar chemical characteristics, will show an even better biodegradability.

Because the cost for crude oil will increase rapidly over the next few decades and because of the complete biodegradability of CGP that has been proved in several preceding studies (Richter et al. 1999; Obst et al. 2002, 2004, 2005; Hejazi et al. 2002), CGP and CGP derivatives can be seen as an attractive future source of nitrogen and carbon-rich bio-based raw materials. Furthermore, CGP or modified CGPs could be used also for the production of defined mixtures of amino acids with various monomer compositions that could be easily released by chemical or enzymatic hydrolysis of the polymer. The monomers in turn could be used as ingredients of a variety of already

established or newly developed amino acid rich commodity products or as basic chemicals for chemical syntheses like, for example, the production of nylon-like material.

Because during the last few years many efforts were made in the construction of CGP-producing recombinant bacteria which show good growth and high CGP production rates in complex but also in mineral media (see before), it is now possible to produce CGP and several CGP derivatives in sufficient amounts to allow testing of the material properties for potential technical applications.

7 Outlook and Perspectives

In the future CGP production rates in bacteria will further increase as bacterial strains, media and cultivation conditions are optimized and cheap waste materials from agriculture or horticulture like, for example, protamylasse (Elbahloul et al. 2005b) with complex amino acid and sugar compositions, are employed as substitutes for currently used media which have to be enriched with arginine or casamino acids to reach high production rates (see before). Finally, establishment of cost-effective production of CGP in plants by creation of highly productive transgenic plant lines as principally already shown by Neumann et al. (2005) will certainly be a great challenge for researchers during the next decade. Its realization would be another milestone on the way from the basic characterization of CGP-producing bacteria and CGP, respectively, to its large-scale production and its introduction to the market.

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Insecticidal Protein Crystals of *Bacillus thuringiensis*

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Abstract The spore-forming bacterium *Bacillus thuringiensis* bears plasmids encoding genes for insecticidal proteins typically synthesized during sporulation. These proteins crystallize forming large polyhedral parasporal inclusions that make up as much as 30% of the cellular dry weight. When ingested by insects and certain other arthropods, these inclusions dissolve and the proteins are cleaved by proteases releasing active toxins that bind to specific receptors on the host's midgut membrane. Activated toxins then oligomerize, inserting into this membrane where they form cation-selective channels that cause cell lysis and host death. Two types of crystal proteins are recognized, Cry proteins, the most common, and Cyt proteins. Cry proteins are generally either 60–80 or 130–150 kDa, the former being truncated versions of the latter. Cyt proteins are approximately 28 kDa, and are unrelated to Cry proteins, having an affinity for midgut membrane lipids. Cry and Cyt proteins are the active ingredients of many commercial insecticides. More importantly, several Cry proteins are the basis of transgenic insecticidal crops such as Bt cotton and Bt corn, now a multibillion dollar global industry. In this chapter, we summarize the role Cry and Cyt proteins play in the biology of *B. thuringiensis*, and then focus on the synthesis and structure of the crystals they form. We also show how their synthesis can be manipulated with recombinant DNA techniques to increase crystal size and improve insecticidal activity.

1 Introduction

Over the past century, the insecticidal bacterium *Bacillus thuringiensis* Berliner has emerged from being an interesting minor pathogen of caterpillars to an environmentally safe insecticide for control of pests of major crops and vectors of human diseases to the primary source of proteins used in genetically engineered crops to protect them from insect damage. Transgenic crops based on insecticidal crystal proteins of *B. thuringiensis* are now an international industry with revenues of several billion dollars per year. Although several proteins and other compounds produced by *B. thuringiensis* contribute to its insecticidal activity, by far the most important components are the proteins that form parasporal crystalline inclusions during sporulation. These inclusions contain Cry and Cyt proteins selectively toxic to caterpillars, beetles, or the larvae of mosquitoes and blackflies. In this chapter, we briefly review key discoveries that led to commercialization of this bacterium, and follow this with sections on how crystal proteins kill insects and genetic control of crystal protein synthesis and assembly. Along the way we review the taxonomic status of *B. thuringiensis* as a species separate from

B. cereus, as well as the methods used to identity and classify its subspecies and crystal proteins. We close with sections on the use of recombinant DNA technology to produce new types of bacterial insecticides based on these interesting insecticidal crystal proteins.

2 A Brief History of *B. thuringiensis*

The species that we recognize today as *B. thuringiensis* Berliner was originally discovered in Japan over a century ago by Shigetane Ishiwata (1901) as the cause of the sudden (*sotto*) death disease of silkworms, larvae of the silkworm moth, *Bombyx mori*. A little over a decade after Ishiwata's discovery, the German bacteriologist Ernst Berliner (1915), unaware of Ishiwata's paper, described a similar bacterium as the cause of disease in larvae of the flour moth, *Ephestia kuhniella*. The species name "*thuringiensis*" is derived from Thuringia, the German state where the diseased flour moth larvae were found. In his description, Berliner noted the presence of parasporal inclusions, as did several subsequent researchers, but their role in disease remained unknown until the 1950s. Even though little was understood about the basic biology of *B. thuringiensis* in the 1930s, it was shown to be highly pathogenic for larvae of certain species of lepidopterous pests. As synthetic chemical insecticides had not yet been developed, a preparation of sporulated *B. thuringiensis* cells known as Sporeine was used in France for insect control just prior to the outbreak of World War II. There was little interest in using *B. thuringiensis* as an insecticide during World War II; however, after the war Edward Steinhaus (1951) of the University of California began the modern era of research on this species by showing that it had potential for controlling the alfalfa caterpillar, *Colias eurytheme*. His studies were followed soon thereafter by others which demonstrated that the bipyramidal parasporal body produced by *B. thuringiensis* was responsible for the rapid death of caterpillars (Hannay 1953; Angus 1954).

Fermentation studies during the 1950s determined that large-scale culture of *B. thuringiensis* was possible at relatively low cost, accelerating its development as an insecticide. After many failures, an isolate known as HD1 of *B. thuringiensis* subsp. *kurstaki* (Fig. 1) was cultured by Howard Dulmage of the USDA and was shown to have broad spectrum of activity against lepidopterous pests, while being safe for most nontarget invertebrates, including bees and beneficial predatory and parasitic insects, as well as for humans and other vertebrates (Dulmage 1981; for a review on safety see Glare and O'Callaghan 2000). By the early 1970s, commercial formulations of *B. thuringiensis* subp. *kurstaki* (HD1) with names such as Dipel and Thuricide were in use to control many lepidopterous pests, such as the cabbage looper (*Trichoplusia ni*), corn earworm (*Helicoverpa zea*), and tobacco bud-

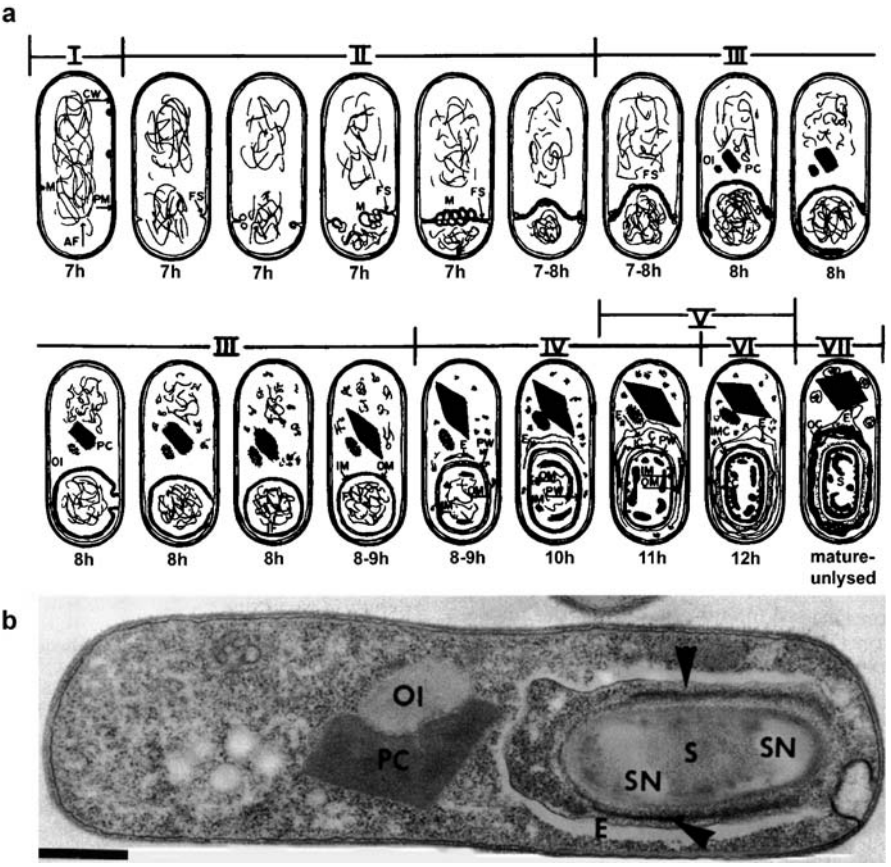

Fig. 1 Sporulation in *Bacillus thuringiensis* Berliner. **a** The standard stages of sporulation in *Bacillus* species indicating the relative timing of the appearance of parasporal bodies as they assemble in the HD1 isolate of *B. thuringiensis* subsp. *kurstaki* (H3a3b3c). **b** Electron micrograph of a sporulating HD1 cell at stages IV–V. *PC* developing bipyramidal crystal in which Cry1Aa, Cry1Ab, and Cry1Ac coassemble. *OI* ovoidal inclusion containing Cry2A. This inclusion typically assembles on one side of the short axis of the bipyramidal crystal, apparently facilitated by the 29-kDa protein (ORF2) of the *cry2Aa* operon. *E* exosporium, *S* spore, *SN* spore nucleoid, *arrowheads* spore coat. *Bar* 500 nm. (From Bechtel and Bulla 1976)

worm (*Heliothis virescens*) in vegetable and field crops, and major forest pests, mainly the gypsy moth (*Lymantria dispar*) and spruce budworm (*Choristoneura fumiferana*). During the late 1970s and early 1980s, years after its initial commercial success, the application of new molecular biological techniques to research on *B. thuringiensis* demonstrated that HD1's broad spectrum of insecticidal activity was due to its complex parasporal body, which was shown to consist of two crystals (Fig. 1) that together contain four

proteins, Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa, each with a different lepidopteran target spectrum and specific activity.

By the mid-1970s, 13 subspecies of *B. thuringiensis* had been described on the basis of the analysis of hundreds of isolates. None of these had any significant insecticidal activity to insect species outside the order Lepidoptera. Then in 1976, Goldberg and Margalit (1977) discovered a new subspecies, subsequently named *B. thuringiensis* subsp. *israelensis*, in the Negev desert of Israel, highly toxic to larvae of a wide range of mosquito species. This subspecies was subsequently shown to also be insecticidal for larvae of other species of flies in the dipteran suborder Nematocera, including blackflies and chironomid midges. The parasporal body of this subspecies is spherical and composed of four major proteins, Cry4A, Cry4B, Cry11A, and Cyt1A, and thus is considerably different from the bipyramidal crystals produced by subspecies toxic to lepidopterans. Though their parasporal bodies differed, comparative studies of *B. thuringiensis* subsp. *kurstaki* and *israelensis* showed that strong sporulation-dependent promoters accounted for the large amount of insecticidal protein produced during sporulation (Fig. 2).

The broad spectrum of activity *of B. thuringiensis* subsp. *israelensis* against biting flies led to its rapid commercialization, and products such as Vectobac, Bactimos, and Teknar for control of nuisance and vector mosquitoes

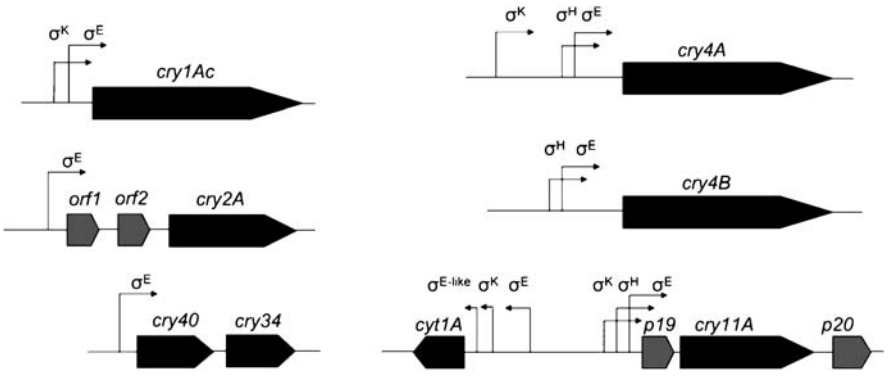

Fig. 2 Promoter and gene arrangements for various genes and operons of *B. thuringiensis*. The most typical promoters are those for which expression is directed by sigma factors, σ^E and σ^K, both active during sporulation. An exception to this is found in *cry3A* genes, for which expression is controlled by σ^A, active during vegetative growth. On the *left*, the top two schematics show gene and operon arrangements for *cry1Ac* and *cry2Aa* in the HD1 isolate of *B. thuringiensis* subsp. *kurstaki*. Immediately below this the operon structure for the *cry40-cry34* operon of *B. thuringiensis* subsp. *thompsoni*. On the *right*, the genes and operons with their promoters are shown for the *cry4A*, *cry4B*, *cry11A*, and *cyt1A* genes, which encode, respectively, the four major proteins of *B. thuringiensis* subsp. *israelensis*. σ^H, active during the transition from vegetative growth to sporulation, plays a minor role in the expression of these genes. (Illustration modified from Baum and Malvar 1995)

and blackflies. Owing to their high efficacy and narrow target spectrum, these products replaced many broad-spectrum chemical insecticides used for mosquito and blackfly control in developed countries, and are currently under development for control of the major anopheline vectors of malaria is Africa and South America. Moreover, products based on *B. thuringiensis* subsp. *israelensis* proved of particular importance in the Onchocerciasis Control Program in West Africa, which significantly reduced onchocerciasis, commonly known as river blindness, a debilitating human eye disease caused by *Onchocerca volvulus*, a nematode transmitted by blackflies of the *Simulium damnosum* complex (Guillet et al. 1990).

Not long after the discovery of *B. thuringiensis* subsp. *israelensis*, a third pathotype highly toxic to larvae and adults of coleopterous insects, i.e., beetles, was discovered in Germany (Krieg et al. 1983). This isolate, which produces a thin rhomboidal parasporal crystal composed of Cry3A, was originally named *B. thuringiensis* var. *tenebrionis*, but is known formally as *B. thuringiensis* subsp. *morrisoni*, strain tenebrionis. Owing to its toxicity to certain important coleopteran pests, such as the Colorado potato beetle (*Leptinotarsa decemlineata*), this isolate was developed as a bacterial insecticide for control of beetle pests. Unlike *B. thuringiensis* subsp. *kurstaki* and *israelensis*, however, the efficacy of commercial products based on the original and similar isolates of *B. thuringiensis* toxic to beetles was not as effective as new chemical insecticides, such as the neonicontinoids, and thus the products were not a commercial success.

Insecticides based on the aforementioned *B. thuringiensis* isolates are the most successful of the various bacterial, fungal, viral, and other pathogens developed as insecticides, with current worldwide annual sales estimated to be $100 million. Although successful, the most significant scientific, technological, environmental, and commercial success resulting from research on the parasporal crystals of *B. thuringiensis* is the development of insecticidal "Bt" transgenic crops based on Cry proteins. These crops, primarily Bt cotton (based on Cry1Ac) and Bt corn (based on Cry1Ab), first released for commercial use in the USA in 1996, and later in countries such as Australia, Argentina, China, and India, have annual revenues in the range $3–4 billion. Environmental benefits are derived from the insecticidal specificity of these crops, which unlike synthetic chemical insecticides, kill only target species and closely related insects, as well as from reductions in chemical insecticide usage (O'Callaghan et al. 2005). In the USA, approximately 50% of the corn and cotton are now Bt crops. Moreover, insecticidal transgenic crops are expected to increase in sales worldwide at a rate of 10–20% per year as new varieties of Bt crops are developed (Shelton et al. 2002). Use of these crops remains controversial in many countries, especially in Europe, and while this chapter is not the place to discuss this new pest control technology, it should be realized that Bt as well as other types of transgenic crops trace their origin to study of the crystal proteins produced by *B. thuringiensis*.

3
Taxonomic Affiliation of *B. thuringiensis*

Over the past few decades, there has been an ongoing discussion regarding the validity of *B. thuringiensis* as a species separate from *B. cereus*. On the basis of the sequence of the 16S ribosomal subunit gene, these two species along with *B. anthracis*, the causative agent of anthrax, could be considered variants of the same species. However, if a wider range of genes and characteristics of the genome using random markers are examined, several distinct clusters are apparent that separate *B. thuringiensis* and *B. cereus* from *B. anthracis*. Thus, *B. anthracis* is not a close relative of *B. thuringiensis*, and of the thousands of isolates of the latter species collected, none have been shown to cause any human illness. It is much more difficult, if not impossible, to reliably separate *B. thuringiensis* isolates from those of *B. cereus*, as they share many overlapping characteristics (Baumann et al. 1984; Radnedge et al. 2003; Hill et al. 2004). Even so, isolates of certain subspecies of *B. thuringiensis* do tend to cluster together (Hill et al. 2004). Despite the latter finding, the evidence is overwhelming that few if any genes coding for Cry or Cyt proteins are located in the chromosome of most *B. thuringiensis* isolates—typically they appear to be borne on large transmissible plasmids. Thus, for the sake of simplicity, *B. thuringiensis* can be thought of as *B. cereus* bearing plasmids that code for Cry and Cyt proteins that typically are synthesized during sporulation under the control of sporulation-dependent promoters. If an isolate of these two species is obtained from soil, plants, or other sources such as grain dust, insect feces, or diseased insects, and during sporulation produces parasporal crystals, whether insecticidal or not, it is considered *B. thuringiensis*. If the isolate does not produce crystals, it is either *B. cereus* or another species. If a strain that bears plasmids coding for Cry or Cyt proteins loses these plasmids during serial passage, though it is commonly referred to as an acrystalliferous strain of *B. thuringiensis*, if it were analyzed by any commonly employed biochemical identification technique, it would be identified as *B. cereus*. So, bacilli that are easily isolated and cultured from substrates such as those noted before, and which produce ovoid spores along with parasporal protein crystals, are classified as *B. thuringiensis*. Before delving into the specific types of crystalline inclusions produced by *B. thuringiensis*, with this background we can proceed with brief discussions of how isolates of this species are identified and classified, and the role these crystal proteins play in its biology.

3.1
Identification and Classification of *B. thuringiensis* Subspecies

During the 1950s and 1960s, hundreds of isolates of *B. thuringiensis* were obtained that were markedly different in their growth characteristics, produc-

tion of various enzymes and secondary compounds, and toxicity to various species of lepidopterans. Though it was realized that most of the toxicity was due to crystal proteins, as modern molecular genetics techniques had not yet been developed, and it was not known that these proteins were encoded on plasmids, the enzymatic and biochemical methods by which isolates were differentiated and described proved unreliable. This was because isolates that had different plasmid complements of endotoxin-bearing genes could have similar biochemical and enzymatic properties. In the end, though not a perfect system, most researchers adopted an identification and classification system based on the serovariety of the H-antigen, i.e., the immunological properties of the flagellar antigen. This system, originally developed at the Pasteur Institute, Paris, France, by de Barjac and Bonnefoi (1962, 1968), provided consecutive H-antigen numbers for newly identified flagellar serovarieties that were given subspecific names. For example, H1 is *B. thuringiensis* subsp. *thuringiensis*, H3a3b3c is *B. thuringiensis* subsp. *kurstaki*, H7 is *B. thuringiensis* subsp. *aizawai*, H14 is *B. thuringiensis* subsp. *israelensis*, and H60 is *B. thuringiensis* subsp. *pingluonsis*. In H-antigen numbers that include lowercase letters, these indicate epitopes shared with other serovarieties/subspecies.

At present, over 70 serovarieties/subspecies have been described from around the world (Lecadet et al. 1999). There is reasonably good correlation between this identification and classification system and insecticidal activity. For example, if an isolate is serotyped as subspecies *kurstaki* (H3a3b3c), it typically exhibits toxicity to lepidopterous insects, whereas many if not most isolates that serotype as subspecies *israelensis* (H14) turn out to be highly toxic to mosquitoes. Nevertheless, isolates of several other subspecies, such as *morrisoni* (H8a8b), *darmstadiensis* (H10a10b), *kyushuensis* (H11a11b), *jegathesan* (H28a28b) and *medellin* (H30), are also toxic to mosquitoes. This system has worked better than any of the others proposed, and remains in use today, as it allows researchers to put a "handle" on new subspecies and describe their properties. However, it is not perfect because the flagellar antigen does not correlate directly with the endotoxin-encoding plasmid complement of many isolates. Thus, isolates HD1 and HD73, for example, are both subspecies *kurstaki* (H3a3b3c), but the former bears plasmids that produce Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa, whereas the plasmids in the latter only produce Cry1Ac. Because HD73 only produces Cry1Ac, it has a much narrower insecticidal spectrum than HD1. Similarly, isolates of subspecies *morrisoni* (H8a8b) can have markedly different target spectra depending on their plasmid complements, with most isolates being toxic to lepidopterous species. However, the PG-14 isolate of this serotype is toxic to mosquitoes, and the tenebrionis isolate (also referred to as a variety) is toxic to beetles.

3.2
Identification and Classification of Endotoxin Crystal Proteins

In the early 1980s, Schnepf and Whiteley (1981) cloned the first *cry* gene and demonstrated that it was carried on a plasmid. Subsequently, research by numerous investigators showed that most if not all *cry* genes were carried on plasmids (see Schnepf et al. 1998). As more and more *cry* genes were cloned, and later *cyt* genes, the focus of understanding the insecticidal activity of new and known isolates of *B. thuringiensis* shifted primarily to identification and expression of plasmid-encoded genes of various subspecies and isolates of *B. thuringiensis*. The specific names of different Cry and Cyt proteins are based on the degree of relatedness within each of these types as determined by the nucleotide and deduced amino acid sequences of the encoding genes (Hofte and Whiteley 1989; Crickmore et al. 1998). The system is hierarchical in that all Cry1 proteins are more closely related to one another than, for example, they are to Cry2 proteins, and all Cry1A proteins are more closely related to one another than they are to Cry1B proteins. To a considerable extent, the system reflects the host spectrum, with most Cry1 proteins being toxic to lepidopterans, whereas most Cry3 proteins are toxic to coleopterans, and Cry4 proteins to dipterans. However, there are exceptions. For example, certain Cry2 proteins such as Cry2A are toxic to both lepidopterans and dipterans, whereas some Cry1B proteins can be toxic to lepidopterans and coleopterans. At a higher level of resolution, for example, Cry1Aa versus Cry1Ab, the lowercase letter translates into a smaller difference between two sequences, but even a minor difference in amino acid sequences can yield a significant difference in host spectrum and specific toxicity within a host group (Hofte and Whiteley 1989). Thus, the system is only partially predictive in that once a new gene sequence is determined, the specific host spectrum and toxicity must be determined through bioassays.

4
Role of Crystal Proteins in the Biology of *B. thuringiensis*

The high toxicity of many Cry proteins to insects provides strong evidence that these proteins evolved to kill these and certain other types of invertebrates, such as nematodes. The advantage for bacteria bearing plasmids encoding endotoxin proteins that kill the host is that these bacteria are provided with a rich source of nutrients for vegetative growth and reproduction. Phylogenetic analyses of Cry proteins indicate most have evolved from a common ancestral protein that evolved variants over time with insecticidal activity specific for different types of insects (Crickmore et al. 1998; de Maagd et al. 2003). The overwhelming majority of Cry proteins are toxic to lepidopterous insects, suggesting that either these were the first hosts, or that the biology of

this group provided one or more traits that facilitated the greatest radiation of this protein type. As far as is known, many fewer Cry proteins are toxic to either dipterous or coleopterous species. As far as we know, comparatively few are toxic to nematodes.

A key feature of all Cry and Cyt proteins is that they are stomach poisons, and thus must be ingested to be toxic. Precisely how these proteins kill invertebrates remains a very active field of study, with the consensus being that they form cation-selective channels in the microvilli of midgut (stomach) epithelial cells, which leads to osmotic lysis of these cells and subsequent death of the host (for reviews see Schnepf et al. 1998; de Maagd et al. 2003). Key events in this process are briefly summarized here, as these are relevant to understanding the biology of crystalline inclusions formed by endotoxin proteins.

After a sensitive insect ingests a protein crystal carrying one or more Cry proteins, the crystal dissolves in the digestive midgut juices. This requires alkaline conditions for the overwhelming majority of Cry proteins, and most sensitive lepidopterous and dipterous insects have a midgut pH in the range 8–11. Exceptions to this are the Cry3 proteins, which typically dissolve under neutral or slightly acidic conditions, the typical midgut pH of their coleopteran hosts. The correlation between crystal dissolution properties and host midgut pH constitutes some of the evidence that the function of Cry proteins is to kill insects, and that they coevolved with their hosts. In addition to midgut conditions, dissolution and insecticidal activity are affected by the composition and ratios of different toxins in complex parasporal bodies (Aronson et al. 1991; Aronson 1995; Chang et al. 2001). After dissolution, Cry proteins are cleaved by midgut proteases that release the active toxin. In protoxins of 135 kDa, such as Cry1 molecules, the toxin resides in the *N*-terminal half of the molecule, whereas in protoxins of 65–80 kDa, most of the molecule is toxin. In both types, activation requires cleavage of peptides from both the *N*- and *C*-termini. Once activated, the molecules bind to specific receptors, typically glycoproteins or glycolipids, and insert in the microvillar membrane where they oligomerize forming cation-selective channels. This results in an influx of cations such as potassium, which leads to a compensating influx of water that within minutes can lead to osmotic cell lysis. Soon thereafter the target host is paralyzed, apparently by entry of alkaline midgut juice into the body cavity, which raises the blood pH and disrupts nerve conductance. The host typically dies within a day or two of initial intoxication.

The structures of several activated Cry toxins, including Cry1Aa, Cry2Aa, Cry3Aa, and Cry4Ba, have been resolved (Li et al. 1991; for reviews of Cry protein structure see Schnepf et al. 1998; de Maagd et al. 2003), and these provide important insights into the molecular basis of host specificity and insecticidal activity. On the basis of X-ray crystallographic analyses, all activated Cry1–Cry4 molecules consist of three domains: domain 1, a bundle of seven α-helices which apparently form the channel in the microvillar membrane;

domain II, a binding domain containing three β-sheets, the loops of which recognize and bind to specific sugar residue patterns on glycoprotein and/or glycolipid receptors; and domain III, a β-sandwich that provides structural support for the molecules, and also contains regions responsible for binding to midgut receptors. In addition to these classic types of Cry proteins, several others of lower mass, for example, in the range 14–50 kDa, are known that form parasporal crystals, but little is known about their biochemistry or mode of action (Crickmore et al. 1998).

Cyt proteins differ from Cry proteins in that they do not appear to require a glycoprotein or glycolipid receptor, but instead have high affinity for microvillar membrane lipids (Thomas and Ellar 1983; Li et al. 1996). These proteins are also protoxins that must be ingested and activated by proteolytic cleavage in the midgut of sensitive insects, after which they insert themselves into microvilli. Two hypotheses exist regarding their mode of action: (1) like Cry proteins, they form cation-selective channels (Li et al. 1996), and (2) they do not form channels per se, but act as detergents causing lipid faults in the microvillar membrane (Butko 2003). Regardless of their mode of action, Cyt proteins, which only occur in subspecies of *B. thuringiensis* toxic to mosquitoes such as *israelensis* and *kyushuensis*, are important as they synergize mosquitocidal Cry proteins by assisting membrane insertion, and can delay the development of resistance to these (see Park et al. 2005 for relevant literature).

Cry and Cyt proteins are the most important component of the insecticidal activity of *B. thuringiensis*, but several other proteins as well as other compounds secreted during vegetative growth can contribute to toxicity and assist these bacteria in overcoming insect species, such as larvae of the genus *Spodoptera*, which are not very sensitive to crystal proteins. Some of the more important proteins are the vegetative insecticidal proteins and various phospholipases and proteases. In addition, Zwittermicin (an antibiotic), and the β-exotoxin (an adenine analog that inhibits the mRNA, polymerase complex) are synergistic for crystal proteins. β-Exotoxin is teratogenic, and thus is not allowed in commercial formulations of *B. thuringiensis* insecticides in most countries. These proteins and other molecules are secreted during vegetative growth after spores germinate in the midgut of infected insects. Interestingly and importantly, only Cry proteins are used in insecticidal transgenic crops, showing that these alone are highly insecticidal for key target pests.

In terms of the biology of *B. thuringiensis*, an interesting question is why Cry and Cyt proteins have evolved to crystallize forming inclusions. The most probable answer is for stability, both within the cell during sporulation, and especially after cell lysis. If these proteins remained soluble after synthesis, they would be quickly degraded by cellular proteases during sporulation or after lysis, by insect proteases in dead or dying insects, or by proteases produced by other microorganisms in the environment. Stability in the environment is likely an essential property of endotoxins, as the larval stages of many insect species are only present during certain seasons

of the year. Interestingly, a similar biology evolved in the most common types of viral pathogens of insects. Environmentally resistant crystalline protein inclusions that occlude and protect virus particles occur in evolutionary unrelated types of viruses that attach insects, including baculoviruses, cytoplasmic polyhedrosis viruses, and entomopoxviruses. Like the parasporal bodies of *B. thuringiensis*, the virion-containing crystalline occlusion bodies produced by these viruses dissolve, releasing infectious virions under midgut pH conditions characteristic of their insects' hosts, typically the larval stages of lepidopterous, coleopterous, or dipterous insects (Tanada and Kaya 1993).

5
Control of Crystal Protein Synthesis and Crystal Assembly

One of the most fascinating aspects of the biology of *B. thuringiensis* is the high level of crystal protein synthesis and assembly achieved during sporulation. For most Cry and Cyt proteins, the most important genetic determinants responsible for this high level of synthesis are the strong sporulation-dependent promoters that control transcription. However, there are several other factors that play significant roles in determining the size of the parasporal inclusions that are produced. These include elements upstream and downstream of *cry* and *cyt* genes, associated accessory or "helper" proteins, which typically accompany certain *cry* genes as part of an operon for proteins of the 65–80-kDa mass type, and a factor often overlooked, the stability of the protein itself. In a simple case, such as the synthesis of Cry1Ac in the HD73 isolate of *B. thuringiensis* subsp. *kurstaki*, two promoters control the synthesis of what is a very stable protein. At the other end of the spectrum is synthesis and assembly of the parasporal body of *B. thuringiensis* subsp. *israelensis*, composed of a complex of three types held together by a reticulated envelope of unknown composition. This parasporal body contains four major proteins, with the net synthesis and size of each inclusion being controlled by one or more promoters, transcript stability, stability of each protein, and posttranslational accessory proteins. Each of these factors is discussed in general in the following subsections, with a more detailed discussion of how they affect crystal size being found under the description of different types of parasporal bodies.

5.1
Sporulation-Dependent Promoters

With the exception of Cry3 proteins, all Cry and Cyt proteins are produced during sporulation under the control of one or two strong promoters under control of mRNA polymerases directed by σ^E and σ^K factors (for reviews see

Agaisse and Lereclus 1995; Baum and Malvar 1995; Schnepf et al. 1998). During sporulation, the cell develops into two major compartments, the forespore and the mother cell (Fig. 1). Promoters that direct endotoxin synthesis operate in the mother cell, and thus this is where Cry and Cyt proteins are synthesized and assemble forming parasporal crystalline inclusions. Designation of these as σ^E and σ^K is based of their relatedness to the corresponding σ factors of *B. subtilis*, to which their relatedness is, respectively, 88 and 85% (Adams et al. 1991). These promoters are also referred to in the literature as BtI (σ^E) and BtII (σ^K), as they are not identical to the corresponding promoters of *B. subtilis*. The σ^E-dependent promoter is active during hours t_2–t_6 during the stationary phase after the end of exponential growth, whereas σ^K is active from t_5 onward. These are relative periods of promoter activity, with actual periods dependent on growth conditions and media composition (Chang et al. 2001). For most *cry1* genes, these promoters are overlapping, which modulates transcription for efficient assembly of newly synthesized molecules into the developing crystal (Sedlak et al. 2000; Chang et al. 2001). The strong activity of these promoters during sporulation has been estimated to produce as many as 1×10^6 —2×10^6 molecules of Cry protein, approximately 500 ng per milliliter of culture medium, constituting 25% of the dry weight of sporulated cells (Agaisse and Lereclus 1995). In addition to σ^E and σ^K, σ^H, a sigma factor active in the vegetative cell, directs the expression of several *cry* genes along with σ^E and/or σ^K. In cases where only a single promoter is present, transcription is usually by the σ^E polymerase.

An atypical case of parasporal crystal synthesis is found in Cry3A, where *cry3Aa* expression is under control of σ^A, a sigma factor active during vegetative growth in the predivisional cell (for reviews see Agaisse and Lereclus 1995; Baum and Malvar 1995). This results in a crystal smaller than the typical Cry1-type crystal, and even this level of synthesis requires additional transcript stabilization during translation (Agaisse and Lereclus 1996).

5.2 Stabilizing Sequences at the 5′ and 3′ Gene Regions

In the noncoding regions of many *cry* and *cyt* genes, there are DNA sequences that when transcribed increase the stability of the transcript, leading to high levels of endotoxin protein synthesis and, consequently, larger crystals. The most common of these is an inverted repeat that apparently forms stable stem–loop structures that impede the action of 3′-5′ exoribonucleases (Wong and Chang 1986). Much rarer, located in the region 600–560 bases upstream of the coding region of several *cry3A* genes, between the promoter and transcription start site, is a Shine–Dalgarno sequence (GAAAGGAGG) referred to as STAB-SD (Agaisse and Lereclus 1996). This sequence apparently stabilizes *cry3* transcripts by binding to the 3′ end of 16S ribosomal RNA, thereby

slowing the degradation of the transcripts during translation by 5′-3′ ribonucleases (Agaisse and Lereclus 1996; Schnepf et al. 1998).

5.3 Posttranslational Accessory Proteins

Most Cry1 proteins of 135–150 kDa as well as many others, but not all, crystallize readily after synthesis, forming large bipyramidal crystals. However, many of those of 65–80 kDa, but again not all, require accessory proteins for optimal net synthesis and crystal formation. The best studied of these are the 29-kDa protein encoded by *orf2* of the *cry2Aa* operon operon of *B. thuringiensis* subsp. *kurstaki* isolate HD1 and the 20-kDa protein encoded by *orf3* of the *cry11Aa* operon operon of *B. thuringiensis* subsp. *israelensis*. A variety of evidence suggests that the Cry2Aa ORF2 protein, which consists primarily of an 11-fold repeat of 15 amino acids (Widner and Whiteley 1989), serves as a template for the ordering of Cry2Aa molecules as they are synthesized (Crickmore and Ellar 1992; Ge et al. 1998; Staples et al. 2001). When *orf2* is deleted from the *cry2Aa* operon, Cry2A is synthesized and forms crystalline aggregates in the mother cell, but not the typical cuboidal crystalline inclusions characteristic of the HD1 isolate (Fig. 3). Thus, the tandem repeats that occur in the 29-kDa ORF2 protein may provide a scaffolding matrix for more efficiently organizing assembly of the Cry2A crystal (Ge et al. 1998), much like the ice nucleation proteins of *Pseudomonas fluorescens*, which also contain a series of amino acid tandem repeats that form a crystallization scaffold for water (Kajava and Lindow 1993).

The 20-kDa protein encoded by *orf3* of the *cry11A* operon has a more generalized effect. Although it only occurs in this operon, the 20-kDa protein can increase net synthesis of many different proteins, including Cry11Aa, Cry2Aa, Cry3a, Cyt1Aa, and even the *B. sphaericus* binary (Bin) endotoxin protein. The mechanism by which this protein increases synthesis is not known, but it is often referred to as a chaperone-like protein, as it appears to bind to endotoxin proteins during their synthesis. Visick and Whiteley (1991) first reported this effect on endotoxin synthesis, showing the 20-kDa protein enhanced Cyt1A synthesis in *Escherichia coli*. Later it was shown to markedly increase net synthesis in *B. thuringiensis* of Cyt1A (Wu and Federici 1993), and to be essential for Cry11A crystal formation (Fig. 4). Placing the 20-kDa protein gene under the control of *cry1Ac* promoters enhanced the synthesis and size of Cry11A crystals by 1.7-fold in comparison with crystals formed when the wild-type Cry11Aa operon was expressed. In addition, the 20-kDa protein has been shown to significantly increase net synthesis of Cry4Aa in *E. coli* (Yoshisue et al. 1992), and Cry2Aa *in B. thuringiensis* (Ge et al. 1998).

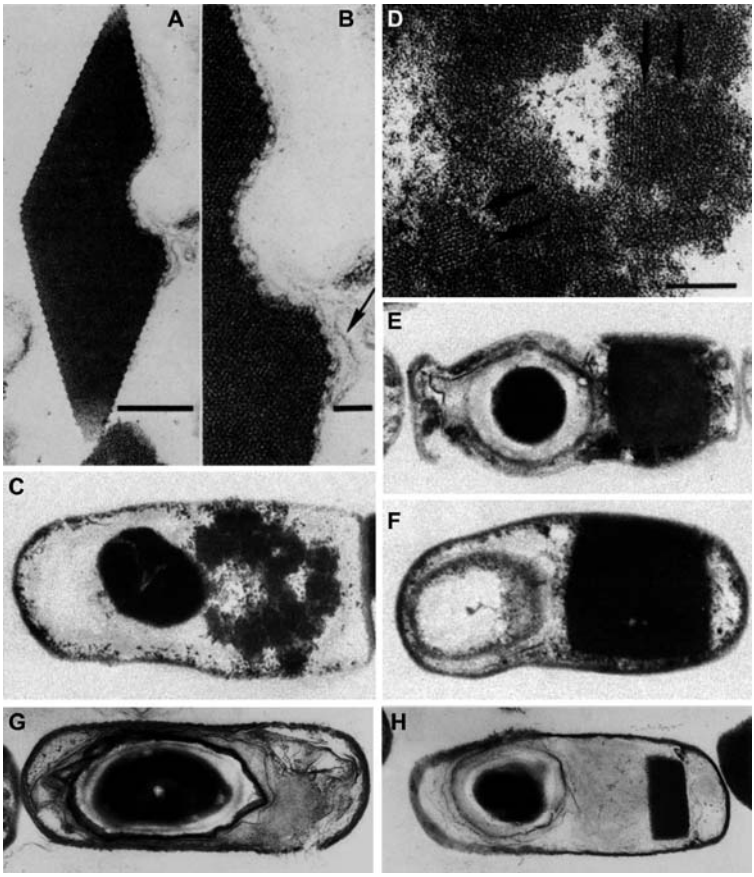

Fig. 3 Examples of the effects of accessory proteins on the synthesis and assembly of Cry2Aa and Cry11Aa. **a**, **b** Bipyramidal crystal of the HD1 isolate of *B. thuringiensis* subsp. *kurstaki* showing the site where the Cry2A molecules assemble near the short apex. The *arrow* in **b** denotes the possible 29-kDA ORF2 scaffolding protein that facilitates the assembly of Cry2A molecules at this site. **c** Recombinant strain of *B. thuringiensis* in which the *cry2A orf2* gene has been deleted from the *cry2A* operon. Note that Cry2A accumulates but does not form the cuboidal crystal typical of this protein. **d** Enlargement of the protein aggregates shown in **c**. The *arrows* denote the crystalline lattice of Cry2A molecules, indicating they crystallize even though they do not form a typical crystal. **e** Recombinant strain of *B. thuringiensis* that expresses the wild-type *cry2A* operon. Synthesis of the wild-type operon alone yields a typical cuboidal Cry2A crystal. **f**–**h** Enhancing effect of the 20-kDa protein on the net synthesis of Cry2Aa and Cry11A in recombinant strains of *B. thuringiensis*. **f** Expression of the *cry2Aa* operon in the presence of a plasmid that expresses the 20-kDa protein gene of the *cry11A* operon. Note that the Cry2A crystal is much larger than that produced by the wild-type operon shown in **e**. **g** Expression of a *cry11A* operon deletion mutant in which the *orf3* gene coding for the 20-kDa protein has been deleted. Note that no Cry11A protein accumulates. **h** Expression of the wild-type *cry11A* operon showing a typical Cry11A crystal. *Bar* in **a** 200 nm, in **b** 50 nm, in **d** 100 nm. **c**, **e**–**h** are approximately ×20 000

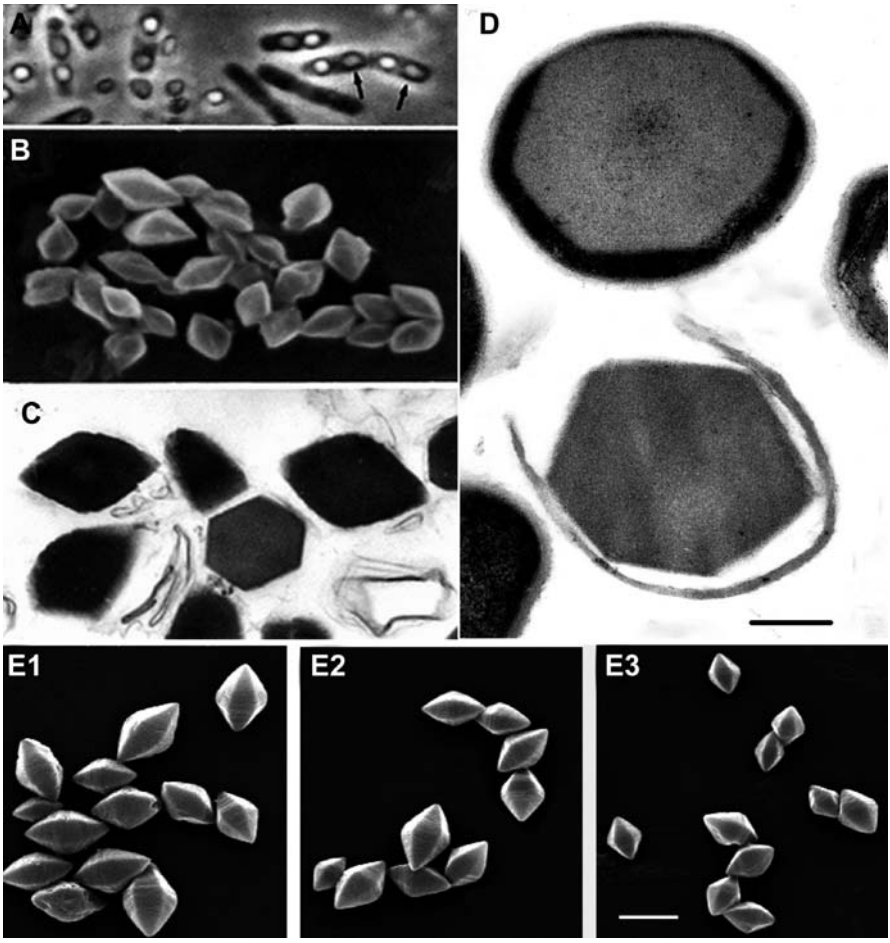

Fig. 4 Synthesis of Cyt1A in *B. thuringiensis* strain CryB. **a** Sporulated cells showing single Cyt1A crystals in sporulated cells. Synthesis was achieved using a construct that contained *cyt1A* under control of its own promoters along with the *orf3* gene encoding the 20-kDa protein gene of the *cry11A* operon under the control of the σ^E and σ^K promoters of *cry1Ac*. CryB is an acrystalliferous strain used for expression of *B. thuringiensis* genes. **b**, **c** Respectively, scanning and transmission electron micrographs of purified bihexagonal Cyt1A crystals. As in the case of Cry1 bipyramidal crystals, Cyt1A crystals average 1 μm in length when produced alone in cells. **d** Cross section through two cells bearing Cyt1A crystals, one just prior to lysis (*top*), the other at lysis (*bottom*). **e** Synthesis of Cyt1Aa crystals using different promoters to control crystal size. Purified crystals produced under the control of (**e1**) all three wild-type promoters, σ^E-, σ^K-, and σ^E-like, (**e2**) the σ^K- and σ^E-like promoters, and (**e3**) the σ^E-like promoter alone. *Bar* in **d** 150 nm, in **e** 500 nm

5.4
Cry Protein Domains Responsible for Crystallization

Unlike most proteins, an important property of Cry proteins is that they crystallize readily. Thus, most Cry molecules contain domains that facilitate their crystallization. Unfortunately, these have not been identified for most of these proteins. Cry proteins of 130–150 kDa crystallize readily in vivo without the aid of accessory proteins. Molecular and biochemical evidence indicates that the domains responsible for their crystallization are located in the *C*-terminal half of the molecule (Bietlot et al. 1990; Aronson 1993). The *C*-terminal half of the molecule is highly conserved among many Cry1-type proteins, yet this portion of the molecule is not required for toxicity, being degraded proteolytically during toxin activation. Without the *C*-terminal half of the molecule, these proteins do not crystallize (Wabiko et al. 1985; Park and Federici 2000). The *C*-terminal half of these molecules is cysteine-rich, and the disulfide bonds formed between these residues are thought to aid crystallization and crystal stability. On the other hand, Cry3 and several Cry2 proteins, which lack the *C*-terminal half characteristic of Cry1 proteins and have few cysteine residues, also crystallize readily. In these, it is thought that salt bridges assist crystallization and stability. In Cry3A, there is some evidence that at least some of the regions responsible for crystallization are located in domain 1 (Park and Federici 2000, 2004). In general, however, the peptide sequences and interactions among these that facilitate crystallization are very poorly understood.

6
Types of Parasporal Bodies and Crystal Inclusions

Owing to its economic importance as a source of insecticidal proteins, over 100 000 isolates of *B. thuringiensis* have been collected over the past 40 years by researchers in industrial, governmental, and academic laboratories worldwide. Among these, many different types of parasporal bodies are produced, some containing a single Cry protein, whereas others contain as many as 12 different proteins, including mixtures of Cry and Cyt proteins (Lecadet et al. 1999). Interest in most of these isolates has focused on identification and cloning of genes encoding proteins with novel target spectra and/or higher toxicity than that of known proteins. Comparatively little research has been devoted to study of the crystalline Cry and Cyt inclusions that compose these parasporal bodies. Nevertheless, a sufficient number of the more economically and well known subspecies and isolates have been studied to provide an overview of parasporal body diversity and their different degrees of complexity. In the following subsections, using representative examples, we begin with properties and assembly of simple paraspo-

ral bodies composed of a single crystal consisting of a single protein, and progress to those of higher complexity. For each of these, we provide some information regarding their economic relevance. For several, we also show how endotoxin synthesis can be manipulated and increased using recombinant DNA technology to construct novel combinations that yield improved bacterial insecticides.

6.1
Parasporal Body of *B. thuringiensis* subsp. *Morrisoni*, DSM 2803

The DSM 2803 isolate of *B. thuringiensis* subsp. *morrisoni*, strain tenebrionis, was originally isolated in Germany from dead pupae of *Tenebrio molitor*, a beetle species common in stored grain (Krieg et al. 1983). This isolate produces Cry3Aa (67 kDa), and interest in it was immediate once it proved insecticidal for larvae and adults of the Colorado potato beetle, *L. decemlineata*, a major pest of potatoes that had become resistant to most chemical insecticides. However, as noted in Sect. 1, this and similar isolates that produced Cry3 proteins discovered subsequently were not commercially successful owing to use of a new neonicotinoid insecticide, imidocloprid, which proved effective for long-term control of many beetle pests. Interestingly, a plant-optimized *cry3A* gene was used to construct transgenic potatoes resistant to the Colorado potato beetle. However, these also have not been commercially successful owing to both the use of imidocloprid and several fast-food chains deciding not to use transgenic potatoes for French fries owing to public perception, unwarranted as it was, that these may not be safe for human consumption. Despite this failure, genes based on Cry3 proteins have been engineered into corn varieties to control several species of corn rootworms, *Diabrotica* species, and commercial lines of this new type of Bt crop are just now entering the market.

The inclusion produced by the original and other *B. thuringiensis* Cry3 isolates is typically a small, thin rhomboidal crystal (Figs. 5, 6), even though only a single crystal is produced per cell (Adams et al. 1994; Park et al. 1998). To a large extent, the small size appears to result from the expression of *cry3* genes during vegetative growth and the stationary phases (presporulation cell), a unique property among *B. thuringiensis cry* genes. Alignment of *cry3* genes in the region surrounding nucleotide position – 129 from the translational start site for *cry3A* allowed the assignment of putative – 10 and – 35 promoter sequences for these genes, showing that they resembled promoters whose transcription is dependent on the σ^A form of RNA polymerase (Sekar et al. 1987; De-Souza et al. 1993; Agaisse and Lereclus 1994; Baum and Malvar 1995). Subsequent studies noted in Sect. 5.2 showed that the STAB-SD sequence just downstream from the 5′ end of the major *cry3A* transcript (T-129) stabilized the transcript, assisting net Cry3A synthesis (Agaisse and Lereclus 1994, 1996).

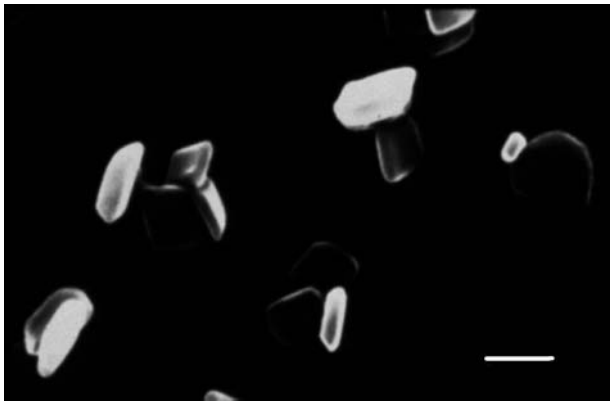

Fig. 5 Scanning electron micrograph of the thin rhomboidal crystals produced by *B. thuringiensis* subsp. *morrisoni*, strain tenebrionis, isolate DSM 2803. These crystals are toxic to beetles and are unusual in that they are produced during vegetative growth. *Bar* 1 μm

Evidence that the crystal's small size was not due to instability during and after translation comes from studies in which Cry3A synthesis was greatly improved by mutating or engineering strains for increased synthesis. For example, Tn5401 disruption of the *spoOf* gene with Tn5401 (Malvar and Baum 1994) or duplication of *cry3A* achieved by γ-irradiation (Adams et al. 1994) resulted in, respectively, increases in Cry3A yields and crystal size from threefold to eightfold. Moreover, very high yields of Cry3A were obtained by placing *cry3* including the STAB-SD mRNA-stabilizing sequence under the control of the strong *cyt1A* sporulation-dependent promoters of *B. thuringiensis* subsp. *israelensis* (Park et al. 1998). The yield of Cry3A obtained per unit medium using *cyt1A* promoters to drive *cry3A* expression without STAB-SD was substantially higher than the yield obtained with the wild-type DSM 2803 strain, but was not nearly as high as the yield obtained with the γ-irradiation mutant NB176 strain produced by Adams et al. (1994). Using the yield of Cry3A produced by the wild-type DSM 2803 strain as a standard with a value of 1, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis showed that Cry3A production by the 4Q7 (acrystalliferous) strain of *B. thuringiensis* subsp. *israelensis* containing the *cyt1A* promoters to drive *cry3A* expression but lacking the STAB-SD sequence was 2.3-fold that by DSM 2803, whereas Cry3A production by the NB176 strain was 8.8-fold that by DSM 2803. When the STAB-SD sequence was added to downstream from the *cyt1A* promoter construct used to drive expression of *cry3A*, Cry3A yield increased to 12.7-fold that produced by DSM 2803 (Fig. 6), and 1.4-fold that produced by the mutant NB176 strain (Park et al. 1998). In sporulated cultures of DSM 2803, the Cry3A inclusions were small and difficult to detect until the cells had lysed (Fig. 6a). In 4Q7 cells transformed

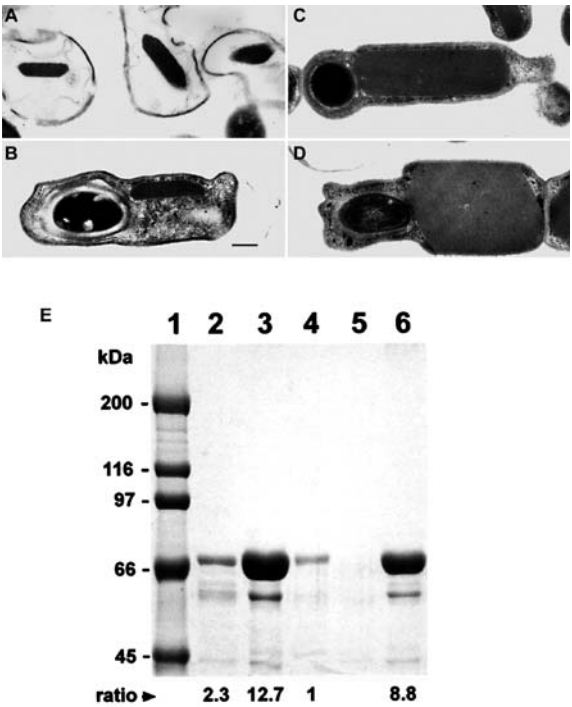

Fig. 6 Enhanced synthesis of Cry3A obtained by use of sporulation-dependent promoters and the Shine–Dalgarno (STAB-SD) messenger RNA stabilizing sequence. **a** Typical size of wild-type Cry3A crystals in sporulated cells of *B. thuringiensis* subsp. *morrisoni* strain tenebrionis. **b** Sporulated cell of *B. thuringiensis* in which expression of *cry3A* is under the control of the three sporulation-dependent promoters of *cyt1Aa* (see Fig. 2). **c**, **d** Respectively, longitudinal and cross sections through Cry3A crystals in sporulated cells of *B. thuringiensis* in which the expression of the *cry3A* gene was under the control of *cyt1A* promoters, and the transcript included the STAB-SD sequence for transcript stabilization. The combination of *cyt1A* promoters and the STAB-SD sequence yielded at least tenfold more protein per cell than wild-type DSM 2803 isolate of *B. thuringiensis* subsp. *morrisoni*. Aside from the significant increase in Cry3A yield, these results show that the small size of the crystals in the wild-type strain is due primarily to the control of expression by σ^A, not an inherent property of Cry3A. All micrographs are of the same magnification; *bar* in **b** 300 nm. Analysis of Cry3A production of wild-type, **e** mutant, and engineered strains of *B. thuringiensis* by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Sedimented crystals, spores, and cellular debris obtained from equal volumes of culture medium at the end of sporulation were loaded into each lane. *Lanes 1* molecular mass markers, *2 B. thuringiensis* 4Q7 transformed with pPFT3A (*cry3A* without the STAB-SD sequence under the control of *cyt1Aa* promoters), *3* 4Q7 transformed with pPFT3As (*cry3A* with the STAB-SD sequence under the control of *cytA* promoters), *4* wild-type *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) DSM 2803, *5* 4Q7 transformed with pHT3101, 6 NB176, the mutant strain of *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) with a higher *cry3A* copy number. The ratios at the *bottom of the lanes* were determined by densitometry scanning of the gel; they indicate the ratio of Cry3A per unit of glucose–yeast salts in comparison to that produced by the DSM 2803 strain. *Bar* in **B** = 400 nm

with the plasmid containing *cry3A* gene under the control of *cyt1A* promoters, however, a distinct Cry3A crystal was easily observed, which in cross section generally appeared larger than the crystals in DSM 2803 cells (Fig. 6b). The high level of additional Cry3A production obtained by including the STAB-SD sequence in the construct resulted in much larger Cry3A crystals than those observed in DSM 2803, NB176, or 4Q7 cells transformed with the plasmid containing *cry3A* under the control of *cyt1A* promoters (Fig. 6c, d). These large crystals were rectangular to rhomboidal and were easily observed in sporulated cells, where they occupied most of the cell. The Cry3A crystals in 4Q7 cells harboring the *cry3A* gene under the control of *cyt1A* promoters combined with the STAB-SD sequence were so large that the spore was often dislocated to the cell periphery and the cell shape was essentially distorted to take on the shape of the enclosed crystal (Fig. 6c, d).

Despite the success of these various groups in improving Cry3A yields, the lack of a significant market resulting from the implementation of imidocloprid, as noted before, has made further development of these strains uneconomic, at least for the near future.

Subsequent studies showed that the *cyt1A*/STAB-SD expression construct could be used to enhance Cry2Aa and Cry11Aa net synthesis, but only one-fold to twofold over that of the wild-type operons (Park et al. 1999). These results indicate that protein stability and other factors affect net yield of these smaller Cry proteins, not only the number and strength of promoters used to drive expression and transcript stabilizing sequences.

6.2
Parasporal Bodies of *B. thuringiensis* subsp. *kurstaki*, HD73 and HD1

These two isolates are good representatives of the range of parasporal body complexity that exists among isolates of *B. thuringiensis*, even within a single subspecies, toxic to lepidopterous insects. HD73 produces a single inclusion composed of a single protein, Cry1Ac, and HD1 two inclusions, one consisting of a single protein, Cry2Aa, the other of three proteins, Cry1Aa, Cry1Ab, and Cry1Ac.

6.2.1
B. thuringiensis subsp. *kurstaki*, HD73

Under the direction of two sporulation-dependent, overlapping promoters, σ^E and σ^K (Figs. 2, 7), this isolate produces a single large bipyramidal crystal, such as that illustrated in Fig. 7c, composed solely of Cry1Ac. Cry1Ac is of particular interest because it was one of the first proteins used to construct transgenic plants, specifically Bt cotton, resistant to insect pests. This protein is highly toxic to larvae of the tobacco budworm (*H. virescens*), a pest that has a broad crop host range, but which is particularly damaging to cotton, and the

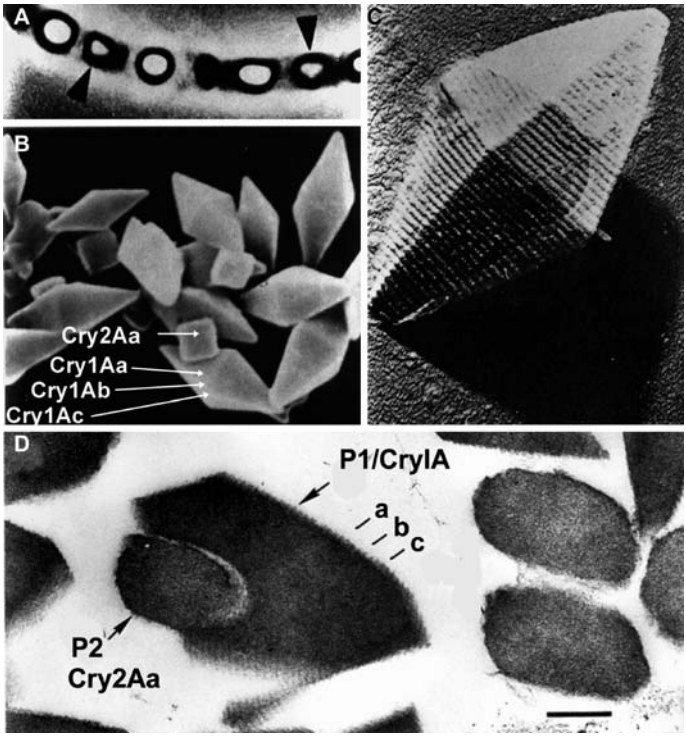

Fig. 7 Typical example of sporulated cells of *B. thuringiensis* and parasporal protein crystals. **a** Phase-contrast micrograph of cells from a sporulated culture of *B. thuringiensis* just prior to lysis. Parasporal protein crystals (*arrowheads*) lie adjacent to oval spores. **b** Scanning electron micrograph of typical Cry1 and Cry2 crystals purified from a sporulated culture of *B. thuringiensis* subsp. *kurstaki*, isolate HD1. The parasporal body of this isolate consists of a bipyramidal crystal that contains Cry1Aa, Cry1Ab, and Cry1Ac, which cocrystallize, and a separate "cuboidal" crystal composed of Cry2Aa molecules. **c** Carbon replica of a typical bipyramidal Cry1-type protein crystal exhibiting the lattice of Cry1A molecules that compose the crystal. The HD73 isolate of *B. thuringiensis* subsp. *kurstaki* only expresses a single *cry1Ac* gene, and its parasporal body contains only a single crystal, such as this one, which measures approximately 1 μm from point-to-point along the longitudinal axis. **d** Transmission electron micrograph through a parasporal body of the HD1 isolate of *B. thuringiensis* subsp. *kurstaki* illustrating the embedment of the cuboidal Cry2A crystal (*P*2) in the bipyramidal crystal (*P*1). *Bar* in **d** 200 nm. (Micrograph in **c** by Hannay and Fitz-James 1955)

pink bollworm, *Pectinophora gossypiella*, another important cotton pest (see Shelton et al. 2002 for a review). The mature bipyramidal crystal in an isolate such as HD73 typically is 1 μm in length by 500 nm at its short axis. The assembly of Cry1Ac molecules to form a crystal is a complex process in which transcription is attenuated by overlapping promoters to achieve orderly synthesis and crystallization. When the σ^K-dependent promoter is inactivated

by mutation, for example, transcription by the σ^E polymerase is increased as much as fivefold, with a corresponding increase of Cry1Ac (Sedlak et al. 2000). However, excess Cry1Ac does not crystallize readily, with most of it being degraded in the σ^K mutant prior to crystallization, yielding crystals only a quarter to a third the size of wild-type crystals.

6.2.2 *B. thuringiensis* subsp. *kurstaki* HD1

The parasporal body of this isolate is one of the most complex known of *B. thuringiensis* isolates toxic to lepidopterous insects. As noted earlier, the HD1 isolate was the first commercialized and remains the one most widely used in products used worldwide for the control of lepidopteran pest of vegetable crops, field crops, and in forestry. The Cry1Ab protein is also the source of plant-optimized genes used to construct transgenic Bt corn varieties resistant to lepidopterous pests.

The parasporal body of HD1 consists of what are now two well-known crystal types, a large bipyramidal crystal composed of Cry1Ac, Cry1Ab, and Cry1Ac, and a smaller cuboidal crystal composed of Cry2Aa (Figs. 1, 7b, d). The *cry1Ab* gene is encoded on a 44-MDa plasmid that is less stable than others, and is periodically lost from cultures. Loss of this plasmid, and thus the Cry1Ab protein, can affect both the solubility and the toxicity of *B. thuringiensis* isolates to certain insect species (Aronson et al. 1991).

Given the composition of this parasporal body, the synthesis of its multiple Cry proteins and their assembly into the two different crystals is a complex process. Synthesis of all three Cry1 proteins is directed by the same dual overlapping σ^E and σ^K promoters (Sedlak et al. 2000), whereas Cry2Aa synthesis is directed only by σ^E (Fig. 2). Synthesis and assembly of these proteins are also likely modulated by competition for amino acids, as their duel promoters operate during the same overlapping periods. The co-crystallization of all three proteins into a single bipyramidal crystal probably results from the high degree of similarity among their *C*-terminal halves, the domain considered the most responsible for crystallization of Cry1 proteins (Bietlot et al. 1990; Aronson 1993). Thus, two principal factors are responsible for the formation of the bipyramdial crystal: (1) dual overlapping promoters that control efficient simultaneous synthesis as assembly of the three Cry1A molecules, and (2) similarity among their *C*-terminal halves.

The Cry2Aa protein (65 kDa) is one of the more interesting Cry proteins with respect to insecticidal spectrum because it is toxic to both lepidopterous larvae and mosquitoes (Widner and Whiteley 1989). Synthesis and assembly of Cry2Aa to form the cuboidal inclusion differ from that of the Cry1A proteins in that its synthesis is only under the direction of a single promoter, σ^E (Fig. 2), and assembly requires the ORF2 protein encoded by the *cry2Aa* operon (Ge et al. 1998), though this protein is not required for assembly of

all Cry2 proteins. The Cry2Aa inclusion typically assembles on one side of the bipyramidal crystal at the short apex (Figs. 3a, b, 7b), with assembly at this site apparently determined by the presence of the ORF2 protein (Moar et al. 1989). Assembly there results in the partial embedment of the Cry2A crystal in the Cry1A bipyramidal crystal (Fig. 7d). Such a trait may have evolved to hold the crystals together to increase the insect spectrum of activity. This protein does not co-crystallize with the Cry1A proteins, as it lacks the large *C*-terminal domain that facilitates crystallization.

6.3 Parasporal Body of *B. thuringiensis* subsp. *israelensis*, ONR 60A

Next to the HD1 isolate of *B. thuringiensis* subsp. *kurstaki*, the ONR 60A isolate of *B. thuringiensis* subsp. *israelensis* H-14 is the most widely used bacterial insecticide in the world. Its primary use is for mosquito and blackfly control, but products are also marketed for other types of nematocerous fly larvae, such as mushroom fly larvae. Unfortunately, *B. thuringiensis* subsp. *israelensis* is not effective for house fly, horse fly, or fruit fly control.

The parasporal body of this isolate, and of most other isolates of this subspecies, is unusual in that it is spherical and the inclusions within it are individually enveloped in a multilamellar fibrous envelope (Ibarra and Federici 1986a), additional layers of which hold the different crystal inclusions together (Fig. 8). The Cry and Cyt toxins as well as other proteins involved in production of this complex body are encoded on a 127.9-kb plasmid, which codes more than 100 potential proteins (Berry et al. 2002). Many of these proteins, even if functional, are probably not required for endotoxin synthesis and inclusion assembly. However, the complexity of the parasporal body and its process of assembly suggest a higher order of regulation than occurs for most Cry proteins, making this plasmid unique among toxin-encoding plasmids of *B. thuringiensis*.

6.3.1 Control of Endotoxin Gene Expression in *B. thuringiensis* subsp. *israelensis*

Gene expression in *B. thuringiensis* subsp. *israelensis* differs from that of *cry1* genes in that none of the promoters are overlapping. The same types of sporulation-dependent promoters, however, are responsible for expression, with the majority of transcription of all four major endotoxin genes being directed by σ^E and σ^K factors (Fig. 2). In addition, σ^H, active during vegetative growth, contributes to *cry4A* and *cry11A* expression (Yoshisue et al. 1993, 1995), as does the atypical third σ^E-like promoter of *cyt1Aa*. The *cry4Aa*, *cry4Ab*, and *cyt1Aa* genes are monocistronic, whereas *cry11Aa* is *orf2* (Fig. 2), flanked by *orf1* and *orf3* of a three-gene operon (Dervyn et al. 1995).

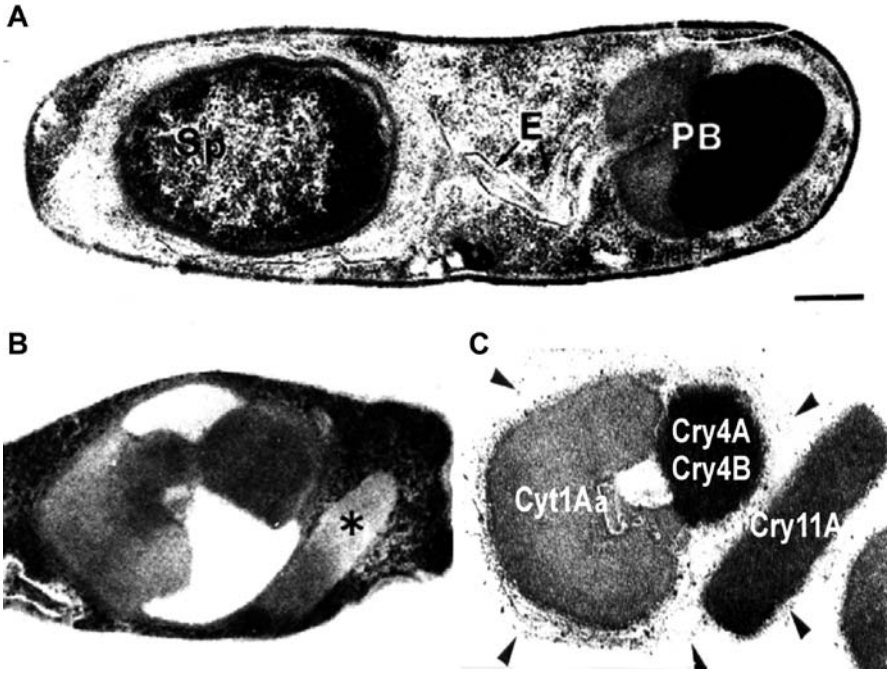

Fig. 8 Transmission electron micrographs of a sporulating cell of *B. thuringiensis* subsp. *israelensis* and parasporal bodies characteristic of this subspecies. **a** Sporulating cell illustrating the developing spore (*Sp*) and parasporal body (*PB*). The parasporal body, composed primarily of Cry4A, Cry4B, Cry11A, and Cyt1A proteins, is assembled outside the exosporium membrane (*E*). **b** Portion of sporulating cell just prior to lysis. The Cry11A crystal (*asterisk*) lies adjacent to the Cyt1A and Cry4A and Cry4B inclusions. **c** Purified parasproal body showing the components of the parasporal body. In this subspecies, the individual protein inclusions are enveloped in a multilamellar fibrous matrix (*arrowheads*) of unknown composition, which also surrounds the crystals holding them together. A typical mature parasporal body of this subspecies measures 500–700 nm in diameter. *Bar* in **a** 100 nm

6.3.2 Cyt1Aa Synthesis and Inclusion Formation

The novelty of Cyt1A when first discovered attracted many researchers to study its properties and mode of action, as it differed so much from Cry endotoxins, the only known type of *B. thuringiensis* endotoxin known as of the late 1970s. This toxin was fairly easy to purify from solubilized parasporal bodies, and it was demonstrated that its primary affinity was for the lipid portion of cell membranes (Thomas and Ellar 1983). However, efforts to express the cloned gene were largely unsuccessful until it was determined that the 20-kDa ORF3 protein encoded by the *cry11A* operon aided synthesis of Cyt1A (Visick and Whiteley 1991). By including the 20-kDa protein gene expressed under

the control of *cry1Ac* promoters in *cyt1A* expression plasmids, it became possible to produce Cyt1A alone in large quantities (Wu and Federici 1993). When these constructs were transformed into acrystalliferous *B. thuringiensis* strain, each cell produced a large crystal of Cyt1A, comparable in size to crystals of Cry1 proteins (Fig. 4). By light microscopy, the Cyt1A crystals appeared bipyramidal, but scanning and electron microscopy revealed each crystal consisted of 12 facets, making them bihexamidal (Fig. 4c,d).

Expression of *cyt1A* is directed by three sporulation-dependent promoters, making it unusual among endotoxin genes (Fig. 2). This is one of the factors that contributes to the large amount of Cyt1A synthesized per cell. When only one or two promoters are used for expression, the resulting Cyt1A crystals are smaller, successively, than when all three are used for expression (Fig. 4e). Interestingly, in light of the evidence that promoters controlling expression of *cry1A* genes are overlapping to ensure efficient Cry crystal assembly (Sedlak et al. 2000; Chang et al. 2001), promoters controlling *cyt1A* expression are not overlapping (Waalwijk et al. 1985; Ward et al. 1986). This implies that Cyt1A is stable and does not have a high turnover rate, perhaps because at 28 kDa it is of much lower mass than Cry1 proteins.

The role of the 20-kDa protein in Cyt1A synthesis has not been determined, although it has been suggested that it acts like a chaperone, and by binding to this protein during synthesis prevents it from disrupting the inner membrane of bacterial cells.

6.3.3
Cry11Aa Synthesis and Inclusion Formation

The synthesis and development of Cry11Aa crystals are under the control of a three-gene operon, the expression of which is directed by three promoters (Fig. 2). Of the genes in the operon, only the *cry11A* gene (*orf2*) and the 20-kDa protein gene (*orf3*) appear to be essential for Cry11A crystal formation (Dervyn et al. 1995). Whether synthesized in the wild-type isolate of *B. thuringiensis* or alone, in transmission electron microscopy, the Cry11A crystal appears "bar-shaped" (Figs. 3h, 8, 9a–c). Scanning electron microscopy, however, shows the crystal is actually bitrapezoidal in shape (Fig. 9d, e), and measures about 900 nm in length, by 600 nm across, by 170 nm in thickness.

The role of the 19-kDa protein (*orf1*) is not clear, but as in the case of Cyt1A, the 20-kDa protein appears to act like a chaperone, facilitating net synthesis and formation of Cry11A crystals (Fig. 3g, h). When the 20-kDa protein gene was eliminated from expression constructs, no obvious Cry11A crystals were observed in cells (Wu and Federici 1995).

Of the four major proteins the parasporal body of *B. thuringiensis* subsp. *israelensis*, the Cry11A protein is the most toxic to the widest range of mosquito species; thus, increasing the size of the crystals and net synthesis

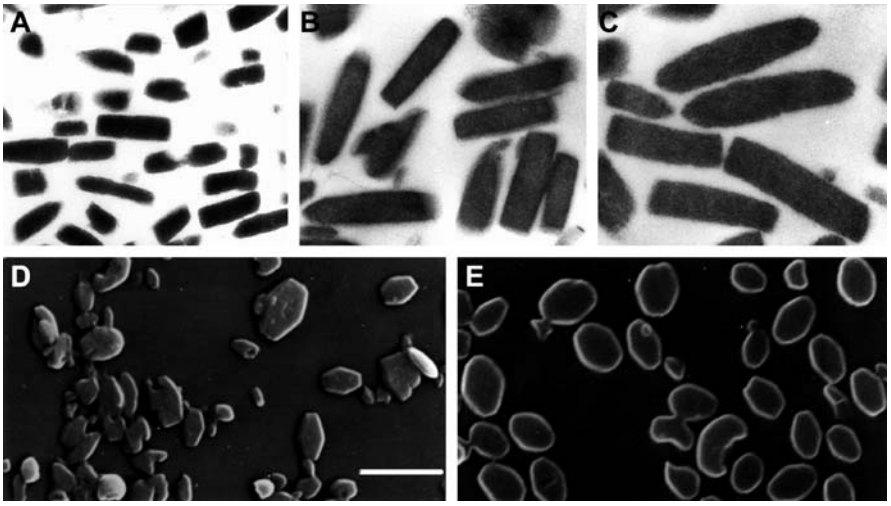

Fig. 9 Effect of the 20-kDa protein encoded by the *cry11A* operon on Cry11A synthesis. **a–c** Transmission electron micrographs through purified **a** wild-type crystals, **b** crystals produced by expression of the wild-type operon alone, and **c** crystals produced by a construct in which expression is under the control of wild-type promoters (σ^E, σ^H, and σ^K), with *orf3* expression directed by *cry1Ac* promoters (σ^E and σ^K). **d, e** Scanning electron micrographs of the thin bitrapizoidal Cry11A crystals purified from **d** wild-type *B. thuringiensis* subsp. *israelensis* cells, and **e** cells in which Cry11A synthesis is driven by wild-type promoters and 20-kDa protein synthesis by *cry1Ac* promoters. *Bar* in **d** 1 μm

of Cry11A is of interest. However, unlike Cry3A, efforts to enhance synthesis using *cyt1A* promoters and the STAB-SD sequence met with only limited success, with increases in the range of 1.5-fold (Park et al. 1999).

6.3.4 Cry4A and Cry4B Synthesis and Inclusion Formation

Although these proteins contribute to the broad mosquitocidal spectrum of *B. thuringiensis* subsp. *israelensis*, they represent only 10–15 percent of the parasporal body mass. Expression of *cry4A* is directed by three promoters (Fig. 2), whereas *cry4B* expression is directed by two (Yoshisue et al. 1993, 1995). In both cases one of these is σ^H, active during vegetative growth. Both proteins are less stable than typical Cry proteins and much more difficult to synthesize in the absence of each other, especially Cry4A (Delecluse et al. 1993), or the 20-kDa protein. They apparently co-crystallize together forming the dense semispherical-to-spherical inclusion characteristic of the parasporal body of this subspecies (Fig. 8).

Although speculative, it appears that the multiple promoters, including σ^H active during vegetative growth, and the 20-kDa protein, all which contribute

to Cry11A and Cry4A synthesis, evolved to facilitate of the synthesis and crystallization of less stable proteins. These features may have been essential to form protein conformations that are toxic and specific to larvae of mosquitoes and related species.

6.4
Parasporal Body of *B. thuringiensis* subsp. *morrisoni*, PG-14

This isolate is similar to *B. thuringiensis* subsp. *israelensis* ONR 60A, but differs in that the spherical parasporal inclusion body (Fig. 10) also contains

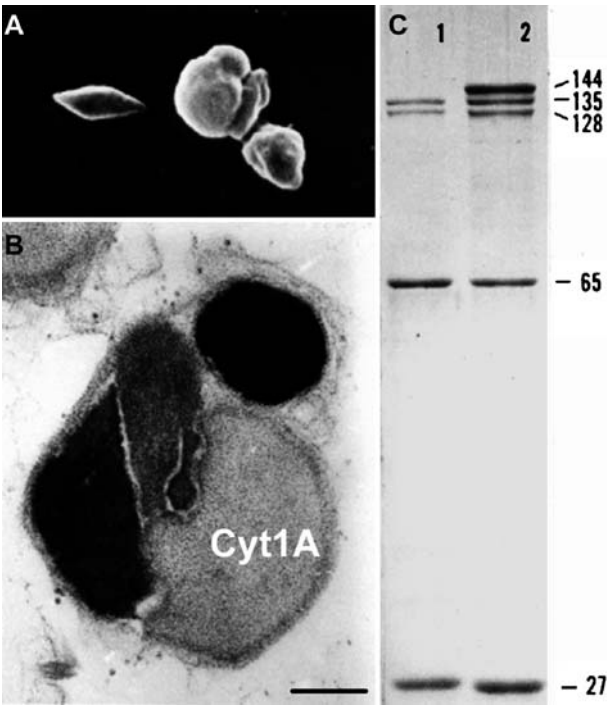

Fig. 10 Parasporal bodies characteristic of the PG-14 isolate of *B. thuringiensis* subsp. *morrisoni*. This parasporal body of this isolate contains the separate crystal inclusions composed of Cry4A and Cry4B, Cry11A, and Cyt1A characteristic of *B. thuringiensis* subsp. *israelensis*, and in addition a Cry1-type bipyramidal crystal composed of a 144-kDa protein toxic to lepidopterous insects. **a** Scanning electron micrograph illustrating the spherical parasporal body and a bipyramidal crystal. **b** Transmission electron micrograph of the parasporal body showing the various protein crystals held together by the surrounding multilamellar fibrous envelope. **c** Polyacrylamide gel comparing the protein composition of (*lane 1*) *B. thuringiensis* subsp. *israelensis* with that of (*lane 2*) the PG-14 isolate of *B. thuringiensis* subsp. *morrisoni*. Note the additional Cry1 protein of 144 kDa in the PG-14 isolate. *Bar* in **b** 150 nm

a 144-kDa Cry1 protein that produces a bipyramidal crystal (Ibarra and Federici 1986b). Plasmid deletion mutants of this strain have shown that Cry4A, Cry4B, Cry11A, and Cyt1A are encoded on one plasmid, apparently similar to the 127.9-kb pBtoxis plasmid of *B. thuringiensis* subsp. *israelensis*, and the 144-kDa protein on a different plasmid (Padua and Federici 1990). That the 144-kDa crystal develops and is occluded inside the parasporal body along with the Cyt and Cry crystals suggests that the molecular determinants responsible for directing the trafficking and envelopment of these proteins are encoded by pBtoxis.

7
Genetic Recombination of Parasporal Inclusions to Improve Toxicity

The various Cry and Cyt proteins and genetic elements identified that control and affect their synthesis provided opportunities to develop new combinations of different types of inclusions to generate improved insecticides. In the following subsections, we describe a few of these, with emphasis on those in which the parasporal inclusions are prominent.

7.1
Combinations of Cry3A and Cyt1A Crystals for Coleopterous Insects

Because both Cry3A and Cyt1A are toxic for certain beetle species (Federici and Bauer 1998), an obvious strain to engineer would be one that produced both proteins. To do this, the pWF43 plasmid (Wu and Federici 1993) used to produce Cyt1A alone was transformed into the DSM 2803 isolate of *B. thuringiensis* subsp. *morrisoni* strain tenebrionis. Because expression of *cry3A* is under the control of the σ^A polymerase and that of *cyt1A* is under the control of σ^E and σ^K in this recombinant strain, Cry3A was synthesized during vegetative growth and Cyt1A during sporulation. As a result of the temporal difference in the synthesis of Cry3A and Cyt1A, the Cry3A crystal synthesized first wound up embedded in the Cyt1A crystal (Fig. 11). Whether this association of the crystals has any applied value is unknown, as the strain has not been evaluated for commercial development owing to the lack of a commercial market for bacterial insecticides for beetle control. Nevertheless, it provides an example of the types of engineering "tricks" that can be used to manipulate parasporal body composition.

7.2
Combinations of *B. thuringiensis* and *B. sphaericus* Inclusions for Mosquitoes

Two insecticidal bacteria are currently used in commercial formulations to control mosquito larvae, *B. thuringiensis* subsp. *israelensis* and *B. sphaericus*.

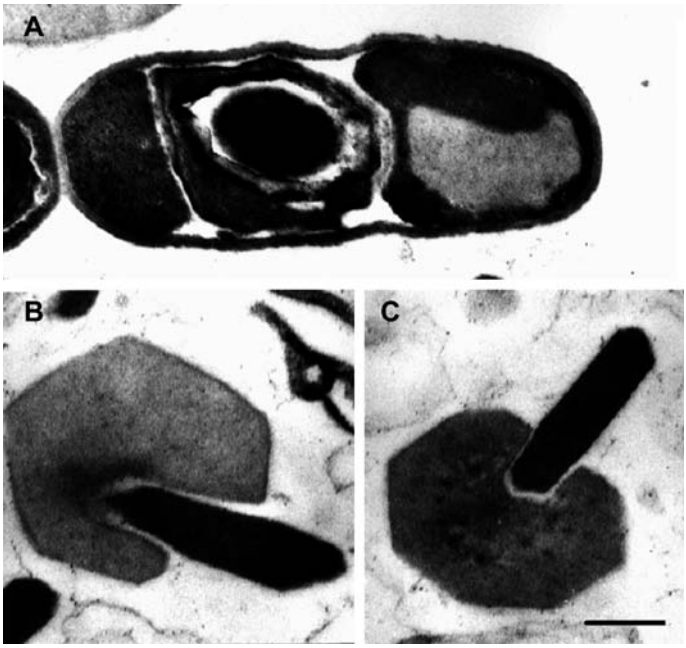

Fig. 11 Recombinant strain of *B. thuringiensis* subsp. *morrisoni* strain tenebrionis that produces Cyt1A. Cry3A is under the control of σ^A, a sigma factor active during vegetative growth, whereas Cyt1A is under the control of its sporulation-dependent promoters; thus, Cry3A is synthesized first, and Cyt1A subsequently. This results in the crystallization of Cyt1A around the already formed crystal of Cry3A. **a–c** Transmission electron micrographs of **a** sporulated cells showing parasporal crystals of Cry3A (*bar shape*) and Cyt1A, and **b**, **c** purified parasporal bodies showing Cry3A crystals partially embedded in Cyt1A crystals. *Bar* in **c** 150 nm

The principal toxin of the latter species is a binary toxin composed of two proteins, one is the binding domain and the other the toxin (Baumann et al. 1991). In cases where *B. sphaericus* has been used intensively for mosquito control, high levels of mosquito resistance have developed (Yuan et al. 2000), as it acts as a single toxin, whereas little if any resistance has evolved to the multitoxin complex of *B. thuringiensis* subsp. *israelensis*. Laboratory studies have shown that Cyt1A of *B. thuringiensis* subsp. *israelensis* can both delay and overcome resistance to *B. sphaericus* (Wirth et al. 2000), suggesting that recombinant bacteria which combined the endotoxins of these two species would result in improved bacterial insecticides for mosquito control. Later, after more details about the *B. sphaericus* binary toxin, we provide an example of how recombinant techniques were used to combine the endotoxin inclusions of these two species to produce an improved bacterial insecticide for mosquito control.

7.2.1
Basic Biology of the *B. sphaericus* Bin Toxin

Attempts to find mosquitocidal bacteria have yielded numerous toxic isolates of *B. sphaericus*, but until 1983, none were as toxic as *B. thuringiensis* subsp. *israelensis*. In that year, Weiser (1984) collected an isolate of *B. sphaericus*, known as 2362, in Nigeria, that is very toxic to larvae of many species of the genus *Culex*, as well as certain species of aedine and anopheline mosquitoes. Ironically, 2362, which was isolated from an adult blackfly, is not toxic to larvae of this medically important group of insects. The efficacy of 2362 is sufficiently high that a product, named VectoLex, based on this isolate entered the market for mosquito control in 2000. An apparent advantage of *B. sphaericus* 2362 is that it has higher residual activity than *B. thuringiensis* subsp. *israelensis*.

The mosquitocidal activity of *B. sphaericus* is due to several protein toxins produced during vegetative growth and sporulation. The most important toxin is the Bin toxin synthesized during sporulation (Baumann et al. 1991; Berry et al. 1993; Humphreys and Berry 1998). All high-toxicity strains of *B. sphaericus* produce Bin toxin. This toxin is referred to as binary because it is composed of two separate proteins, BinA, a 42-kDa toxin domain, and BinB, a 51-kDa binding domain (for reviews see Charles et al. 1996; Charles and Nielsen-LeRoux 2000). During alkaline extraction, both are activated by proteolytic cleavage yielding proteins of, respectively, 39 and 43 kDa (Baumann et al. 1991). Bin proteins cocrystallize, and have a strong affinity for one another, making purification of one protein in the presence of the other difficult. In vivo, the Bin crystal, small and quasi-cuboidal in shape, forms during stage III of sporulation on the internal surface of the exosporium membrane (Fig. 12a). Thus, unlike the parasporal body of *B. thuringiensis* subsp. *israelensis*, Bin toxin remains associated with the spore after cell lysis (Yousten and Davidson 1982; Broadwell and Baumann 1986; Baumann et al. 1991).

Like *B. thuringiensis* subsp. *israelensis*, *B. sphaericus* 2362 acts as a stomach poison and thus must be ingested to be toxic. The activated toxin binds to the microvillar membrane of midgut epithelial cells, after which it is internalized inside the cell, causing midgut lysis and host death 24–48 h after initial intoxication (Baumann et al. 1991).

7.2.2
Increasing Yield of the *B. sphaericus* Bin Toxin

As noted earlier, the parasporal crystals produced by *B. sphaericus* are small compared with those typically produced by *B. thuringiensis*. This suggested that it might be possible to increase the size and yield of this toxin using *cyt1A* promoters and the STAB-SD sequence, as was done for the Cry3A

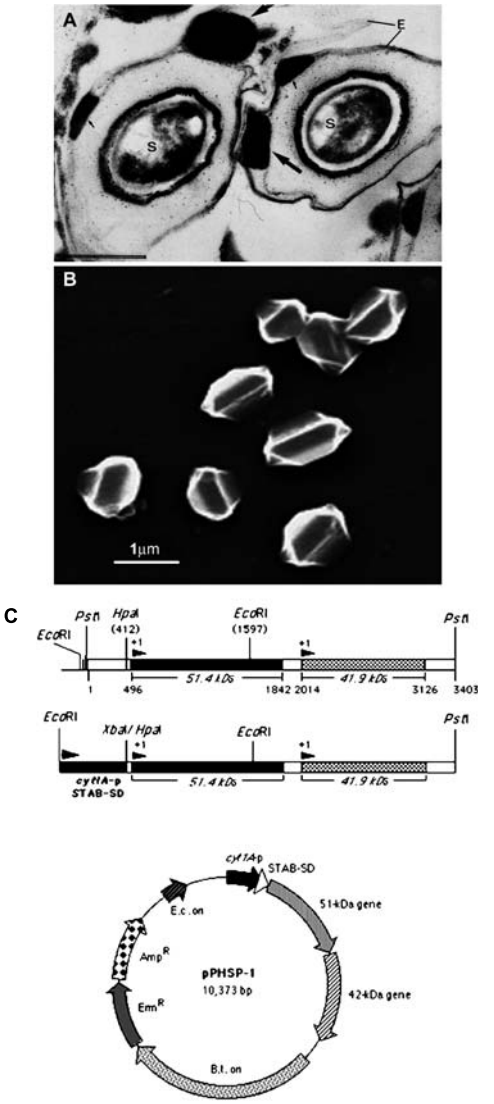

Fig. 12 *B. sphaericus* and recombinant crystals of its binary toxin produced in *B. thuringiensis*. **a** Sporulated cells of wild type *B. sphaericus* cells showing *B. sphaericus* binary (*Bin*) crystals (*arrows*) adhering to the inside of the exosporium membrane. **b** Scanning electron micrograph of purified crystals of the *B. sphaericus* binary toxin produced in *B. thuringiensis* using *cyt1A* promoters and the STAB-SD transcript stabilizing sequence using the construct illustrated in **c**. **c** Arrangement of the wild-type *bin* operon (*top*) and the engineered operon (*middle*) with control of expression under *cyt1A* promoters and translation stabilized with the STAB-SD sequence. The final plasmid, pPHSP-1 (*bottom*) was used to transform various crystalliferous and acrystalliferous strains of *B. thuringiensis* to overproduce the *B. sphaericus* Bin toxin and improve their mosquitocidal toxicity. (**a** Courtesy of J.-F. Charles, Institut Pasteur, Paris, France)

toxin. The plasmid pPHSP-1 was constructed by placing the *bin* operon under control of the *cyt1A* promoters and STAB-SD (Fig. 12), and was then transformed in to acrystalliferous cells of the 4Q7 strain of *B. thuringiensis*. This construct/strain combination yielded large crystals of the binary toxin, one per cell produced outside the exosporium membrane (Fig. 12b). Measurements on scanning electron micrographs showed mean dimensions for these Bin crystals of 0.80-μm × 0.80-μm wide by 1.0-μm long, yielding a volume of 0.64 μm^3. Crystals produced in wild-type *B. sphaericus* 2362 averaged 0.42-μm × 0.42-μm wide by 0.58-μm long, yielding a volume of 0.10 μm^3. This resulted in an approximate size increase of more than sixfold over the wild type for crystals produced using the *cyt1A*-p/STAB-SD expression system in *B. thuringiensis* 4Q7 (Park et al. 2005).

7.2.3 Combinations of the *B. sphaericus* Bin Toxin and Cry and Cyt Toxins

The development of highly effective recombinant *B. thuringiensis* subsp. *israelensis* strains with novel combinations of toxins is of considerable interest owing to their potential utility in mosquito control. An example of such a recombinant is a *B. thuringiensis* strain that synthesizes the *B. sphaericus* Bin toxin, Cyt1A and Cry11B (Park et al. 2003). This strain is significantly more toxic to *Culex quinquefasciatus* than either *B. thuringiensis* subsp. *israelensis* IPS-82 or *B. sphaericus* 2362.

To construct this strain, two plasmids were used for expression of toxin genes in *B. thuringiensis* subsp. *israelensis* 4Q7 (Fig. 13a, b). The first, p45S1, contained *bin*, *cyt1A*, and an erythromycin resistance gene (*erm*) and the second, pPFT11Bs-CRP, contained *cry11B* and a chloramphenicol resistance gene (*chl*). The results showed a high level of endotoxin production in strains transformed with p45S1, pPFT11Bs-CRP, or with both plasmids (Fig. 13c, d). In this strain, the amount of Cyt1A produced by 4Q7 containing both p45S1 and pPFT11Bs-CRP decreased by 50% compared with that of 4Q7 containing only p45S1, whereas no significant differences in yield were observed for Bin and Cry11B. Bioassays showed that the combination of Cry11B, Cyt1A, and Bin is significantly more toxic to *Cx. quinquefasciatus* larvae, but not to those of *Aedes aegypti*, than the wild-type strain of *B. thuringiensis* subsp. *israelensis* or *B. sphaericus* 2362 that serve as the active ingredients in commercial bacterial larvicides used for mosquito control. This strategy could be useful for generating individual *B. thuringiensis* strains that produce various combinations of insecticidal proteins to assess their potential synergistic or antagonistic interactions.

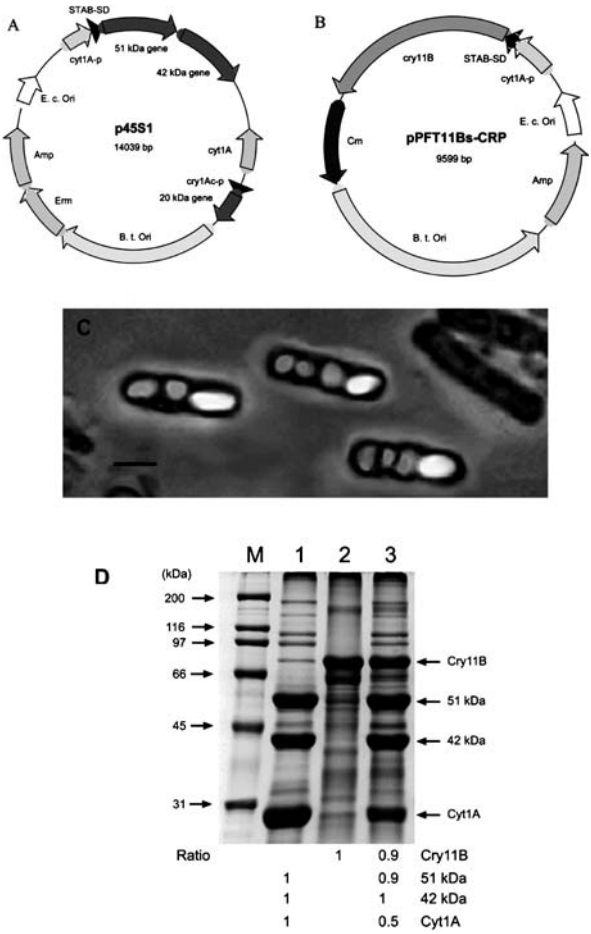

Fig. 13 Construction de novo of a unique mosquitocidal strain of *B. thuringiensis* that produces individual endotoxin crystals of the *B. sphaericus* Bin toxin, Cyt1A, and Cry11B. **a** Vector bearing the *cyt1A* and *B. sphaericus bin* genes. **b** Vector bearing the *cry11B* gene from *B. thuringiensis* subsp. *jegathesan*. These were transformed successively into the 4Q7 acrystalliferous strain of *B. thuringiensis* subsp. *israelensis* to yield the final recombinant strain that produces *B. sphaericus* Bin, Cyt1A, and Cry11B. **c** Phase-contrast micrograph of sporulated recombinant cells of *B. thuringiensis* 4Q7 containing individual crystals of Cyt1A, Cry11B, and the *B. sphaericus* Bin toxin. The spore is on the *right* in each of these cells. **d** Analysis of endotoxin content in wild-type and recombinant strains of *B. thuringiensis*. *M* molecular size marker, *lane 1 B. thuringiensis* subsp. *israelensis* 4Q7 producing *B. sphaericus* Bin toxin and Cyt1A (4Q7/p45S1), *lane 2 B. thuringiensis* subsp. *israelensis* 4Q7 producing Cry11B (4Q7/pPFT11Bs-CRP), *lane 3 B. thuringiensis* subsp. *israelensis* 4Q7 producing Cry11B, Cyt1A, and *B. sphaericus* Bin toxin (4Q7/p45S1-11B). The numbers at the base of lane 3 indicate the approximate ratio of each toxin produced in the Cry11B, Cyt1A, Bin recombinant in comparison to, respectively, the Cyt1A plus Bin recombinant (lane 1) and the Cry11B recombinant (lane 2). Equal amounts of culture medium were loaded in each well. *Bar* in **c** 1 μm

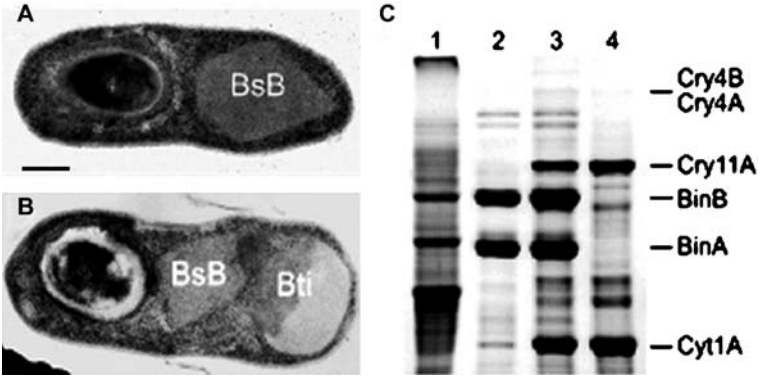

Fig. 14 Recombinant cells of *B. thuringiensis* subsp. *israelensis*. **a** An acrystalliferous strain (4Q7) containing a single crystal of the *B. sphaericus* 2362 Bin toxin produced using the plasmid pPHSP-1 (Fig. 12), which produces the Bin toxin using *cyt1A* promoters and the STAB-SD sequence. **b** The IPS-82 strain of *B. thuringiensis* subsp. *israelensis* transformed with plasmid pPHSP-1. This strain produces the *B. thuringiensis* subsp. *israelensis* parasporal body and the *B. sphaericus* Bin toxin. **c** Comparative endotoxin yields produced per unit medium by wild-type and recombinant strains. *Lane 1* wild type control strain of *B. sphaericus* 2362, *lane 2* recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 strain transformed with pPHSP-1 that produces the *B. sphaericus* Bin toxin, *lane 3* recombinant *B. thuringiensis* subsp. *israelensis* IPS-82 strain transformed with pPHSP-1—this strain produces the full complement of *B. thuringiensis* subsp. *israelensis* toxins plus *B. sphaericus* Bin, *lane 4* wild-type *B. thuringiensis* subsp. *israelensis* IPS-82 control strain. *Bar* in **a** 300 nm

7.2.4 Synthesis of *B. sphaericus* Bin Toxin in *B. thuringiensis* subsp. *israelensis*

When pPHSP-1 was used to synthesize the *B. sphaericus* 2362 Bin protein in *B. thuringiensis* subsp. *israelensis* IPS-82 along with the normal complement of *B. thuringiensis* subsp. *israelensis* proteins, this strain yielded *B. sphaericus* 2362 Bin crystals that averaged 0.77-μm × 0.77-μm wide by 1.0-μm long, yielding a volume of 0.59 μm^3 (Fig. 14). These crystals were not significantly different in size from those produced by the acrystalliferous 4Q7 strain (Fig. 12). Moreover, synthesis of the *B. sphaericus* 2362 toxin in *B. thuringiensis* subsp. *israelensis* 4Q7 and IPS-82 demonstrated a substantial increase in toxin yield per unit medium as assessed by scanning densitometry (Fig. 14). The wild-type *B. sphaericus* 2362 strain produced approximately 100 μg/ml of the 42- and 51-kDa binary toxin proteins, whereas the yield of these proteins in 4Q7 was 223 μg/ml, that of IPS-82 was 302 μg/ml (Park et al. 2005). Both recombinant strains of 4Q7 and IPS-82 were much more toxic than either wild-type *B. thuringiensis* subsp. *israelensis* or *B. sphaericus* to several species of mosquitoes, including *Cx. quinquefasciatus* and *Cx. tarsalis*, vectors of West Nile Virus.

8
Other Types of Inclusions Produced by *B. thuringiensis*

Our focus has been on the parasporal insecticidal crystals of *B. thuringiensis*, but it is worth noting that the search for novel insecticidal isolates of this species has resulted in the discovery of many noninsecticidal strains that produce crystal proteins with other potentially useful properties. For example, a new class of proteins known as parasporins is under study for their possible use as cytolytic agents for human cancer cells (Ohba 1996; Katayama et al. 2005). Like endotoxin proteins, parasporins are synthesized during sporulation and crystallize forming parasporal inclusions (Fig. 15). Pre-parasporin molecules are released upon proteolytic cleavage with trypsin. Several of these, such as parasporin-1, and heterodimer composed of 15- and 56-kDa fragments, are under evaluation against human cancer cells in vitro (Katayama et al. 2005).

In addition to these, there are many other uncharacterized parasporal inclusions produced by *B. thuringiensis*, with an example of one shown in Fig. 15b. Some of these may be insecticidal or toxic to other animals that they have not been tested against. That a *B. thuringiensis* strain would invest so much energy and other resources into the synthesis of these inclusions suggests that they have a function. It is our task to determine just what the function is!

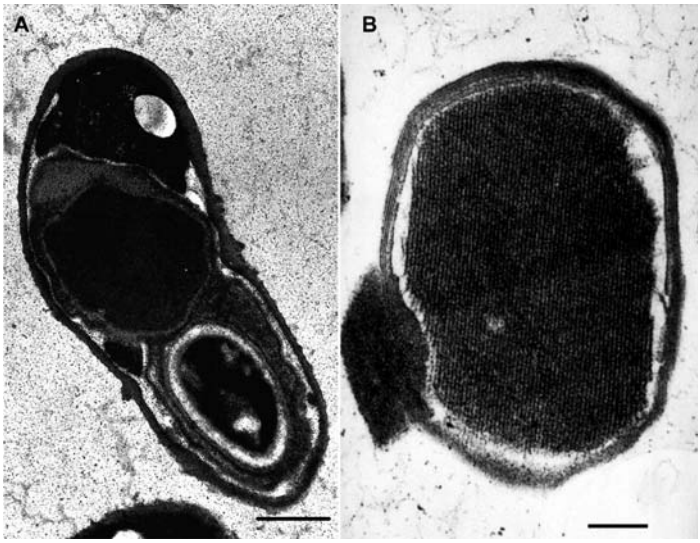

Fig. 15 Atypical parasporal bodies produced by *B. thuringiensis*. **a** Isolate from mulberry leaves in Japan that produces parasporal proteins, which when cleaved with trypsin yield peptides cytolytic for human cancer cells. This particular strain is the source of parasporin-1, a heterodimer of 15- and 56-kDa proteins. **b** A novel uncharacterized parasporal body from a soil isolate of *B. thuringiensis*. *Bar* in **a** 250 nm, in **b** 100 nm. (**a** Courtesy of M. Ohba, Kyushu University, Fukuoka, Japan)

9 Future Research

The impetus for most research on parasporal inclusions of *B. thuringiensis* over the past 30 years was the possibility of finding isolates with new insecticidal proteins. Several small biotechnology companies were created to meet this challenge in the 1980s, and these were successful for a while. However, the development of transgenic Bt crops, especially Bt cotton and Bt corn, which targeted the same commodity markets for pest control, led to the failure of most of these companies, or their acquisition by larger agrochemical companies that moved into the transgenic crop business. Over the past decade, this has shifted most research away from *B. thuringiensis* per se and onto the studies of the mechanism of action and safety of Cry proteins produced by transgenic crops to nontarget organisms, including humans and other nontarget vertebrates, invertebrates, and soil microflora and microfauna. Economics always drives the level of research in specific areas, and while it is unlikely that there will be a major focus on the inclusions produced by *B. thuringeinsis*, studies on improving endotoxin synthesis have the potential to yield new insecticides, such as the *B. thuringiensis*/*B. sphaericus* recombinants described herein that were recently developed for control of nuisance and vector mosquitoes. Another interesting aspect of endotoxin inclusion biology, which has received relatively little study, is the mechanisms underlying crystallization. These molecules have evolved to crystallize, and thus this presents the possibility that if the underlying mechanisms were understood, domains of endotoxins proteins might be useful for crystallizing proteins that are difficult to crystallize. Lastly, new types of activities, such as the cytolytic activity of parasporins for cancer cells, indicate the "noninsecticidal" inclusions produced by *B. thuringiensis* may have other potentially important uses, or may eventually be found to have other pesticidal properties.

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Protein Inclusion Bodies in Recombinant Bacteria

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Abstract Fast and high-level expression of heterologous proteins in bacterial hosts often results in the accumulation of almost pure aggregates (inclusion bodies, IBs) of the target protein. Although knowledge of the pathways and influential factors of protein folding *in vivo* has increased for many years, the complexity of the cellular networks does not allow easily the prediction of favourable conditions for production of correctly folded proteins. Therefore, IB-based production is still a potential and straightforward strategy for the production of complex recombinant proteins. IB-based processes combine the advantages of a high concentration of the target protein produced in well-characterized bacteria such as *Escherichia coli*, efficient protocols for IB isolation, purification and *in vitro* protein refolding without the need of elaborate coexpression systems and time-consuming trial-and-error expression optimization. Recent advances in understanding the molecular physiology of IB formation and in resolubilization enable a streamlined development of fermentation processes to obtain a high-quality product. In addition, simple strategies have been established to improve the purification and renaturation of disulfide bond containing proteins allowing for a fast transfer of those processes to industrial production scale.

1 Introduction

Thousands of DNA sequences from various sources have been expressed in bacteria such as in *Escherichia coli* and many of the target proteins have been obtained in their correct three-dimensional structure. However, in as many as 40% of the cases the product accumulates in the form of insoluble aggregates (Fig. 1; Mayer and Buchner 2004).

Mostly, investigators then start to optimize the conditions to obtain the soluble and correctly folded form of the product. For this production strategy a large number of new host strains, vectors and experiences have been described in the literature and it also has resulted in efficient processes. However, despite the amount of accumulated knowledge this way is still based on long-lasting trial-and-error approaches and even experienced scientists may not predict successful and feasible process strategies.

Especially a low yield is mostly obtained for eukaryotic secreted proteins when produced in *E. coli*, even though such proteins are often a target for drug research. Recent progress in understanding the protein folding in eukaryotes, especially in the endoplasmic reticulum, has revealed the high complexity of this compartment and the significant difference to the periplasm

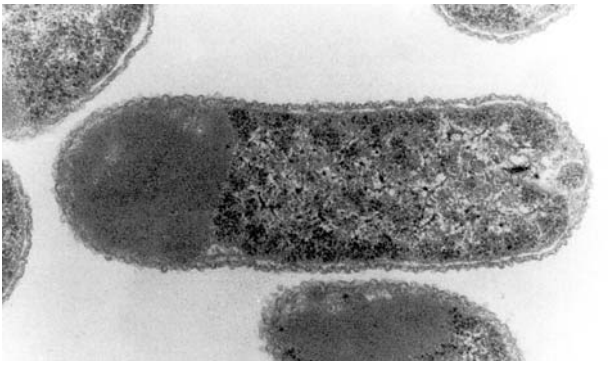

Fig. 1 Electron micrograph of negatively stained *Escherichia coli* cells displaying inclusion bodies (*IBs*)

of bacteria. In particular, it becomes better understood that a simple coexpression of eukaryotic foldases in bacterial hosts will not guarantee that these proteins will work in the same way. Their action is not limited to the redox situation which has been considered for a long time, but extends to their interplay with other proteins and the availability of ATP in the eukaryotic endoplasmic reticulum.

Therefore, inclusion body (IB) based processes belong today to the usual repertoire of producing target proteins, especially if the product is structurally complicated, for example it has a number of disulfide bonds or a multidomain structure. IB-based processes are also a feasible alternative if the product is a domain fragment of a protein, for example from a receptor, or if it is either very large or a small peptide. An IB-based process demands the solubilization and *in vitro* refolding of the target protein during the downstream processing, which might be considered to be difficult. However, generally the production of IBs is comparably easy and high yields are obtained, mostly on the order of 10 to more than 50% of the total cell protein. Also the initial isolation of the IBs, which contain the target protein in almost pure form, is easy by simple cell disruption and centrifugation. Consequently, *in vitro* optimization of the folding in a defined environment seems more straightforward for proteins which are not easily obtained in a soluble form *in vivo*, and is performed more easily than in a complex cellular system with a large number of interacting parameters and reaction sequences.

This review will mainly focus on the characteristics of IBs which are produced in the bacterium *E. coli*, although principally IBs can be obtained also in other organisms, even including eukaryotic hosts (Lefebvre-Legendre et al. 2005; Outeiro and Lindquist 2003). Although the formation of IBs has been reported extensively in many recent reviews (e.g. Baneyx and Mujacic 2004; Fahnert et al. 2004; Hoffmann and Rinas 2004; Sorensen and Mortensen 2005a), new results with respect to the composition and formation process

of IBs have improved our understanding of their nature and will be reported here specifically.

2
Protein Structure Determinants for Aggregation

Expression of recombinant proteins at a high rate often results in the accumulation of IBs, independent of whether the protein is of homologous or heterologous origin. This formation of insoluble aggregates owing to the high, mostly non-natural expression of proteins has been considered for a long time to be an unspecific process driven by contacts between partially folded or unfolded peptides. However, studies in recent years have put this dogma into question. The methodological progress has been greatly influenced by the investigation of protein aggregation in human disorders, such as Alzheimer's and Parkinson's diseases. Aside from clear differences also homologies were found between the underlying principles of amyloid fibril formation in these disorders and the IBs in bacterial systems. These results have entirely changed the picture. Although more studies are needed to understand this aggregation at the molecular, structural and physiological level, the studies performed so far have revealed that also protein aggregation in bacteria is a more specific process than previously expected driven by distinct characteristics of the target protein.

2.1
Protein Folding and Sequence Features

Generally the function of most proteins is based on their solubility in connection with a specific three-dimensional structure, whereas insoluble aggregates are usually toxic (Bucciantini et al. 2002), with exception of proteins devoted to specifically function in well-ordered aggregated structures, such as silk and spider web proteins. Therefore, it seems that both amino acid sequences of the proteins and the components of the cellular protein synthesis machinery have evolved to prevent uncontrolled protein aggregation (Dobson 2003). Thereby the soluble state is connected to one specific configuration, although a protein principally can reach different intermediate states prone to aggregation during the protein folding process. Factors which either destabilize the native structure or decelerate the folding pathway contribute to a higher aggregation.

Protein aggregation is a specific process which starts with a slow nucleation phase, possibly through self-assembly of protein monomers via a nucleation-dependent pathway (Ventura 2005).

Several observations indicate that the formation of IBs is likely to arise from specific and selective mechanisms, similar to amyloid fibril polymeriza-

tion. Generally the IBs formed are relatively pure in respect to the aggregated protein. That is why they are very useful for protein production if a process exists to refold them *in vitro* into their native structure, as discussed later. Other cellular proteins found in IBs often result from coisolation during the enrichment process more than from integration into the original IBs (Georgiou and Valax 1999), especially of proteins connected to the membrane fraction.

A large number of results allow the conclusion that IB formation depends on the specific folding behaviour rather than on general characteristics of a protein such as size, fusion partners, subunit structure and relative hydrophobicity (Rudolph 1996). Especially folding-rate-limiting structural characteristics such as disulfide bonds result in proteins which easily aggregate. Also in normally fast folding proteins, mutations which cause a slower folding increase aggregation (Rinas et al. 1992; Schulze et al. 1994). Additionally, also mutations which destabilize the folded state of a protein increase aggregation by producing partially unfolded stretches which result in intermolecular interactions (Idicula-Thomas and Balaji 2005). This effect has been documented for a number of proteins, such as P22 tailspike protein, single-chain antibodies, interferon-γ, colicin A, CheY, immunoglobulin domains and interleukin-1β.

Interestingly, with respect to proteins which form amyloids, mutations which suppress amyloid formation also show a lower aggregation potential in *E. coli* and accumulate more as soluble protein (Idicula-Thomas and Balaji 2005; Wigley et al. 2001). Also vice versa, if a soluble protein is genetically modified so that it yields increased amyloid propensity, it accumulates in IBs during recombinant expression (Sirangelo et al. 2002; Wigley et al. 2001). De novo designed amyloid proteins which displayed amyloid properties *in vitro* also formed IBs in *E. coli* (West et al. 1999; reviewed by Ventura 2005).

No sequence or structural similarity is apparent between any of the proteins which accumulate extracellularly in amyloidoses. However, despite their heterogeneity and regardless of their origin, all polypeptides involved in amyloid fibrils display similar features, namely (1) binding to various dye molecules such as Congo red and thioflavin-T, (2) similar fibrillar morphologies and (3) aggregated proteins organized in a cross-β-sheet architecture (Carrio et al. 2005; Nilsson 2004) For all of these proteins it is characteristic that the organized structures formed are highly pure with respect to the protein species involved. Interestingly, also other proteins which are unrelated to any known human disease have been found to organize *in vitro* into higher-ordered amyloid-like structures (Fandrich et al. 2001; Guijarro et al. 1998; Pallares et al. 2004; Ventura et al. 2004); therefore, the ability to form these structures was concluded to be a generic property of the polypeptide backbone. The specific amino acid sequence determines the propensity of aggregation for a specific protein species (Dobson 1999).

As a consequence of such results, computational tools have been developed which calculate the propensity to aggregate for a protein (Fernandez-

Escamilla et al. 2004; Lopez et al. 2005; Lopez and Serrano 2004; Ventura et al. 2004) and might be valuable future tools to assess the behaviour of proteins also in *E. coli*.

Beyond the sequence-based folding characteristics in the cell also the vectorial nature of protein synthesis, the high protein concentration (molecular crowding), the presence and action of partner proteins, such as molecular chaperones, foldases and proteases, the availability of metal ions and other cofactors as well as the potential for transport and posttranslational modification are all factors likely to influence the propensity for protein aggregation.

2.2 Disulfide Bonds

The formation of the correct disulfide bonds is usually the rate-limiting reaction for the formation of the native structure of disulfide bond containing proteins under otherwise optimum conditions. Especially in the reducing redox environment of the bacterial cytosol disulfide bonds form very slowly or not at all and therefore disulfide bond containing proteins will easily aggregate. Mutations which lead to a more oxidizing intracellular milieu have been described, such as the knockouts of thioredoxin reductase (*trxB*) and glutathione reductase (*gor*). These mutants promote the formation of disulfide bonds and thereby decrease aggregation (Bessette et al. 1999; Derman et al. 1993; Proba et al. 1995). Although the *gor* and *trxB* gene products are central in the known major reductive pathways in the *E. coli* cell the mutations are not lethal and suppressor mutants with an oxidized cytosol can be easily selected. The results from Bessette et al. (1999) with a truncated form of tissue plasminogen activator (*t*-PA) with nine disulfide bonds and coexpression of oxidoreductases such as DsbC or other proteins with a thioredoxin fold were a breakthrough for the *in vivo* synthesis of proteins with a high number of disulfide bonds. The *gor trxB* double mutants have been applied in the past few years for the production of many other proteins in their native form, including proteins with a lower number of disulfide bonds such as Fab antibody fragments, human collagen prolyl 4-hydroxylase, and domains of membrane proteins (Cassland et al. 2004; Dutta et al. 2001, 2002; He et al. 2004; Jurado et al. 2002; Kersteen et al. 2004; Lauber et al. 2001; Lehmann et al. 2003; Levy et al. 2001; Li et al. 2005; Liu et al. 2002; Miertzschke and Greiner-Stoffele 2003; Neubauer et al. 2005; Premkumar et al. 2003; Schuhmann et al. 2003; Shimizu et al. 2005; Venturi et al. 2002; Xiong et al. 2005; Zhao et al. 2003)

Other disulfide-bond-rich proteins containing more complex structures (e.g. disulfide knots) have not been successfully produced *in vivo* in their correct fold in high amounts yet. *In vivo* production of BMP2 has been thoroughly investigated under different conditions and with various fusions. Although a soluble product was obtained *in vivo* as a fusion to maltose binding protein by Fahnert (2001) a high yield of active protein was gained only

by *in vitro* renaturation. In the case of another disulfide-knot-containing protein, human nerve growth factor (hNGF), the natural prosequence was shown to support the folding of the protein and an efficient *in vitro* refolding process from *E. coli* IBs was established (Rattenholl et al. 2001a, b).

Proteins consisting of different subunits, such as antibody Fab fragments – containing both intermolecular and intramolecular disulfide bonds – have been traditionally produced as IBs. Both separate expression of the heavy and light chains and coexpression systems have been used and *in vitro* refolding protocols have been developed (Buchner and Rudolph 1991a, b; De Bernardez et al. 1999). However, also the production of single-chain antibody fragments and Fabs in the *E. coli* periplasm became a standard strategy (Hayhurst 2000; Hayhurst and Georgiou 2001; Hayhurst and Harris 1999; Horn et al. 1996; Humphreys et al. 1996, 1997, 2002, 2004; Kujau et al. 1998; Kujau and Riesenberg 1999; Plückthun et al. 1996; Raffai et al. 1999) although the optimization process which includes the expression of cofactors is still time-consuming and the yields vary depending on the antibody.

Small proteins with disulfide bonds such as human proinsulin are mainly produced as IBs as preprotein, a fusion protein (Nilsson et al. 1996) or by separate expression of the different chains. Although the formation of disulfide bonds is limited in bacteria, the problems to produce proinsulin do not arise from insufficient disulfide bond formation in general, but mainly arise from incorrectly formed ones and the low stability of the product (Winter et al. 2000).

2.3 Membrane Proteins

Overexpression of membrane proteins has always been a complicated challenge. Such proteins aggregate easily, can be even toxic and difficult to express owing to their hydrophobic nature. Several *E. coli* membrane proteins such as OmpF, OmpC, PhoE and LamB have been successfully produced in sufficient amounts (about 80% of the total cellular membrane protein) (Ghosh et al. 1998).

However, the production of heterologous membrane proteins seems to be more problematic. A breakthrough came in 1996 when Miroux and Walker (1996) succeeded in isolating specific *E. coli* mutants, allowing the production of membrane proteins. If membrane proteins are overproduced in these mutants, new intracellular membranes proliferate containing the recombinant product in the correct conformation (Arechaga et al. 2000). The authors reported the soluble production of the β subunit of F_1F_0 ATP synthase, containing one transmembrane span, in the *E. coli* BL21(DE3) mutants C41 and C43. Also Shanklin (2000) expressed an *E. coli* membrane protein, the acyl–acyl carrier protein synthase (80.6 kDa) efficiently in the C41 mutant. In this case a smaller amount of this protein (one third) was also correctly integrated

in membranes of the BL21(DE3) strain. Promising results have also been reported by others for the expression of active membrane-bound cytochromes in these mutants (Mulrooney and Waskell 2000; Saribas et al. 2001) and a few other eukaryotic membrane proteins (Chapot et al. 1990; Grisshammer et al. 1993; Quick and Wright 2002).

Also IB production with subsequent renaturation of urea-dissolved IBs in phosphate buffer containing *n*-dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulfonate (SB12) has been successfully applied in a few cases. Jansen et al. (2000) produced *Neisseria meningitidis* PorA using the *E. coli* BL21 pET system in high amounts as very pure IBs at 37 °C and established an efficient *in vitro* refolding protocol for this outer-membrane protein. A few other membrane proteins from prokaryotes have been refolded (Charbonnier et al. 2001; Kumar and Krishnaswamy 2005), but the number of successful examples is still low. From larger membrane proteins such as G-protein coupled membrane receptors only extracellular binding domains have been expressed in *E. coli* as IBs and refolded (Chauhan et al. 2005; Grauschopf et al. 2000).

2.4 Glycosylation

Many therapeutically interesting proteins from eukaryotes are glycosylated. Glycosylation is known to affect the folding behaviour and especially the solubility (Idicula-Thomas and Balaji 2005; Zhang et al. 1998). Therefore, glycosylated proteins may be prone to aggregation if produced non-glycosylated in *E. coli*.

Often glycosylation is not a prerequisite for function of the protein but it influences its activity and degradation characteristics, such as thermostability (Solovicova et al. 1996); therefore, many proteins can be produced in bacterial hosts lacking the eukaryotic glycosylation system for therapeutic applications. Recombinant proteins produced in *E. coli* may even have beneficial properties compared with the glycosylated forms. For example, a non-glycosylated recombinant variant of human *t*-PA obtained by refolding from *E. coli* IBs showed a longer half-life and a lower clearance rate in rats (Martin et al. 1992; Mattes 2001).

3 Structure and Composition of IBs

Being extremely common during the bacterial production of recombinant proteins, IBs have been in general poorly studied regarding their structure and the mechanics of aggregation of the protein composing them. The poor solubility of recombinant proteins and specifically the formation of these deposits of insoluble protein often represent a serious obstacle for the produc-

tion of readily usable protein products (Baneyx and Mujacic 2004; Sorensen and Mortensen 2005a). Since, in addition, minimization of IB formation is not a straightforward task, IB formation has been usually seen as an unavoidable complication associated with the prokaryotic nature of the production system itself. Interestingly, IBs are also the source of relatively pure polypeptides, since they are easily separable from cellular debris by low-speed centrifugation (Georgiou and Valax 1999) and can be isolated with a reasonably purity by simple detergent-washing procedures (Carrio et al. 2000). This has also been exploited for large-scale-addressed production purposes, provided a convenient, *in vitro* refolding procedure had been developed. In this case, the enhancement of IB formation can be easily achieved by conditions favouring protein aggregation, such as high temperature, high rate of recombinant gene expression and the absence of relevant proteases in the producing strain. Again, these strategies have been empirically embarked on by very little, if any, physiological investigation of the biology of the aggregation event itself.

In the context of arising concern of conformational diseases, such as those caused by prions or involving amylodgenesis, IBs have been turned into exciting models for the *in vivo* study of protein aggregation. Recent insights about their structural and physiological traits have transformed the classic picture of IBs and provided intriguing clues to understand the mechanics of their formation.

3.1
Morphology, Structure and Molecular Organization

Under optical microscopy, cytoplasmic IBs are usually seen as refractile structures, present in low number (generally one or two) per cell, usually with a polar distribution, and with a volume often comparable to that of the cell cytoplasm itself (Bowden et al. 1991; Carrio et al. 1998). Electron microscopy usually pictures IBs as electrodense particles (Carrio and Villaverde 2005), generally amorphous, but in some cases showing icosahedra-like shapes. Scanning microscopy of purified IBs shows cylinders with a surface tending to be rough (Bowden et al. 1991; Carrio et al. 2000), a fact that could be indicative of a porous structure. On the other hand, the morphological evolution of IBs during *in vitro* trypsin digestion shows that the proteolytic attack is not surface-restricted (Carrio et al. 2000). Instead, the enzyme penetrates the bodies and in the first phases of the disintegration they are fragmented into smaller pieces of protease-resistant protein (Carrio et al. 2000). Thus, IBs appear as being formed by multiple cores of protease-resistant polypeptides, each of them surrounded by protein species more sensitive to proteases, that are initially lost during proteolytic attack, revealing the particulate nature of IBs. IB subunits could have an independent origin in the cell, although this extent has not been proved experimentally. However, protein synthesis is not

punctually localized in the bacterial cytoplasm and IBs are expected to result from aggregation of protein species produced in distant ribosomal sets. Such IB subunits could be aggregation intermediates or independent nucleation cores that finally cluster. In this respect, in GroEl140 mutants lacking a functional GroEL protein at 42 °C, IBs produced at this temperature are smaller and much more abundant than in the wild type (Carrio and Villaverde 2003). Although the molecular basis of this observation is not completely solved, GroEL could act, either directly or indirectly, by condensing small aggregates formed dispersedly in the cell. In this context, GroEL can stimulate *in vitro* protein aggregation as revealed by both *E. coli* β-galactosidase (Ayling and Baneyx 1996) and the prion protein models (De Burman et al. 1997; Stockel and Hartl 2001).

The *in vitro* digestion analysis of IBs renders two additional observations relevant to the molecular organization of IB proteins. Firstly, the relative composition of protease-resistant and protease-sensitive species changes during IB formation, indicating inner molecular reorganization and therefore both structural and evolving flexibility of these aggregates (Carrio et al. 2000). Secondly, protein digestion occurs as a cascade process, in which protease target sites are sequentially exposed for cleavage (Cubarsi et al. 2001; Cubarsi et al. 2005). Interestingly, the sequential cleavage takes place on protein that remains associated with IBs during their progressive fragmentation. This fact indicates a packaging scheme loose enough to allow for aggregated polypeptides to undergo conformational modifications while being IB components.

Fourier transform IR (FTIR) analysis and other structural approaches have revealed an increase in the β-sheet structure of IB protein relative to that of the soluble version (Ami et al. 2003; Przybycien et al. 1994), which adopts an antiparallel, intermolecular organization (Carrio et al. 2005). In addition, many amyloid-like traits observed in IBs such as binding of amyloid-tropic dyes or a sequence-dependent seeding process (Carrio et al. 2005) suggest that IB proteins are not completely disorganized but are packaged in a rather regular way. The recent finding of recombinant proteins forming amyloid-like fibres in *E. coli* and occurring as loose aggregates in the soluble cell fraction (de Marco and Schroedel 2005) prompts us to speculate that such structures could be among the IB precursors.

Interestingly, a certain extent of native-like secondary structure has been also observed by FTIR analysis in IBs formed by different proteins (Ami et al. 2005; Oberg et al. 1994; Umetsu et al. 2005), coexisting with the dominating characteristic β-sheet. In this context, enzymatic activity associated with enzyme-based IBs that could be compatible with the occurrence of at least a fraction of native protein has also been reported (Garcia-Fruitos et al. 2005a; Kuczynska-Wisnik et al. 2004; Tokatlidis et al. 1991; Worrall and Goss 1989).

Such active protein species, which are probably trapped by unfolded domains not affecting the active site, are able to confer at least some IB-cata-

lysing properties that could prompt us to consider the use of IBs as catalysers in enzymatic processes (Garcia-Fruitos et al. 2005b). The cultivation conditions can have an influence on the structure of the target protein which is entrapped in the IBs. A highly interesting strategy was published recently by Jevsevar et al. (2005), who developed a strategy for a bioprocess for human G-CSF on the basis of IBs which had a close to native state like structure. The accumulation of this kind of "nonclassical" IB was promoted by slow expression and lower temperature. This new way of optimizing the folding state of the target protein in the IBs resulted in a simpler and very efficient downstream purification procedure without the need to use high guanidinium chloride (GdmCl) or urea concentrations.

The molecular basis for the coexistence of active, properly folded protein species and cross-β-sheet-organized polypeptides and the possible potential of IBs which contain the target product in a close-to-native conformation needs further investigation.

3.2 Compositional Analysis

Early proteomic analyses revealed that the recombinant protein itself is the main component of IBs (Rinas et al. 1993; Rinas and Bailey 1992; Valax and Georgiou 1993), representing up to 95% of the total embedded protein (Carrio and Villaverde 2002; Villaverde and Carrio 2003). Interestingly, in the case of proteolytically unstable IB-forming proteins, stable digestion fragments are also abundant in the aggregates (Corchero et al. 1996). An important part of those fragments could be generated from in situ proteolysis (Carbonell et al. 2002; Corchero et al. 1997; Vera et al. 2005), while others can result from site-limited protein digestion in the soluble fraction and further deposition (Carrio et al. 1999). Many cellular proteins, including some membrane proteins belonging to the Omp family, are also found associated with IBs (Jürgen et al. 2000; Rinas and Bailey 1992). Most of these polypeptides are mere contaminants retained by unspecific attachment during the purification process (Georgiou and Valax 1999). Traces of cell material such as phospholipids and nucleic acids have also been identified (Valax and Georgiou 1993). The small heat shock proteins IbpA and IbpB seem to be a common component of protein aggregates in *E. coli* (Allen et al. 1992; Carrio and Villaverde 2002; Hoffmann and Rinas 2000). These chaperones play an important and immediate role in mediating the release of IB proteins (Carrio and Villaverde 2003), especially at high culture temperatures (Lethanh et al. 2005). Minor amounts of main chaperones such as DnaK and GroEL are also occasionally observed as IB components (Carrio and Villaverde 2002; Jürgen et al. 2000), and they might also have a functional role in IB processing. In particular, the surface-restricted localization of DnaK (Carrio and Villaverde 2005) indicates that this chaperone is not passively trapped by interaction with ag-

gregating polypeptides but that it probably displays disaggregation activities at the body's interface for protein transfer to the soluble cell fraction (Carrio and Villaverde 2001). The role of this chaperone in disassembling aggregated proteins (Mogk and Bukau 2004; Schlieker et al. 2002; Weibezahn et al. 2004) is compatible with this hypothesis.

The homogeneity of IBs concurs with a highly selective aggregation process. In eukaryotic cells, intermolecular interactions between the hydrophobic patches leading protein aggregation is a sequence-specific event (Rajan et al. 2001). Also, *in vitro* aggregation of denaturated, IB-forming proteins occurs by interactions between homologous regions (Speed et al. 1996). Recently, it was shown that isolated VP1LAC IBs can direct the aggregation of soluble forms of homologous VP1LAC, but not of other IB-forming proteins (Carrio and Villaverde 2005). This fact indicates that aggregation of IB-forming proteins is a sequence-selective event that can largely account for the high protein purity found in the particles. In this respect, the precipitation of minor amounts of other unrelated proteins such as pre-β-lactamase (Rinas and Bailey 1993) during IB formation could be observed as the result of chaperone undertitration during recombinant protein synthesis, a fact that could indirectly enhance the aggregation tendency of many cellular, or as in this case, high-rate-produced plasmid-encoded polypeptides.

4
Dynamics of IB Formation and Disintegration

Many newly synthesized *E. coli* cell proteins require assistance to reach their native conformation, specifically during thermal or other conformational stress (Deuerling and Bukau 2004). During the folding process, many folding intermediates appear sequentially and represent evolving conformational states prior to the native form, as thoroughly investigated in the case of the tailspike protein of phage P22 (Goldenberg et al. 1983; King et al. 1996; Speed et al. 1995, 1997). In these intermediates, hydrophobic patches, buried in the native conformation but important for the folding process (Betts et al. 2004), are exposed to the solvent and can interact with each other, leading to protein aggregation. Such intermolecular interactions through "sticky" regions can also involve thermodynamically stable misfolded protein forms (Betts and King 1999). When plasmid-encoded recombinant proteins are produced, both the unusually high gene dose and the use of inducible promoters drive a high, non-physiological protein synthesis rate that cannot be regulated by the cell itself. In this situation, cell foldases are exposed to a substrate load that is higher than manageable, and misfolded species and folding intermediates aggregate and accumulate in the insoluble cell fraction usually as IBs. Protein aggregation seems to be also favoured in the case of recombinant polypeptides of eukaryotic or viral origin. Protein synthesis in eukaryotic cells occurs

more slowly than in prokaryotic cells, and the structure of eukaryotic proteins might have been adapted to cotranslational folding in a vectorial manner (Ellis and Hartl 1999). The extremely fast translation process in prokaryotic cells (Sorensen and Pedersen 1991) presumably imposes an additional constraint for proper folding of eukaryotic proteins.

For a long time IBs have been considered as a dead end of the cellular protein network (mainly composed by folding modulators and proteases) surveying the conformational quality of cellular proteins, and protein aggregation as a passive event. However, more recent studies have shown that in the absence of either total or recombinant protein synthesis, IBs are disintegrated in a few hours and the protein contained is refolded and eventually further proteolysed (Carrio and Villaverde 2001; Le Thanh and Hoffmann 2005). Therefore, IBs are reservoirs of misfolded protein when the high substrate load for chaperones and proteases does not allow them to prevent the occurrence of misfolded proteins (Carrio and Villaverde 2002). The steady volumetric growth and biomass increase of IBs while recombinant cells are productive (Carrio et al. 1998) then results from an asymmetric balance between protein deposition and removal, which can allow IB disintegration if no additional misfolded protein is produced in the cells (Carrio and Villaverde 2001). Protein removal from IBs is largely impaired in cell mutants deficient in IbpA, IbpB, DnaK, GroEL, GroES, ClpA (Carrio and Villaverde 2003; Lethanh et al. 2005) and proteases Lon and ClpP (Vera et al. 2005), proving a role for all these heat shock proteins in the disintegration process. The details of the disintegration mechanics as integrated in the global cell stress response are discussed in Sect. 4.3.

On a molecular level, protein aggregation as IBs is compatible with a pseudo-first-order kinetics model as monitored under *in vivo* conditions (Hoffmann et al. 2001). This is indicative of a seeding behaviour of IB formation, in agreement with the *in vitro* spontaneous aggregation of IB-forming polypeptides after seeding with isolated IBs that entrap on their surface soluble, homologous polypeptides (Carrio and Villaverde 2005). All these findings, together with the low number of IBs per cell and the existence of IB intermediates as discussed before, allow us to picture the dynamics of IB formation as follows.

Numerous nucleation cores are generated in the cell during recombinant protein production that act as seeding elements to which misfolded polypeptides are surface-entrapped with exquisite sequence specificity, as during amyloid formation. Such clusters of insoluble protein grow concentrically and they probably originate in the soluble cell fraction. In this context, the existence of "soluble" aggregates was recently reported (Sorensen and Mortensen 2005b) and these structures are now the subject of careful structural analysis (de Marco and Schroedel 2005). IB intermediate(s) would get attached together and finally collapse into high-order structures. The involvement of cell elements in this process cannot be excluded as discussed before. Also,

minimizing solvent-exposed hydrophobic patches could enhance the cell performance by reducing the amount of toxic protein species, as suggested by the negative impact on cell viability of IB formation in the absence of functional GroEL or DnaK (Gonzalez-Montalban et al. 2005).

4.1 Protein Synthesis and Aggregation

The folding kinetics of a protein and the occurrence of different conformational species during this process affect the protein aggregation propensity. While the correctly folded protein is rarely subject to aggregation, folding intermediates and misfolded proteins aggregate mainly driven by surface-exposed hydrophobic patches which are buried in the native structure. As the folding may be a comparably slow process, aggregation is favoured by a high concentration of unfolded protein. Therefore, aggregation in the bacterial cell is very much dependent on the synthesis rate of a protein.

The synthesis rate is influenced by a number of parameters, including the gene copy number, the level of messenger RNA (mRNA), the strength of the ribosome binding site, the amount of attracted ribosomes, the elongation rate and the distribution of the ribosomes on the mRNA, the last two being mainly a function of the codon bias (Sorensen and Pedersen 1991).

Although the rate of translation has a profound effect on the *in vivo* folding reaction and the accessibility of chaperones, it has not been possible yet to tune the translation process; hence, in practice the rate of synthesis is mostly controlled at the induction level. Aggregation occurs mainly owing to application of high inducer concentrations (Kopetzki et al. 1989; Schein and Noteborn 1988) and at cultivation temperatures above 18–25 °C.

Gene copy number: The gene copy number is determined by the origin of replication of the plasmid and is mostly in the range of 20–50 copies per cell in the case of medium-copy-number plasmids or even more than 100 copies for high-copy-number plasmids.

The copy number basically affects the sum of the cellular components engaged in the synthesis process of a recombinant protein and therefore may provoke a significant impact on the metabolic load of the synthesis of a product. At the start of a screening process for product optimization a low copy number of a stably maintained vector is strongly recommended, since signal amplification is more easily and more accurately controlled at the level of transcription and translation than at the level of copy number control. Furthermore, the gene copy number does not only depend on the plasmid origin, but also changes during a process depending on the strain, the growth medium, the growth rate and the cultivation temperature. Proper control of the exact copy number is rarely possible for vectors with a higher copy number.

Transcriptional control: Most optimization strategies aim to properly control the transcription rate. This includes the choice of an inducible promoter

and the variation of the inducer concentration based on a given expression system. The inducer concentration influences the number of mRNA molecules produced per unit time, but does not affect the synthesis rate of a single mRNA molecule. The *E. coli* RNA polymerase proceeds with a rate of about 40–50 nucleotides per second, lowered by pausing sites leading to transient arrest of the transcription especially under detrimental environmental conditions and resulting in an up to 50% reduction of the total transcription rate (Vogel et al. 1992). In the case of *E. coli* RNA polymerase the transcription rate is on the order of the translation rate of ribosomes, varying depending on the codon bias between approximately 15 nucleotides per second for rare codons and 62 nucleotides per second for abundant codons (Sorensen and Pedersen 1991). These rates for transcription and translation suggest that ribosomes stack closely to each other behind the *E. coli* RNA polymerase if the recombinant genetic construct contains a strong ribosome binding site. Furthermore, ribosome tailing is proposed to occur by the relatively low ribosome release rate at the stop codon. Therefore, the synthesis time of a single protein molecule is limited either by the transcription rate of the RNA polymerase or by the codon usage if rare codons with low translation rates are contained at higher levels in the sequence of a recombinant gene.

T7 RNA polymerase in connection with a T7 promoter is often applied as an alternative strong expression system (Studier et al. 1990). This polymerase synthesizes the mRNA with a fourfold to fivefold higher transcription rate (about 230 nucleotides per second) compared with *E. coli* RNA polymerase (Golomb and Chamberlin 1974; Iost et al. 1992; Lyakhov et al. 1998). As the initiation rate of both RNA polymerases is similar (*E. coli* RNA polymerase one to three per second; T7 RNA polymerase one per second) the higher accumulation of proteins in the T7 systems is most probably due to the high elongation rate of the enzyme. That is why in T7 RNA polymerase systems the synthesis rate of the single protein molecule is not limited by the formation rate of the mRNA, but is only limited by the codon usage of the gene.

Translation: As discussed before, a high expression rate of the recombinant protein should be optimal for the accumulation of IBs; therefore, a strong ribosome binding site is beneficial. In contrast a weaker ribosome binding site could improve the yield of correctly folded product by lowering the overall translation efficiency, which eventually should decrease the aggregation between different product molecules but may also result in a lower mRNA stability.

The rate of synthesis of a single polypeptide depends on the elongation rate in the translation process. The elongation rate is mainly affected by the codon bias and varies between approximately 15 nucleotides per second for rare codons and 62 nucleotides per second for abundant codons (Sorensen and Pedersen 1991). The codon bias varies between different organisms and is well reflected by the respective transfer RNA (tRNA) population (Dong et al. 1996). If an mRNA from a heterologous target gene is overexpressed in *E. coli*,

differences in codon bias of the gene to the codon bias of *E. coli* can impede translation. In practice the presence of a small number of rare codons does not affect the target protein synthesis very much. However, the accumulation of a recombinant gene product can be very low if the gene contains clusters and/or numerous rare *E. coli* codons. It is well known that such an accumulation of unfavourable codons within a gene causes reading errors (e.g. frameshifts, hops), mRNA instability and degradation (Coburn and Mackie 1999; Deana et al. 1996), misincorporation of other amino acids (Calderone et al. 1996) and even death of the cells (Zahn 1996). The codon usage of the first 20 amino acids is crucial for the expression level of the product (Niemitalo et al. 2005; Parker 1989; Rosenberg et al. 1993; Spanjaard et al. 1990).

Problems with the codon bias of a recombinant target gene can be solved by redesigning the codons through synthetic genes, by site-directed mutagenesis or by a higher expression of the rare tRNAs. Especially coexpression of tRNAs, which was first applied by Brinkmann et al. (1989), has become a generally applied tool since corresponding vectors became commercially available.

An improvement of the codon bias to abundant codons by adapting the sequence of the recombinant gene or introducing extra copies of rare tRNAs, for example *argU*, *ileY* and *leuW*, into the host cells (Kleber-Janke and Becker 2000) increases the probability of aggregation by a high specific synthesis rate and by affecting the protein domain folding rate (Cortazzo et al. 2002). A further factor to be considered is the relatively slow ribosome release rate at the stop codon (Freistroffer et al. 1997). This causes ribosome tailing at the 3′ end of the mRNA (Niemitalo et al. 2005), which might be suggested to increase interactions between the ribosome-bound peptides as a first step to aggregation.

4.2 Stress Responses to IBs

Stress imposed on the cellular system owing to the production of IBs is caused by (1) the high synthesis rate of a recombinant protein and (2) the cellular response to aggregating protein.

Strong induction of recombinant protein expression is an artificial stress redirecting the metabolism of a bacterium to the production of a specific target protein. Although the metabolic network of a cell has some flexibility, the effect of strong induction of a gene with a strong promoter and a strong ribosome binding site is considerable with respect to necessary energy resources (Dasilva and Bailey 1986) and depletion of transcriptional and translational factors and may impose a stress situation. Although the strong synthesis of a recombinant protein can principally induce a number of different stress signals connected to cellular regulons such as the stringent response (Cserjan-Puschmann et al. 1999), the general stress response and the SOS

response (Benito et al. 1995), it depends on how strongly the recombinant protein is synthesized whether the corresponding stress gene expression pattern can be found, because all stress responses are connected to the synthesis of new proteins. At a very high synthesis of the recombinant product, the protein synthesis apparatus including transcription and translation may be fully occupied by the synthesis of the product and no cellular responses are observed, but the cell will lose its ability to divide and will slowly die (Lin et al. 2001). Only a heat-shock-like response is always found if misfolded forms of the target protein accumulate independent of the expression system used (Bahl et al. 1987; Dong et al. 1995; Ito et al. 1986; Parsell and Sauer 1989). The high speed and competitiveness of the heat shock response controlled by σ^{32} can be explained concerning the regulatory mechanism of this response.

As discussed before, IBs result from aggregation of partially folded or misfolded proteins. In connection to bacterial survival, the accumulation of proteins recognized as non-functional or unfolded molecules is critical and mechanisms have been developed to counteract this process. Analogously, in nature unfolded proteins appear during heat stress causing the unfolding and subsequent aggregation of proteins. The bacterial heat shock response is regulated by a sensitive measure of the appearance of unfolded molecules, which is here only briefly summarized. For more details see recent reviews (e.g. Arsene et al. 2000). The regulation of the heat shock response in *E. coli* basically involves the heat shock sigma factor σ^{32} and its binding to the DnaK and DnaJ chaperones. Both chaperones contribute to the downregulation of the σ^{32} activity and stability. After a heat shock, while proteins are inactivated and aggregated proteins appear, σ^{32} is released from the chaperones, leading to higher stability and activity of σ^{32}. Consequently, σ^{32} edging out the housekeeping sigma factor σ^{70} of RNA polymerase and the RNAPσ^{32} holoenzyme initiates transcription from promoters belonging to the heat shock regulon. Among others, the σ^{32}-connected response leads to increased transcription of *dnaK*, of other chaperone genes and of heat-shock-related protease genes such as *lon* and *clP* (Bukau 1993; Yura and Nakahigashi 1999). Therefore, the concentration increases rapidly and transiently of both the Hsp70 chaperone proteins (DnaK, DnaJ and GrpE) and the Hsp60 chaperonins (GroEL and GroES). This transient increase of the chaperones furthermore leads to binding and degradation of σ^{32}, causing the transient character of the heat shock response

Unlike a heat shock primarily causing the unfolding of existing proteins, induction of the synthesis of recombinant proteins may cause the synthesis of misfolded proteins and export-defective secretory protein precursors (Wild et al. 1993). The regulation of the heat-shock-like response to such misfolded recombinant proteins is similar to a heat shock. The accumulation of newly synthesized misfolded proteins also attracts the chaperones and leads to an activation and stabilization of σ^{32} (Bukau and Horwich 1998; Craig and Gross 1991; Straus et al. 1987). However, this response lasts longer (Kanemori et al.

1994; Parsell and Sauer 1989) than the synthesis of the recombinant protein. Furthermore, as σ^{32} must compete for the very effective synthesis of the recombinant protein, the accumulation of new chaperones may be lower than after a heat shock and inactivation of σ^{32} may be retarded. The heat-shock-like response due to IB formation also leads to enhanced proteolytic activities (Goff and Goldberg 1985; Kosinski et al. 1992; Kosinski and Bailey 1991, 1992) and increased levels of Hsp60 and Hsp70 chaperones (Dong et al. 1995; Fahnert 2001; Gill et al. 2000b, 2001; Rinas 1996; Snow and Hipkiss 1987). The formation of IBs also induces *ibpA* and *ibpB* gene expression and incorporation of these proteins in the IBs (Allen et al. 1992; Rinas 1996).

Aside from a potential positive effect of the heat-shock-like response on the folding of a recombinant protein by increasing the level of chaperones, the induction of the heat shock response also can negatively affect the production of recombinant proteins because it is connected to induction of cytoplasmic (Lon, Clp) and periplasmic (DegP, OmpT) proteases (Gill et al. 2000a). Therefore, methods to reduce the heat-shock-like response and thus the proteolytic activities during overexpression of target proteins can produce positive effects. The simplest method is to lower the cultivation temperature (Kosinski et al. 1992; Surek et al. 1991), but positive effects were also obtained by using *htpR* or *lon* mutants (Surek et al. 1991) and by antisense downregulation of σ^{32} (Srivastava et al. 2000, 2001).

4.3 Chaperone Action

Chaperones are conserved proteins in all organisms. They are engaged in the folding of proteins, induced especially under heat stress but also under other extreme situations which lead to aggregation of proteins. Therefore, they are designated "Hsp" and are named according to their approximate molecular mass as Hsp100, Hsp90, Hsp70, Hsp60, and sHsp (small heat shock proteins) with the following representatives in *E. coli*: Clp, HtpG, DnaK, GroEL and IbpA/Ibpb. They function primarily in concert with other proteins, including co-chaperones such as DnaK/DnaKJ/GrpE or GroEL/GroES.

Although the correct folding to the three-dimensional structure of a protein is an intrinsic characteristic, chaperones and foldases decrease the folding time and protect the protein from aggregation. It has been calculated that in *E. coli* only 10–20% of the host's proteins are folded with the help of the Hsp60 and Hsp70 chaperone systems during exponential growth and this increases to about 30% under heat shock conditions (Ewalt et al. 1997; Teter et al. 1999). In contrast, many of the interesting heterologous target proteins fold slowly and therefore benefit from the availability of chaperones.

Although the mechanism of chaperone function has been studied over many years, the whole complex picture of protein folding networks *in vivo* remains fragmentary. Especially, studies with *in vivo* protein aggregates and the

evaluation of the function of the small heat shock proteins only very recently contributed to a better understanding of the whole network in more detail. (for a schematic overview see Fig. 2)

The function of chaperones and foldases is tightly connected to the whole protein folding pathway, starting with the growing polypeptide during translation. In bacteria trigger factor is placed in close proximity to the end of the exit tunnel of the ribosome and shields the unfolded growing protein from the environment by its large hydrophobic cradle, stabilizing unfolded nascent chains, thus preventing unproductive intramolecular interactions (Maier et al. 2005).

This ribosome-associated trigger factor cooperates with the DnaK system during the folding of newly synthesized cytosolic polypeptides (Schaffitzel et al. 2001). DnaK binds to a short linear consensus motif of four to five hydrophobic amino acids (mainly leucine) flanked by basic ones (Rudiger et al. 1997a, b; Schmid et al. 1994) with the help of its co-chaperone DnaJ.

Substrates of the Hsp60 (GroEL) system consist preferentially of partially structured, hydrophobic protein molecules (Houry et al. 1999). In the case of a misfolding (indicated by exposed hydrophobic areas) of the now non-linear, compact protein it can enter the hydrophobic cavity of the GroEL chaperonin. Common to the GroEL substrates are extensive hydrophobic surfaces char-

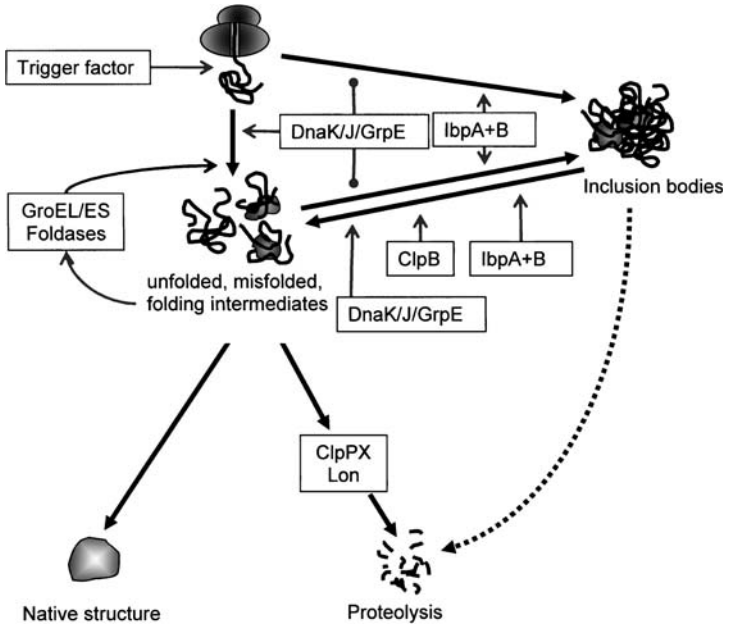

Fig. 2 Simplified scheme of the competitive routes of *in vivo* protein folding adapted for *E. coli* and the production of recombinant proteins (*continuous arrow* positive effect, *dotted arrow* negative effect)

acterized by β-sheets or α-helices (Chaudhuri and Gupta 2005; Houry 2001) and a size of 10–55 kDa. By association with GroES (Hsp10) and consumption of ATP, the conformation of GroEL changes. This causes the bound substrate protein to be released from the cavity surface of GroEL. So it is enabled to fold again according to the principle of the Anfinsen cage (spontaneous self-assembly of polypeptides; Anfinsen et al. 1961) in the hydrophilic milieu of the chaperon's interior. The protein is released after the dissociation of the chaperone. If the correct conformation is not achieved, the protein may enter GroEL again (Coates et al. 1999; Ellis 1994).

During the folding process proteins can be transferred from one chaperone system to the other. Thereby the chaperones do not refold the protein actively to the correct structure, but they lower the aggregation potential by repeatedly binding and releasing the substrate and they allow the protein successively to reach a kinetically favourable state and finally the active conformation.

Irreversibly damaged proteins may be further presented to the Clp chaperones. These proteins are structurally similar to GroEL in having an inner chamber formed by one or two stacked rings of six or seven monomers. ClpB of *E. coli* can apparently act exclusively as a molecular chaperone, whereas other Clp family members such as ClpA and ClpX function both as chaperones and as components of ATP-dependent proteases. Damaged proteins may be either directed to further degradation by proteases such as ClpPX (Wickner et al. 1999) or alternatively they can be resolubilized by the ClpB chaperone in concert with the Hsp70 chaperone system (Goloubinoff et al. 1999; Mogk et al. 1999; Zolkiewski 1999).

The mechanism is that hydrophobic regions of polypeptides are exposed either while the newly made proteins emerge from the ribosome or because of subsequent misfolding or failure to assemble properly. These exposed regions are subject to binding by any of the chaperones or by the ATP-dependent proteases. Chaperone binding and release of folding intermediates may allow proteins to reach their native conformation or may return them to the pool of non-native proteins that can rebind chaperones or proteases. Protease binding followed by ATP-dependent unfolding and subsequent degradation removes the protein from the pool of non-native proteins. However, a high rate of synthesis of a protein with slow folding characteristics may exceed the available resources of foldases, chaperones and proteases. In this context, IBs may represent reservoirs of protease-resistant protein that steadily accumulate until chaperones and proteases become available either for successful protein folding or for proteolysis. This model, recently suggested by Carrio and Villaverde (2003), is supported by data on higher IB accumulation in a *dnaK* mutant (Fig. 2). Contrarily a *groEL* mutant had a lower content of IBs and a higher amount of folded protein, which might be explained by the early function of the Hsp70 chaperone system in the protein folding pathway and the GroEL system as a "second-stage" folding supporter. However, there

are many examples where also GroE overexpression results in more soluble product.

Although the chaperones act most generally to prevent aggregation, they are also able to dissolve aggregates (Glover and Lindquist 1998; Motohashi et al. 1999; Zolkiewski 1999). The controlled aggregation and resolubilization of protein aggregates is connected to the action of two small heat shock proteins, IbpA and IbpB (Allen et al. 1992).

IbpA and IbpB, which are transcriptionally controlled by σ^{32} (Chuang et al. 1993), are found in the insoluble protein fraction after a heat shock and also are detected in IBs from recombinant proteins (Allen et al. 1992). Typically for small heat shock proteins, IbpA and IbpB form oligomers with molecular masses of about 2.0–3.0 MDa, corresponding to 100–150 subunits (Kitagawa et al. 2002; Shearstone and Baneyx 1999)

In vivo, overexpression of *ibpA* and *ibpB* confers heat resistance to *E. coli*, reduces protein aggregation and suppresses the heat sensitivity of an *rpoH* (σ^{32}) mutant (Kitagawa et al. 2000). *In vivo* experiments have shown that IbpA and IbpB are dispensable during mild stress conditions (45 °C) but are required for removal of misfolded proteins upon extreme, long-term heat conditions (50 °C) when aggregation is increased and removal of the aggregates is less efficient. Deletion of the *ibpA*/*ibpB* operon results in a twofold increase of aggregated proteins and a tenfold decrease of cell viability under the extreme conditions (Kuczynska-Wisnik et al. 2002).

As shown for thermally inactivated proteins, IbpA and IbpB bind to misfolded proteins and can stabilize such proteins in a disaggregation competent state, which is characterized by a decrease of the hydrophobicity of the aggregated protein substrates (Matuszewska et al. 2005). In aggregates containing IbpA and IbpB even a higher enzymatic activity of the aggregated substrate proteins was detected than in IbpA-deficient and IbpB-deficient IBs (Kuczynska-Wisnik et al. 2004). Such data support a model which was earlier suggested on the basis of *in vitro* data, assuming that IbpA and IbpB create a reservoir of non-native refoldable proteins (Kitagawa et al. 2002) and further have, like other small heat shock proteins, a role in delivering the proteins to the ATP-dependent DnaK/DnaJ/GrpE system (Ehrnsperger et al. 1997; Kitagawa et al. 2002; Kuczynska-Wisnik et al. 2004; Lee and Vierling 2000; Mogk et al. 2003a; Veinger et al. 1998). The protective role of IbpA and IbpB is supported also by the observation that they slow down disaggregation and degradation of IBs at higher temperature of a protein which can be partially reactivated *in vivo* under non-heat-stress conditions (Lethanh et al. 2005).

During disaggregation of IBs, IbpA and IbpB cooperate with the ClpB-DnaK/DnaJ/GrpE bichaperone system in reversing protein aggregation. *In vitro* the DnaK/DnaJ/DnaE system can dissociate soluble small heat shock protein–substrate complexes, whereas ClpB is additionally required for disaggregation of large, insoluble complexes (Mogk et al. 2003a, b).

A large number of more detailed reviews on the action of chaperones and their action in the protein folding pathway and in connection to aggregation of recombinant proteins have been published recently and are recommended to the interested reader (Deuerling and Bukau 2004; Dougan et al. 2002; Haslbeck and Buchner 2002; Hoffmann and Rinas 2004; Maier et al. 2005; Mogk and Bukau 2004; Nystrom 2002; Schlieker et al. 2002; Slepenkov and Witt 2002; Villaverde and Carrio 2003; Wegele et al. 2004; Weibezahn et al. 2004; Zhang et al. 2002).

4.4 Periplasmic Response to Misfolded Protein

In addition to the induction of the σ^{32} response by proteins overexpressed within the bacterial cytoplasm, a σ^{24} (σ^{E})-dependent heat shock response is induced by misfolded proteins in the periplasm. The response to protein misfolding in the cell envelope is a finely tuned system regulated by a cascade of phosphorylation and dephosphorylation reactions (Missiakas and Raina 1997b). Interestingly, overexpression of periplasmic proteins does not only induce the σ^{E} regulon, but also leads to a significant increase of the expression of the cytoplasmic σ^{32}-dependent chaperones GroEL, GroES, DnaJ and DnaK as revealed by transcriptome analysis (Fahnert 2001). Similarly to the cytoplasm, chaperones and proteases in the periplasm contribute to degradation or refolding of damaged proteins. One example is protease DegP, which consists of a double ring to be entered by a protein before it is proteolytically cleaved. Therefore, the protein has to be partially unfolded or disaggregated, respectively. Interestingly, DegP changes its tertiary structure and activity in a temperature-dependent manner. It is only a protease at high temperatures but may act as a chaperone in the lower-temperature range (Kim et al. 1999; Pallen and Wren 1997).

4.5 Response to Aggregation in Other Bacteria

The response to unfolded proteins is not restricted to *E. coli* but is common in other bacteria too. In *Bacillus subtilis* overexpression of insoluble proteins induces the so-called class I and class III heat shock genes (Jürgen et al. 2001; Mogk et al. 1998). Mogk et al. (1998) found non-native proteins to induce the CIRCE regulon controlled by the HrcA repressor. The authors supposed that high levels of non-native proteins titrate the GroE chaperonins, which prevents reactivation of the HrcA repressor and thus causes induction of class I heat shock genes. Jürgen et al. (2001) have confirmed these results for another recombinant protein and showed additional induction of clpP, clpC and clpE, belonging to the class III heat shock proteins. Interestingly, the authors proved that the Clp proteins ClpC, ClpP and ClpX are associated with the IBs

and also contribute to resolubilization of recombinant aggregates in *B. subtilis* (Krüger et al. 2000). In contrast to *E. coli*, the mRNAs of the proteases LonA and LonB are not induced; possibly the roles of these proteases are taken over in *B. subtilis* by the ClpP protease with its subunits ClpC or ClpX (Jürgen et al. 2001). An additional difference between the responses of *B. subtilis* and *E. coli* is the significant increase of the mRNA levels of genes encoding purine and pyrimidine synthesis enzymes (purB, purC, purM, pyrA, pyrD) and ribosomal proteins (rpsA, rpsB, rplJ) in *B. subtilis* (Jürgen et al. 2001).

5
Impact of the Production Process, the Host and the Target Protein on Aggregation

Heterologeous protein expression in *E. coli* often leads to a high local concentration of molecules with features the host cannot appropriately cater for (e.g. needed specific foldases, redox conditions, folding speed, posttranslational modifications; Schein 1989). The tendency for aggregation of the product thus depends on different factors such as strain, expression plasmid, temperature, pH, medium and compartment. Their corresponding impact is not predictable yet (Georgiou and Valax 1996; Mburu et al. 1999; Murby et al. 1996; Schein 1993; Strandberg and Enfors 1991).

5.1
Rate of Synthesis

The expression rate and the correct folding of the product are considerably determined by the level of gene induction, by the gene codon bias and by the mRNA stability (Pines and Inouye 1999). The rate of synthesis of a single polypeptide depends on the elongation rate in the translation process influenced by the codon bias (Sorensen and Pedersen 1991) as discussed before. Although the rate of translation has a profound effect on the *in vivo* folding reaction and the accessibility of chaperones, it has not been possible yet to tune the translation process; hence, in practice the rate of synthesis is mostly controlled at the induction level. Aggregation occurs mainly owing to application of high inducer concentrations (Kopetzki et al. 1989; Schein and Noteborn 1988) and at cultivation temperatures above 18–25 °C.

5.2
Cultivation Conditions

Cultivation conditions mainly influence the aggregation kinetics by varying the synthesis rate and concentration of the target protein within the host cell. Aggregation is caused by protein–protein interactions. The wrong intermole-

cular contacts are enhanced owing to the so-called crowding effect (van den Berg et al. 1999) characterized by competing interchain and intrachain interactions. Secreted proteins are less likely to aggregate because of the dilution within the periplasm (Schein 1993). Higher temperatures not only increase the speed of all involved reactions but essentially help to cross the thermodynamic threshold necessary for intermolecular reactions and thus aggregation. Basically all parameters (e.g. high cell density, high concentrations of the inducer and a short induction time) (Choi et al. 2000; Schein and Noteborn 1988; Strandberg and Enfors 1991; Wang et al. 1999) resulting in a high concentration of unfolded recombinant protein within the cell especially promote aggregation. Complex media favour the formation of IBs as well (Kopetzki et al. 1989). An optimized yield of IBs can be found by varying the expression level and the input of thermal energy.

5.3 Components of the Cultivation Medium

Certain ingredients of the medium (rich medium in particular) or substances added during the production process because of auxotrophic markers or in order to overcome bottlenecks and molecules produced by the cells themselves in response to the stress of synthesizing and hosting a recombinant target protein considerably affect the solubility of the product and have to be taken into consideration when aiming at IBs (Fahnert 2004). Those agents not only have an influence on the solubility but also on the folding of the target protein; however, as always, the effect is not predictable and can be either beneficial or unfavourable.

Since the outer membrane is highly permeable, components of the medium or added substances affect the periplasm directly and much more than the cytoplasm. Molecules smaller than 600 Da can freely diffuse into the periplasm of *E. coli* (Rosenbusch 1990). An application of this is varying the redox potential (Sect. 5.3) of the medium in order to establish different redox states within the periplasm. This leads to modified conditions promoting efficient redox shuffling and formation of correct disulfide bonds. Wunderlich and Glockshuber (1993) reported a fivefold increase in correctly folded target protein after adding reduced and oxidized glutathione to the medium.

The effects of many different other low molecular weight additives on recombinant proteins were studied and are described as well. Polyols and sucrose (Georgiou et al. 1994) have an impact on the folding owing to their increasing the viscosity of the solvent and the stability of protein solutions by excluded-volume effects. Added cofactors such as zinc (Baneyx et al. 1991), magnesium (Beck and Burtscher 1994) and calcium (Kurokawa et al. 2000) or single amino acids can cope with limits or directly affect the proteins. Glycine influences the folding of proteins prone to aggregation (Kaderbhai et al. 1997). L-Arginine increases the yield of native product (Schäffner et al.

2001; Winter et al. 2000) as do formamide, methyl formamide, acetamide, urea and urea derivatives among others (Schäffner et al. 2001).

Compatible solutes being osmoprotective towards the cells in their natural habitat also have a protein-stabilizing effect when present in the course of recombinant processes or when added to the medium. These substances (amino acids and their derivatives, sugars, polyols, quaternary amines and their sulfur analogues, sulfate esters, *N*-acetylated diamino acids, peptides) are water-soluble, uncharged at neutral pH and can be accumulated in high amounts. They affect the hydration of macromolecules and thus are considered chemical chaperones. The *in vitro* effect of betaine is comparable to that of the chaperone DnaK (Caldas et al. 1999).

Growth on glycerol (Kopetzki et al. 1989) or complex medium (Moore et al. 1993; Winter et al. 2000) can promote solubility and folding of the recombinant product. This effect of complex medium may be caused by an increased expression of foldases (Wei et al. 2001). Many other conditions (e.g. low pH; Kopetzki et al. 1989) and additives (ethanol; Georgiou and Valax 1996; Thomas and Baneyx 1997; Winter et al. 2000; 2-propanol; Kuivila 2002) even cause protein-protective stress responses.

5.4 Chaperones and Foldases

Recombinant protein production is an artificial stress situation for the host cell. In particular, the accumulation of target protein recognized as nonfunctional, unfolded, misfolded and/or aggregated molecules (e.g. IBs) induces the host's stress responses to cope with the situation (Gill et al. 2000b; 2001; Kosinski et al. 1992; Rinas 1996). This is comparable to the cell- and protein-protective heat shock response (Bahl et al. 1987; Dong et al. 1995; Ito et al. 1986; Parsell and Sauer 1989). The chaperones involved are mainly DnaK, DnaJ (Bukau 1993; Yura and Nakahigashi 1999) GrpE, GroEL and GroES (Dong et al. 1995; Ito et al. 1986). Foldases catalyse the formation of disulfide bonds and protein folding (see later). However, these molecules are not only naturally occurring; sometimes they are deliberately introduced (coexpression) into the recombinant production system to increase the level of the target protein or to overcome bottlenecks. This kind of strategy has to be carefully approached with respect to yielding IBs, because chaperones initially prevent aggregation of nascent chains and are even able to unfold misfolded proteins and dissolve aggregates (Glover and Lindquist 1998; Motohashi et al. 1999; Zolkiewski 1999). DnaK can selectively bind to misfolded proteins after having detected areas of four to five hydrophobic amino acids on the surface flanked by basic ones and can guide the protein to the degradation pathway (Bukau and Horwich 1998; Huang et al. 2001). DnaK acts comparably to a detergent by separating hydrophobic regions and hence solubilizing the denatured proteins. DnaK preferably attacks small aggregates

(Diamant et al. 2000). The impact of all chaperone actions depends on the folding environment and even more on the target protein. Again the outcome is not predictable (Nishihara et al. 2000). Chaperones are sometimes limited in their effect in the case of recombinant proteins aggregated owing to, for example, the average charge of the protein, the amount of cysteines and prolines, hydrophobic or hydrophilic parts or the size (Wilkinson and Harrison 1991). Thus, the simultaneous coexpression of a chaperone with the recombinant protein often does not lead to resolubilization. Timing is crucial. The rate-determining steps in folding are oxidation of cysteines (catalysed by disulfide bond forming proteins, Dsbs), the disulfide shuffling (*in vivo* catalysed by DsbC or protein disulfide isomerases, PDIs, in eukaryotic organisms) and the peptidyl–prolyl bond isomerization (by peptide prolyl isomerases, PPIs) (Jaenicke 1998; and see later). Accordingly the cellular processes of the host have to be considered. The complex regulation is far from being understood despite the well-known physiology of *E. coli*.

Coproduction of either DsbA or RotA has been found to increase the yield without preventing the formation of IBs (Joly et al. 1998; Knappik et al. 1993). A coproduction of DnaK and DnaJ was seen to promote solubility independent of the cultivation temperature, whereas GroEL and GroES did not impair IB accumulation above 30 °C (Thomas and Baneyx 1996).

The conflicting data demonstrate that although chaperones and foldases actually have an aggregation preventing or unfolding activity they are not necessarily successful in the suppression of IB accumulation per se. This is caused by the non-physiological character of recombinant protein expression. The host cell is overcrowded with protein often remarkably different to their own. Cellular systems are working at their limits. So the solubilizing effect of the chaperones depends on the target protein and the expression conditions.

5.5 Target Proteins with Disulfide Bonds

IBs can be the result of a lack of or incorrectly formed disulfide bonds since their correct formation is the rate-limiting step in the folding of disulfide bond containing proteins. The reaction requires an oxidizing redox potential of the environment. That is why disulfide bond containing proteins often accumulate as IBs in the reducing conditions in the cytosol of *E. coli*. The oxidizing periplasm is the actual compartment where disulfide bonds are formed. By changing the redox state of the cytoplasm from reducing to oxidizing a new opportunity for producing recombinant disulfide-bonded proteins is created. The prokaryotic cytoplasm is oxidizing in thioredoxin reductase mutant (*trxB*$^-$) strains. In wild-type strains thioredoxin reductase reduces thioredoxin. This then reduces proteins that have accidentally formed disulfide bonds in the cytoplasm in order to re-establish their function (Derman et al. 1993). In the mutants thioredoxin is accumulated in an oxidized

state; hence, it can act as a catalyst for the disulfide bond formation (Stewart et al. 1998). This alternative approach offers a higher production capacity and a different folding environment compared with the naturally oxidizing periplasm (chaperones, proteases, protein concentration, cofactors). The oxidizing effect of the cytoplasm of those mutant strains can even be enhanced by coexpression of thioredoxin or more oxidizing mutants thereof (Bessette et al. 1999). Whether the correct folding of a soluble recombinant target protein can be achieved in the naturally oxidizing periplasm or in the artificially oxidizing cytoplasm cannot be predicted or guaranteed. Therefore, also this system can be used for the production of a recombinant protein as IBs depending on its individual characteristics. In prokaryotes proteins mainly fold posttranslationally owing to the rapid translation. However, there is recent evidence that cotranslational folding of certain proteins might also be occurring (Cortazzo et al. 2002; Kolb et al. 2000; Ying et al. 2005). Contrarily, eukaryotic proteins are considered to fold cotranslationally in domains. This is discussed as being one reason for the often incorrect folding of eukaryotic proteins in the prokaryotic hosts (Ellis and Hartl 1999; Netzer and Hartl 1997). The tendency of IB formation is dependent on the number of cysteines among other parameters. According to this, the cysteine-bond-rich mammalian proteins are reported to be especially difficult to express solubly in *E. coli* (Wilkinson and Harrison 1991). The natural folding environment is also different in many other aspects. In eukaryotes the disulfide bonds are formed within the endoplasmic reticulum. It contains about 100 g of protein per litre. Permeases import ATP from the cytoplasm. It is needed by ATP-dependent chaperones (e.g. BiP, Grp94). Foldases different from the ones in the recombinant host are present as well. The PDIs (e.g. PDI, Ero1p) perform the disulfide shuffling and the PPIs (e.g. cyclophilin B) the peptidyl–prolyl bond isomerization, respectively (Jaenicke 1998). PDI has two active-site motifs (Cys-X-X-Cys, thioredoxin superfamily). One of them is oxidizing and the other one isomerizing. The reoxidation of PDI is probably performed by Ero1 (Debarbieux and Beckwith 1999). There are also the heat shock proteins Hsp70 (e.g. BiP), Hsp40 (e.g. Sec63p), Hsp90 (Grp94) and lectins (calnexin, calreticulin, both of them being glycoprotein chaperones) (Stevens and Argon 1999). Glycosylation improves solubility and is often a prerequisite of correct folding. Cotranslationally introduced glycans hand the protein over to the calnexin/calreticulin system and calreticulin increases the efficiency of glycoprotein folding by protecting the protein from aggregation (Herbert et al. 1996; Vassilakos et al. 1996). In the endoplasmic reticulum ATP is consumed synergistically with disulfide bond formation; there is recent proof that ATP-dependent chaperones (e.g. BiP, Grp94) act synergistically with foldases for oxidative folding (Hosoda et al. 2003; Mayer et al. 2000; Mirazimi and Svensson 2000).

In contrast the presence of ATP is unlikely in the prokaryotic periplasm (Braakman et al. 1992). The catalysis of the formation of disulfide bonds is

more complicated in the periplasm than in the endoplasmic reticulum, because its oxidizing milieu (redox potential) is affected by the environment of the cell. Small molecules are constantly diffusing through the cell envelope (Missiakas and Raina 1997a). The foldases of the periplasm are independent of ATP. Redox proteins catalyse the slow formation of disulfide bonds. These Dsbs also belong to the thioredoxin superfamily. DsbA oxidizes the thiol-groups of proteins within the periplasm and is reoxidized by DsbB. Spontaneous formation of disulfide bonds and even the effective catalysis performed by DsbA is at random. DsbC, DsbE and DsbG repair the non-native bonds and are reduced again by DsbD. It is not known so far when the disulfide bonds are formed cotranslationally or posttranslationally and whether the cysteines involved are chosen specifically (Bessette et al. 1999; Chung et al. 2000; Missiakas and Raina 1997a; Raina and Missiakas 1997; Rietsch et al. 1997; Rietsch and Beckwith 1998).

Owing to the known differences between disulfide bond formation in the natural eukaryotic environment of most target proteins and that in the recombinant prokaryotic host there is no doubt about the likelihood of misfolding and thus the occurrence of IBs in the host.

5.6 Fusion Proteins

Difficulties in recombinant protein production such as inefficient translation initiation and an incomplete removal of the starting methionine can be often solved by fusing the target protein to other proteins. Moreover, fusion proteins are also applied in basic research and biotechnology owing to their inherent adequacy as a purification and immobilization tool and a means of detection (Uhlen et al. 1992). Both eukaryotic (glutathione *S*-transferase from *Schistosoma japonicum*) and prokaryotic (protein A from *Staphylococcus aureus*; maltose binding protein, thioredoxin and DsbA from *E. coli*) proteins are used as partners (Lavallie and McCoy 1995; Winter et al. 2000). The addition of the fusion partner to the target protein enormously affects solubility and folding of the target and the resulting construct, respectively, depending on the nature of the partners. The fusion has to be chosen and constructed accordingly when IB production is aimed at.

The order of the fusion partners often determines the solubility of the product. An *N*-terminal fusion of maltose binding protein leads to a soluble expression in most cases, whereas IBs accumulate in the case of *C*-terminal fusions. The yield of the renaturation of those IBs is higher than that of the unfused target protein (Sachdev and Chirgwin 1998a, b, 2000).

A comparison of six different insoluble target proteins each fused to maltose binding protein, glutathione *S*-transferase or thioredoxin showed maltose binding protein to be the most effective in promoting solubility independent of the expression temperature. Thioredoxin was more effective than

glutathione *S*-transferase (Kapust and Waugh 1999). So the impact is not necessarily correlated with the relative size of the fusion partners. The aggregation preventing the influence of maltose binding protein is caused by both its molecular characteristics and its interaction with the target protein (Furukawa and Haga 2000; Kapust and Waugh 1999; Wang et al. 1999). Comparable to other periplasmic substrate binding proteins (hydrophilic with some hydrophobic amino acids) (Boos and Lucht 1996) maltose binding protein with or without its ligand bound has a chaperone-like effect (Richarme and Caldas 1997). Owing to its interaction with membranes, maltose binding protein is also able to contact the hydrophobic areas exposed by misfolded proteins (Martineau et al. 1990). This shows the different possibilities of how a fusion partner can interact with the target protein and impair IB formation. It cannot be predicted whether and how fusions will result in IBs. The effect has to be studied.

6
Production of IBs and Downstream Functionalization

6.1
Fermentation

The advantage of producing a protein in the form of IBs is that high product yields are commonly obtained and that the recombinant protein can be easily separated and purified. Furthermore, the optimization of the *in vitro* refolding conditions is straightforward.

Prior to fermentation a small-scale optimization procedure is done in shake flasks concerning the host strain and the medium. In connection with the host strain both *E. coli* K-12 strains, such as W3110 (Jensen 1997; Smith and Neidhardt 1983), and B strains, such as BL21 (Studier and Moffatt 1986), have been used. Specific mutants, for instance in connection with the stringent response, have also been included in the optimization procedure, such as K10; in contrast, auxotrophic strains are mostly avoided to allow the use of a simple standard fed-batch protocol.

In shake flasks IBs are mostly produced in complex media, although the yield and the purity of IBs are mostly not decreased in pure mineral salt media. These mineral salt media are beneficial if the production of IBs is considered on the fermentation scale (Fahnert et al. 2004). For high cell density cultivation a process on pure mineral salt medium with glucose or glycerol as the single carbon source is easier and optimization mostly is faster. However, IBs originating from cultivations with pure mineral salt medium may sometimes be more difficult to extract. Therefore, as proposed earlier, complex substrates may be beneficial if added from the beginning of the cultivation or around the point of induction (Fahnert et al. 2004).

The production of IBs in fed-batch fermentation cultivations is mostly highly efficient. Generally cell densities of 30–80 g l^{-1} dry cell weight (320 g wet weight) can be obtained with the addition of only glucose, ammonia and magnesium sulfate during the process.

In principle, most fed-batch fermentations for IBs on the laboratory scale can be carried out within 1 day; an exemplary process is shown in Fig. 3. The fermentation was started with a batch phase at 35 °C and an initial glucose concentration of 5 g l^{-1}. The pH was controlled so as not to drop below 7.0 by addition of 25% ammonia. Air flow (e.g. 0.02–2 vvm) and stirrer speed (e.g. 200–1500 rpm) were controlled in a cascade mode during the batch phase to keep the dissolved oxygen tension above 20%. The foam level was controlled by an antifoam electrode and addition of poly(propylene glycol) 2000. The medium contained all the other ions necessary for growth to high cell densities of above 100 g l^{-1} cell dry weight with the exception of $MgSO_4$ to avoid precipitation of insoluble magnesium phosphates. Therefore, a 1 M solution of $MgSO_4$ was intermittently added to the cultivation by injecting 2 ml l^{-1} for every 2.5 g of cell dry mass additional growth. The batch phase ended when the initially supplied glucose was exhausted at an optical density at 500 nm of about 10, corresponding to about 2.5 g l^{-1} cell dry weight. The fed-batch was performed by continuous feeding of a concentrated glucose solution at a constant rate. The

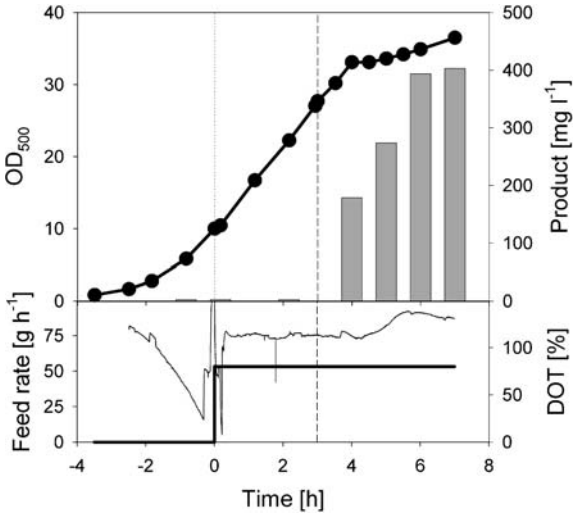

Fig. 3 Fed-batch fermentation for production of IBs with *E. coli* K-12 RB791. The process consists of three phases. An initial batch phase is followed by a constant feeding of a concentrated glucose solution (*thick line in lower graph*) started at 0 h. During the fed-batch phase cell growth proceeds quasilinearly. Isopropyl-*β*-*d*-thiogalactopyranoside was added 3 h after the start of feeding to induce product formation (*grey bars*). The *lower graph* shows the dissolved oxygen tension (*DOT*, *thin black line*) indicating by the increasing DOT that respiration declines at about 2 h after induction

synthesis of the recombinant product was induced 3 h after the start of feeding in the same way as evaluated during the optimization procedure in the shake flasks and the culture was harvested at about 4 h after induction.

The process shown as an example may be applied for most IB processes, with minor modifications. Generally it is possible to increase the cell mass by prolongation of the batch phase, which can be done by addition of 20 $g\,l^{-1}$ of initial glucose. In this case also the feeding rate in the glucose-limited fed-batch can be increased. Also more sophisticated feed profiles, such as exponential feeding, pH-stat or pO-stat principles can be applied.

In view of the discussion when induction should be performed, IB processes generally are less sensitive than processes which aim for a soluble product. In many cases the strong induction which is beneficial for IBs leads to a decrease in cell growth and even may result in cell death. Therefore, induction at higher cell density may provide a higher yield. However, good production is generally only obtained if the specific growth rate at the point of induction is above 0.1 h^{-1}.

For most strong expression systems the fermentation is stopped 2–5 h after induction; however, this can vary depending on the expression system used, the protein of interest, and product stability.

6.2 IB Isolation and Purification

IBs obtained by cytosolic microbial overexpression of a recombinant protein are large particles with an amorphous manifestation. Since IBs are characterized by a relatively high specific density of about 1.3 $mg\,ml^{-1}$ (Mukhopadhyay 1997), they can be harvested after cell lysis by centrifugation (Taylor et al. 1986). The first step of this isolation procedure is maximal lysis of the *E. coli* cells by lysozyme treatment, subsequent repetitive high-pressure dispersion and, finally, incubation with a detergent such as Triton X-100 and high salt (Rudolph et al. 1997). Provided that the initial level of expression is sufficiently high, collection of the IBs by centrifugation or filtration generally yields a rather homogeneous preparation. It may contain the recombinant protein with a purity of up to 90% (Rattenholl et al. 2001a). However, certain host-cell proteins, such as elongation factor EF-Tu, outer-membrane proteins or small heat shock proteins may be enriched in IB isolates (Allen et al. 1992; Hart et al. 1990). These impurities may derive from coprecipitation upon overexpression of the foreign gene product. Most of the impurities commonly identified in IB isolates, however, may originate primarily from incomplete cell lysis and removal of particulate host-cell material.

Therefore, if the recombinant expression and IB isolation result in recombinant material with a high degree of impurities, these IBs can be further purified by additional washing steps. These may utilize EDTA, low concentrations of denaturant such as urea or GdmCl, and detergents such as

Table 1 Purification of inclusion body (*IB*) material

Recombinant protein	Washing step	Refs.
Human prourokinase	0.1% Triton X-100	Orsini et al. (1991)
Arginine deiminase	4% Triton X-100	Misawa et al. (1994)
Human *t*-PA	2% Triton X-100, 5 M urea	Sarmientos et al. (1989)
Horse radish peroxidase	2 M urea	Smith et al. (1990)
Human IGF-1	0.5% sarcosyl	Noguchi et al. (1996)
Bovine growth hormone	2% deoxycholate	Langley et al. (1987)
N-terminal domain of PTH receptor	1.5% LDAO	Grauschopf et al. (2000)

LDAO lauryldimethylamine oxide, PTH parathyroid hormone, *t*-PA tissue plasminogen activator

Triton X-100, deoxycholate, octylglycoside and sarcosyl (Table 1). Furthermore, IBs in their particulate state can be purified by gel filtration.

Alternatively, the recombinant protein can be purified from IBs after solubilization. This is particularly attractive if the protein is expressed as a fusion protein with a purification tag such as a His tag. In this case purification can be achieved by immobilized metal affinity chromatography (IMAC) under denaturing conditions (Grauschopf et al. 2000; Hochuli et al. 1987). More generally, reversed-phase high-performance liquid chromatography may be applied for chromatographic purification of solubilized IBs.

6.3 Solubilization of IBs

In general, IBs do not readily disintegrate under physiological solvent conditions. In most cases for solubilization of IBs rather strong denaturants such as GdmCl are exploited. The denaturants are usually employed at high concentrations (6–8 M) to ensure complete solubilization and unfolding of the IB proteins. However, in a few cases denaturants at low concentrations (1–2 M) proved to be more efficient since other impurities in the particulate fraction were not solubilized under these mildly denaturing conditions (Li et al. 1999).

Another class of denaturants solubilizing IBs are detergents. Cetyltrimethylammonium salts (cetyltrimethylammonium chloride or cetyltrimethylammonium bromide) have been used for solubilization of IBs of human growth hormone (Patra et al. 1998; Puri et al. 1992). Even though detergents might be advantageous in some cases compared with GdmCl and urea, one has to keep in mind that they may interfere with the following protein renaturation and subsequent purification. Furthermore, a variety of other methods can be used for solubilization of IBs (De Bernardez 2001; Vallejo and Rinas 2004).

In the case of proteins containing cysteines, the isolated IBs usually contain a certain amount of non-native disulfide bonds (Schoemaker et al. 1985), which reduce their solubility in the absence of reducing agents. Addition of thiol reagents such as dithiothreitol, glutathione, cysteine or mercaptoethanol in combination with chaotrophs allows reduction of the disulfide bonds by thiol–disulfide exchange (Fischer et al. 1992). Since the reactive species in thiol–disulfide exchange is the thiolate anion, IB solubilization in the presence of reducing agent is usually performed under mild alkaline conditions.

6.4 Refolding of Proteins from IBs

Refolding of solubilized IB proteins requires removal of the denaturant. Most protocols for renaturing proteins apply dilution of the denatured protein in the renaturation buffer. By this method the protein environment is switched to native conditions immediately. Furthermore, dilution of the denatured protein does not only result in low denaturant concentrations but also in low protein concentration during refolding. In some cases, however, other protocols for removing the denaturant, such as dialysis, gel filtration or matrix-assisted folding on an IMAC column, have been established successfully (Fahey et al. 2000a, b; Grauschopf et al. 2000; Rogl et al. 1998).

The most prominent unproductive side reaction of renaturation is aggregation. Since aggregation is a process of second-order or higher-

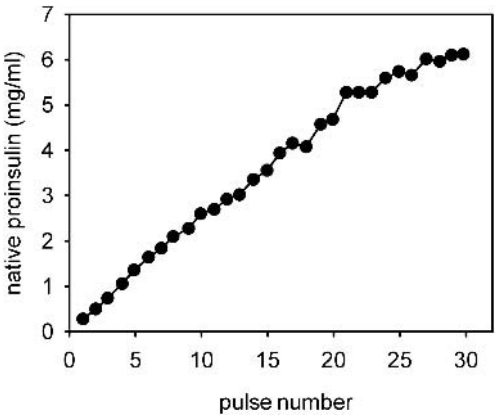

Fig. 4 Stepwise renaturation of proinsulin. Denatured and reduced proinsulin was refolded at 15 °C by stepwise addition of the protein to the refolding buffer [10 mM tris(hydroxymethyl)aminomethane, 10 mM glycine, 1 mM EDTA, pH 10.5, 0.5 mM cysteine, 4.5 mM cystine]. The final concentration was 500 $\mu g\,ml^{-1}$ per pulse. After every 30 min samples were taken, analysed by reversed-phase high-performance liquid chromatography (•) and new protein was added to the renaturation buffer. Thirty pulses were performed. (Adapted from Winter et al. 2002)

order reaction kinetics (Zettlmeissl et al. 1979) low protein concentrations ($10–100\ \mu g\ ml^{-1}$) are strictly required for efficient renaturation. This, however, leads to large reaction volumes in order to produce quantities of renatured IB proteins. To circumvent this technical problem, a stepwise renaturation procedure can be applied (Fig. 4). Because completely folded proteins are usually not prone to coprecipitation with folding proteins, high yields of renatured protein per volume of refolding buffer can be obtained by slow continuous or discontinuous addition of the denatured protein to the refolding buffer (Buchner et al. 1992; Fischer et al. 1992; Winter et al. 2002).

6.5 Disulfide Bond Formation During Protein Renaturation

Folding proteins with concomitant disulfide bond formation include the formation of both the native tertiary structure of the molecule stabilized by non-covalent interactions and the covalent disulfide bonds.

The simplest method promoting disulfide bond formation is oxidation by molecular oxygen, catalysed by metal ions such as Cu^{2+} (Ahmed et al. 1975; Builder et al. 1998). Because of the low efficacy of disulfide bond formation by oxidation with molecular oxygen, thiol–disulfide exchange reactions with low molecular weight thiols in reduced and oxidized form are generally employed for protein disulfide bond formation. Examples for such redox couples are listed in Table 2. Because thiol–disulfide exchange reactions are rapidly reversible, oxido-shuffling reagents increase both the rate and the yield of correct protein disulfide bond formation by rapid reshuffling of improper disulfide bonds.

Table 2 Oxidative protein renaturation

Protein	Thiol	Disulfide	Refs.
Fab fragment	5 mM GSH	0.5 mM GSSG	Buchner and Rudolph (1991a)
N-terminal domain of PTH receptor	5 mM GSH	1 mM GSSG	Grauschopf et al. (2000)
Fab fragment	0.5 mM GSH	Mixed disulfides	Buchner and Rudolph (1991a)
Fab fragment	3 mM DTT	4 mM GSSG	Lilie et al. (1994)
Human *t*-PA	2 mM GSH	Mixed disulfides	De Bernardez et al. (1999)
Human *t*-PA	2 mM GSH	0.2 mM GSSG	Kohnert et al. (1992)
Human IL2	10 mM GSH	1 mM GSSG	Tsuji et al. (1987)
Proinsulin	0.5 mM cysteine	4.5 mM cystine	Winter et al. (2002)

DTT dithiothreitol, *GSH* reduced glutathione, *GSSG* oxidised glutathione

Another approach of oxidative refolding uses a two-step mechanism. Firstly, all cysteines in the denatured protein are converted to mixed disulfides with glutathione. In the subsequent renaturation step, formation of the correct disulfide bonds is catalysed by adding catalytic amounts of the low molecular weight thiol in its reduced form (De Bernardez et al. 1999).

6.6 Improvement of Renaturation

Most large, multidomain proteins refold *in vitro* only with low yields, since unproductive side reactions (especially aggregation) compete with proper folding. In this case, the yield can be improved by speeding up rate-determining folding steps, decelerating aggregate formation and/or destabilizing off-pathway products. This can be achieved by optimizing the folding conditions with respect to buffer composition, ionic strength, pH, folding time, temperature, protein concentration, cofactors, and, in the case of disulfide-bonded proteins, additives which promote disulfide bond formation.

Upon *in vitro* folding of disulfide-bonded as well as non-disulfide-bonded proteins, the yield of correct folding can be improved by supplementing the refolding buffer with low molecular weight additives (Table 3). As shown for numerous proteins, the yield of correct folding can be improved tremendously by adding the amino acid L-arginine to the refolding buffer in relatively high molar concentrations (Buchner et al. 1992; Buchner and Rudolph 1991a; Grauschopf et al. 2000; Lin and Traugh 1993; Rattenholl et al. 2001b). Although containing a guanidino group, arginine has only a minor effect on protein stability. As shown for RNase A arginine only slightly destabilizes the native protein conformation (Lin and Timasheff 1996). On the other hand, this additive strongly enhances the solubility of folding intermediates and decreases the kinetics of aggregation (Reddy et al. 2005; Baynes et al. 2005).

In some cases, additives which strongly stabilize native protein structures are essential for successful folding. Human placental alkaline phosphatase, for example, could only be refolded *in vitro* in the presence of stabilizers such as sulfate or carbohydrates (De Bernardez et al. 1999). For bovine carbonic anhydrase B, stoichiometric amounts of poly(ethylene glycol) have been found to improve *in vitro* structure formation (Cleland et al. 1992). In this case, poly(ethylene glycol) inhibits aggregation by complex formation with a molten globule folding intermediate which is otherwise prone to aggregation.

Other low molecular weight additives such as non-denaturing concentrations of urea or GdmCl, detergents and mixed micelles have been found to promote protein renaturation (De Bernardez et al. 1999). The last ones bind to folding intermediates, thus preventing aggregation. Proper folding requires

Table 3 Renaturation procedure for IB proteins (see text for references)

Step	Conditions
IB solubilization	2 h incubation at 20 °C in 6 M GdmCl 100 mM DTT (or DTE) 0.1 M Tris-HCl, pH 8 $c_p \approx 5$ mg ml^{-1}
Removal of reductant	adjust pH to approximately pH 4.5 dialyse against 6 M GdmCl, pH 4.5
Folding	1 : 200 dilution in Tris-HCl buffer, pH 7.5[a] or pH 8.5[b], 15 °C, 5 mM EDTA in the presence of additives such as – no additive – 0.5 M L-arginine – 1 M Tris – 0.5 M GdmCl – 0.06 mg ml^{-1} laurylmaltoside – 33 mM Chaps – mixed micelles consisting of Triton X-100 and phospholipids – 20% glycerol – metal ions, ligands, etc.[c]

Chaps 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, *DTE* dithioerythritol, *GdmCl* guanidinium chloride, *Tris* tris(hydroxymethyl)aminomethane

[a] For proteins containing cysteines but no disulfide bonds in the native state, add 2 mM DTE to folding buffer.

[b] For proteins containing disulfide bonds in the native form, add 5 mM GSH and 1 mM GSSG to the folding buffer.

[c] If the native protein contains metal ions, EDTA should be omitted from the folding buffer. For refolding add a fivefold molar excess of the respective metal ion to the folding buffer. Similarly, add other ligands that bind to the authentic native protein.

the release of the detergent from the folding intermediate, which is facilitated by the extraction with cyclodextrin (Rozena and Gellma 1995). In this context one has to consider that cyclodextrin also interacts with bulky hydrophobic amino acid side chains (Cooper 1992; Machida et al. 2000; Sharma and Sharma 2001); therefore, cyclodextrin may also increase the solubility of folding intermediates.

As for protein purification, the optimum conditions for *in vitro* folding have to be established on a case-by-case basis. The first round of the development of a folding process comprises a crude variation of the folding conditions such as various additives, protein concentration, pH, temperature, time and ionic strength. The second round of process development involves the fine-tuning of the solvent conditions. After careful optimization, *in vitro* folding of any recombinant protein deposited in IBs will likely be successful.

6.7 Industrial Processes Based on Refolding of IB Proteins—Human *t*-PA

t-PA is a serine protease of the fibrinolytic pathway. It catalyses the proteolytic conversion of plasminogen to the active protease plasmin, which degrades fibrin clots. *t*-PA is a polypeptide of of 527 amino acids which are arranged in five structural domains: a finger domain, an EGF-like domain, two kringle domains and the proteolytic active domain (Ny et al. 1984). *t*-PA contains 35 cysteine residues that form 17 disulfide bonds. Thus, this complex molecule exemplifies all the challenges for protein renaturation. In Fig. 5 the overall process of production of recombinant *t*-PA is summarized. This procedure is based on the *in vitro* renaturation of *t*-PA according to the techniques described before. Essential features of the renaturation comprise the formation of mixed disulfides of the denatured protein with glutathione, the use of molar concentrations of L-arginine as a low molecular weight folding enhancer and a stepwise renaturation protocol (Rudolph et al. 1994; Rudolph and Fischer 1990). These combined techniques allowed high-yield production of *t*-PA from IBs.

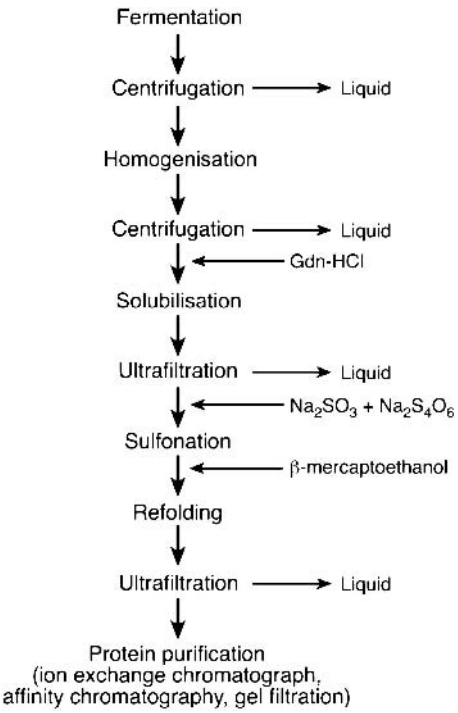

Fig. 5 The production process of tissue plasminogen activator (adapted from Datar et al. 1993)

7 Outlook

Protein aggregates accumulating after expression of target proteins in bacteria have been considered disadvantageous for a long time since the protein does not fold into its native state. However, today the wide range of modern refolding techniques allow for a straightforward optimization of the refolding process, thereby including high-throughput screening tools (Scheich et al. 2004), which may be more straightforward than trying to optimize the cellular system to especially produce complex protein in its active form.

Protein IB has also developed into a highly interesting field of research in recent years. The whole network of protein folding in a cell cannot be understood without considering aggregation reactions. Furthermore, IBs have been shown to share some similarity with amyloid fibrils found in human disorders and therefore might be interesting and easily accessible study objects for the underlying aggregation processes. Thus, IBs are no longer a dead end of inactive proteins, but are a fascinating topic for academic and applied research.

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Part II
Cameos of Selected Additional Inclusions

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Vacuoles

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Abstract Within the filamentous sulfur bacteria there are giant forms with cell diameters of up to several hundred microns. Members of this monophyletic group of bacteria possess a central vacuole, which restricts the active cytoplasm to a thin outer layer. Most of these sulfur bacteria seem to store nitrate in the vacuole, which they use as an electron acceptor for the oxidation of sulfide.

1 Phylogenetic Relationship of Sulfur Bacteria with Vacuoles

Bacterial vacuoles are found in three different genera of sulfur bacteria: *Thioploca*, Beggiatoa and *Thiomargarita* (Maier and Gallardo 1984; Nelson et al. 1989; Schulz et al. 1999). Within these three closely related genera the vacuolated species form one monophyletic cluster among the smaller not vacuolated species of all three genera (Teske and Nelson 2004). Additionally, there are reports of attached vacuolated filamentous sulfur bacteria, which apart from the vacuole resemble morphologically *Thiothrix*. According to their 16S ribosomal RNA (rRNA) sequence these bacteria are the closest relatives to the other vacuolated sulfur bacteria and are only remotely related to the smaller *Thiothrix* species (Kalanetra et al. 2004). Thus, judging from the 16S rRNA sequences available at this moment, the morphological feature of vacuoles in sulfur bacteria has only evolved once.

2 Morphology of Sulfur Bacteria with Vacuoles

The vacuolated sulfur bacteria can reach enormous cell diameters of several hundred microns (Jannasch et al. 1989; Schulz et al. 1999; Fig. 1a) but

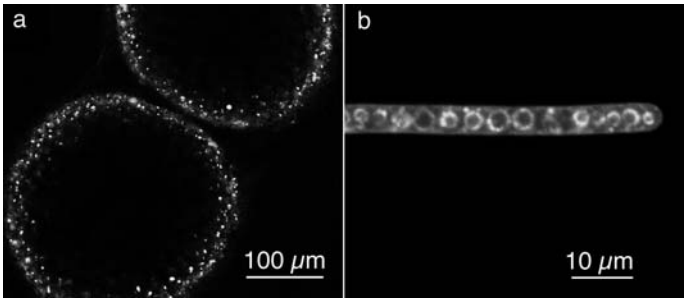

Fig. 1 Confocal laser micrographs of large sulfur bacteria stained for protein. **a** *Thiomargarita namibiensis*: most of the cell appears hollow owing to the large vacuole. About 98% of the cell is taken up by the vacuole. *Bright spots* are reflections from sulfur inclusions. **b** A *Beggiatoa* filament with small circular vacuoles. Only about 10% of the volume of the individual cells is taken up by the vacuole

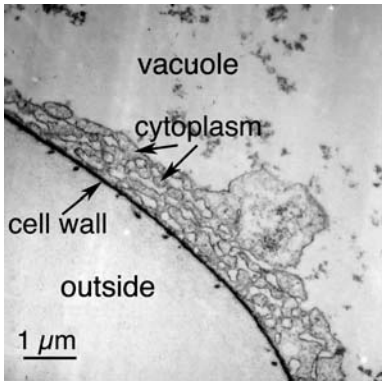

Fig. 2 Transmission electron micrographs showing the spongy cytoplasm surrounding the vacuole of *T. namibiensis* (courtesy of Mariona Hernández Mariné)

the actual cytoplasm is typically restricted to a thin outer layer of 0.5–2 μm. Thus, apart from their large size, the cells are not diffusion-limited as only a thin layer of cytoplasm is actually metabolically active (Schulz and Jørgensen 2001). Also smaller filaments of *Beggiatoa* or *Thioploca* with diameters of more than 3 μm may contain less conspicuous vacuoles (Maier and Murray 1965; Fig. 1b). The vacuole is separated from the cytoplasm by a bilayered membrane, which might originate from an intrusion of the cytoplasmic membrane (Maier et al. 1990). In larger sulfur bacteria the cytoplasm tends to lose continuity and appears as a spongy net surrounding the empty void of the vacuole (Maier et al. 1990; Fig. 2).

3
Vacuoles Used for Nitrate Storage

In *Thioploca*, *Beggiatoa* and *Thiomargarita* cells with vacuoles were found to contain internally accumulated nitrate in very high concentrations of up to 800 mM (Fossing et al. 1995; McHatton et al. 1996; Schulz et al. 1999). This observation led to the conclusion that the vacuole is used to store nitrate. Nevertheless, in the attached, vacuolated filaments resembling *Thiothrix* no internal nitrate could be detected (Kalanetra et al. 2004). Thus, even though they are closely related to the other vacuolated sulfur bacteria, the vacuole of these attached filamentous bacteria must serve a different, yet unknown purpose.

Nitrate may be used as an alternative or as the only electron acceptor by sulfide-oxidizing bacteria. The accumulation of nitrate in high concentrations enables *Thioploca* and *Beggiatoa* to take nitrate as an electron acceptor down into the sediment until they find sulfide, their electron donor, which they can also store internally as elemental sulfur. Because *Thioploca* and *Beggiatoa* are able to store both electron acceptors and electron donors in high amounts and shuttle between the surface of sediments where they find nitrate, and greater depths, where sulfide is available, they can monopolize this energy source, which is only available for larger bacteria that can store nitrate (Fossing et al. 1995; Jørgensen and Gallardo 1999; Schulz and Jørgensen 2001). *Thiomargarita*, the largest known bacterium, has an even greater capacity to store nitrate, but in contrast to the filamentous *Beggiatoa* and *Thioploca* it is not motile. These giant bacteria seem to rely on passively waiting until their loose sediment gets suspended in nitrate-containing water to regain access to nitrate (Schulz et al. 1999).

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The Unique Role of Intracellular Calcification in the Genus *Achromatium*

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Abstract *Achromatium* are found in freshwater and brackish sediments, where, as giant sulfur oxidisers, they play a key role in the carbon and sulfur cycles of the sediments they inhabit. The most striking feature of this genus is its enigmatic precipitation of intracellular calcite. Past explanations for this process have included the dissolution of stored calcite to regulate acidity generated by H_2S oxidation, the use of calcite as a buoyancy-regulating mechanism, the use of calcite as an electron acceptor source in "carbonate respiration", and the use of calcification to generate CO_2 for carbon fixation. However, more recent in situ physiological studies and detailed characterisation of the environments inhabited by these organisms have indicated a possible role for intracellular calcification in the dissolution of sulfide minerals. It is proposed that this unique adaptation of *Achromatium* is a means of overcoming a challenge not faced by other giant sulfur bacteria, namely inherently low levels of free sulfide in their sedimentary environment.

1 Introduction

The involvement of bacteria in the precipitation of calcium carbonate (calcification) is widespread. This is because bacterial activities which increase pH and release bicarbonate as a by-product cause oversaturation with respect to carbonate. Extracellular polymeric substances, cell walls and external

sheathes then provide nucleation sites which facilitate the precipitation process (Wright and Oren 2005). Processes such as ammonification, dissimilatory nitrate reduction, degradation of urea or uric acid and sulfate reduction all, in this way, indirectly facilitate extracellular calcification. Photosynthetic bacteria cause extracellular calcification by the uptake of bicarbonate, release of OH^- ions and consequent formation and precipitation of carbonate from solution. However, there is only one bacterial group that produces intracellular precipitates of calcite. These are the giant uncultured sulfur-oxidising bacteria of the genus *Achromatium*.

Achromatium are found in freshwater and brackish sediments, where they play a key role in the carbon and sulfur cycles of the sediments they inhabit (Gray et al. 1997, 1999a; Head et al. 2000). The most striking feature of this genus is its enigmatic precipitation of intracellular calcite. Despite descriptions of *Achromatium* from as early as 1893 (Schewiakoff 1893), the purpose of intracellular calcification has not been satisfactorily explained. In this review, ecological, physiological and geochemical data on *Achromatium* and its habitats are reassessed. By comparison of the data with the ecology of other colourless sulfur bacteria and by reference to intracellular calcification by other organisms, various past theories and one new theory for the role of calcification in *Achromatium* are discussed.

2
Intracellular Calcite Inclusions in *Achromatium* spp.

Intracellular inclusions, which have been shown by X-ray diffraction analysis to contain calcite, constitute a large part of the volume of *Achromatium* cells (Head et al. 1996). With use of confocal microscopy it has been estimated that the mean calcium content of an individual cell is 2.62 ± 0.34 ng cell^{-1} (mean $\pm$ standard error). Scanning electron microscopy images of *Achromatium* cells have revealed that the size, number and distribution of calcite inclusions vary greatly between individual organism (Head et al. 1996); however, even within individual cells, calcite inclusions vary in size (Fig. 1a, b). This variability in inclusion size suggests that calcite precipitation is a continuous process in active *Achromatium*, a finding which was supported by microautoradiographic analysis of bicarbonate uptake, which demonstrated the rapid incorporation of radiolabelled bicarbonate into the calcite component of the cell carbon (Gray et al. 1999b). Thin-section transmission electron microscopy images, which highlight the internal structure of individual inclusions (Fig. 1c), reveal a laminated structure, a possible membrane and an electron-dense central nucleation point. These features, along with the intensity ratios of D spacings in the X-ray diffraction data, suggest that the calcite is not purely crystalline and is thus precipitated under strict biological control.

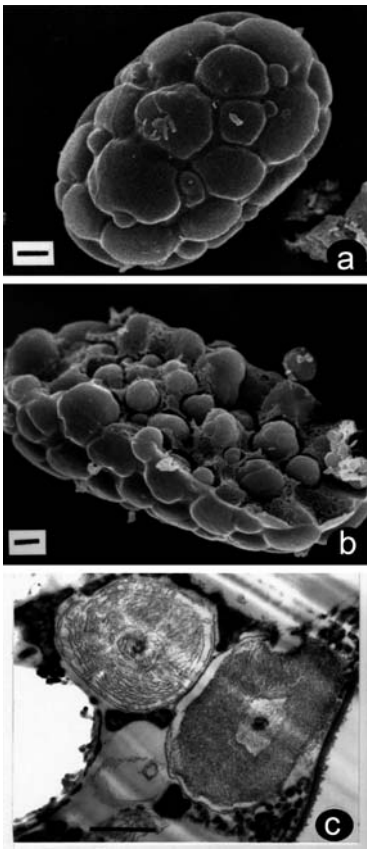

Fig. 1 Scanning and thin-section transmission electron micrographs of *Achromatium* cells from the Rydal Water sediment. **a** Scanning electron micrograph of an oval cell showing calcite inclusions of varying size. The *scale bar* represents 1.25 μm. **b** Scanning electron micrograph of an elongated cell disrupted by grinding in a pestle and mortar showing larger calcite inclusions and smaller sulfur inclusions. The *scale bar* represents 1.25 μm. **c** Transmission electron micrograph of an ultrathin section of an *Achromatium* cell showing two intact calcite inclusions with a characteristic laminated internal structure and probable central nucleation site. The *scale bar* represents 0.625 μm. (Adapted from Head et al. 2000 with the kind permission of Springer)

Although the mechanism of calcification in *Achromatium* is unknown, a number of its features can be deduced from stoichiometric and physiological considerations. For instance, the ability of *Achromatium* cells to precipitate calcite internally is likely to be dependent on the ability to scavenge available Ca^{2+} from the surrounding pore waters (it is assumed that bicarbonate is not limiting in most natural waters). The occurrence of calcite-containing *Achromatium* cells in environments with fairly low dissolved calcium concentrations has been noted in a number of freshwater sediments (Nadson and

Visloukh 1923). Accordingly, it has been inferred that *Achromatium* species must possess an active mechanism for concentrating this cation from the environment (Head et al. 2000). Calcium ATPase mechanisms appear to underlie most biological calcification (McConnaughty and Whelan 1997). For instance, coccolithophorid algae exhibit high Ca^{2+}-dependent V- and P-type ATPase activities (Kwon and González 1994; Araki and González 1998) and a gene sequence of the *vap* subunit of a V-ATPase has been recovered from the calcifying coccolithophorid *Pleurochrysis carterae* (Corstjens et al. 2001). Ca^{2+} ATPases use the energy of ATP hydrolysis to exchange Ca^{2+} ions for protons and, typically, either two or four protons are exchanged for every Ca^{2+} ion transported. In *Achromatium* the formation of calcite most likely occurs by reaction of the accumulated Ca^{2+} with carbonate, but not, it seems, by direct reaction of Ca^{2+} with bicarbonate. This is because calcite is an ionic compound and can only be formed from its component ions (Wright and Oren 2005). On this basis, before calcification can take place, bicarbonate, which is the predominant form of inorganic carbon in near-neutral pH environments, must be converted to carbonate. The possible mechanism and ecological meaning of calcification in *Achromatium* is discussed in more detail later. However, it is clear that the precipitation of intracellular calcite by *Achromatium* is carried out at an energetic cost to the organisms.

3 The Habitat and Physiology of *Achromatium* spp.

An essential requirement for understanding the role of calcification in *Achromatium* is a deeper understanding of the relationship of these organisms with their geochemical environment. *Achromatium* spp., which form a phylogenetically coherent group within the γ-*Proteobacteria* (Gray et al. 1999a), typically, inhabit littoral freshwater sediments. Here, regardless of sulfate-reduction rates and sulfate concentrations, high levels of reactive iron (as oxides of ferric iron and dissolved Fe^{2+}) serve to maintain low levels of dissolved sulfide (less than micromolar quantities) within the sediment (Gray et al. 1997; Gray and Head 1999; Head et al. 2000). These low sulfide conditions are unlike those typically encountered in environments which harbour other genetically related giant sulfur bacteria, where dissolved sulfide is abundant (Schultz et al. 1999).

Studies of the redox chemistry of *Achromatium* sediments have provided an insight into the ecology of these organisms. In one *Achromatium*-dominated freshwater sediment (Rydal Water) in the English Lake District, profiles of pore water sulfate exhibited a maximum at 4 mm. Below this depth sulfate became depleted, accompanied by concomitant increases in precipitated pyrite, sulfur (chromium-reducible sulfur) and iron monosulfides (acid volatile sulfur) (Gray et al. 1997; Fig. 2). This evidence of active sulfate reduc-

tion and the finding that the *Achromatium* constituted 90% of the bacterial biovolume was indicative of the rapid recycling of reduced sulfur (Gray et al. 1997). The role of *Achromatium* in this oxidative process was confirmed by studies which showed that in sediment microcosms, in which sulfate reduction had been inhibited by sodium molybdate, sulfate accumulation was directly proportional to the size of the *Achromatium* population (Gray et al. 1997). Additionally, microautoradiographic studies showed that in uninhibited sediment microcosms ^{35}S-labelled sulfate was rapidly incorporated by *Achromatium* cells into intracellular elemental sulfur (Gray et al. 1999b).

Studies of the depth distribution of *Achromatium* cells in relation to redox-sensitive chemical species showed that they are distributed throughout the zone of sulfate reduction (Gray et al. 1997; Fig. 2). Furthermore, it has been demonstrated, by measurements of sediment oxygen profiles, that a large proportion of *Achromatium* resided below where oxygen was detectable (Head et al. 1996). Recent microcosm experiments (Gray et al. 2004) have confirmed this microaerophilic/anaerobic nature by demonstrating that all the *Achro-*

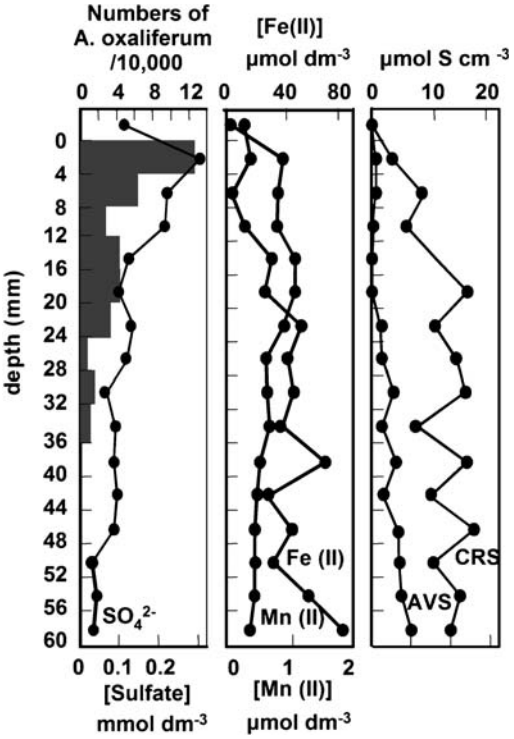

Fig. 2 The vertical distribution of *Achromatium*, dissolved Fe(II), Mn(II), chromium-reducible sulfide (*CRS*) and acid volatile sulfide (*AVS*) in a sediment core from Rydal Water. *Grey bars* denote cell numbers. (Adapted from Gray et al. 1997 with the kind permission of the American Society for Microbiology)

matium spp. present in the Rydal sediment are, at the very least, facultative nitrate reducers.

Ecophysiological studies on *Achromatium* have, therefore, amply demonstrated that despite limitations imposed on their sulfide oxidising potential, i.e. very low levels of dissolved sulfide, *Achromatium* spp. have a clearly defined role in the carbon, sulfur and nitrogen cycles of their environment. However, these organisms cannot rely on the diffusive flux of sulfides as do other giant sulfur bacteria, so it has been suggested that *Achromatium* spp. utilise other reduced sulfur resources (Gray et al. 1997), namely precipitates of iron sulfides that have been shown to form in these iron-rich environments. How this process of sulfide dissolution and take up might be achieved is discussed in more detail later.

4
Past Interpretations of Calcification in *Achromatium* and the Role of Intracellular Calcite Inclusions in the Coccolithophorid Algae

A number of hypotheses relating to the role of intracellular calcification in *Achromatium* have been proposed. For example the dissolution of stored calcite to regulate acidity generated by H_2S oxidation, the use of calcite as a buoyancy-regulating mechanism and the use of calcite an electron acceptor source in "carbonate respiration" (La Rivière and Schmidt 1992; Babenzein 1992; Head et al. 2000). Based on a clearer understanding of the habitat and physiology of *Achromatium* these past interpretations of calcification can now be critically examined. For instance, the use of calcite by *Achromatium* to regulate acidity generated by H_2S oxidation is an unlikely requirement in non-sulfidic, near-neutral, naturally buffered freshwater systems and the precipitation and dissolution of calcite for buoyancy regulation in sediments (Babenzien 1992) is inconsistent with observations which show an inverse relationship between the quantity of calcite and the depth within the sediment (Head et al. 2000). Additionally, the use of calcite as a source of carbonate as an electron acceptor is unlikely when it is considered that *Achromatium* spp. are closely related to other O_2- or NO_3^--consuming sulfur bacteria, not methanogenic Archaea. Perhaps more significantly, these organisms have been shown to carry out nitrate-dependent sulfur oxidation (Gray et al. 1997, 1999a, 2004).

Recently and, more plausibly, it has been proposed that *Achromatium* spp. deposit intracellular calcite to maintain a high internal partial pressure of CO_2 to facilitate carbon fixation by the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) (Head et al. 2000). This proposed use of calcification has also been suggested for the coccolithophorid algae, for example *Emiliania huxleyi* and *Pleurochrysis carterae*. For these unicellular marine algae it is thought that under conditions of high pH, low dissolved CO_2 and high dissolved oxygen concentrations, bicarbonate is converted to CO_2 by the action

of the enzyme carbonic anhydrase (Borowitzka 1982) (Eq. 1) or by export of protons for the extracellular conversion of bicarbonate to CO_2, which is then assimilated (McConnaughty and Whelan 1997). As a consequence, calcification is required to neutralise a raised cytoplasmic pH through the direct or indirect reaction of bicarbonate with hydroxyl ions to form carbonate (Eq. 1), which then precipitates as calcite (Eq. 2). This calcification process occurs within a membrane-bound compartment termed the coccolith-containing vesicle. Support for this mechanism by which calcification is linked to photosynthesis comes from the tight coupling of inorganic and organic carbon produced in the cell, i.e. an approximately one-to-one relationship (Crawford and Purdie 1997) and the fact that calcification rates are affected by light intensity and changes in CO_2 partial pressure. However, an unequivocal link between calcification and photosynthesis has not yet been provided (Paasche 2001) and it has been suggested that calcification and, in some cases its regulation, may impart other benefits to these organisms, for example defence against grazing and parasite invasion or regulation of density and sinking rates based on increased calcification rates (Raven and Waite 2004).

$$HCO_3^- + OH^- \Longleftrightarrow CO_3^{2-} + H_2O \quad (1)$$

$$CO_3^{2-} + Ca^{2+} \Longleftrightarrow CaCO_3 \quad (2)$$

While it has been proposed that calcification is used by *Achromatium* spp. to fix carbon in sedimentary environments (Head et al. 2000), there is now some doubt about the physiological need for such a mechanism. For instance, the pH of an *Achromatium*-containing sediment is typically circumneutral where CO_2 still constitutes a significant component of the dissolved inorganic carbon pool. In addition, recent microautoradiographic studies on carbon metabolism in *Achromatium* populations from different geographical locations have demonstrated that some calcite-precipitating *Achromatium* communities are probably chemolithoheterotrophs and do not utilise bicarbonate for biosynthesis (Gray et al. 1999b). Furthermore, these *Achromatium* populations did not contain homologues of the RuBisCO large subunit gene (*rbcL*), involved in CO_2 fixation (Head et al. 2000). Consequently, the precipitation of calcite by these *Achromatium* populations cannot be linked to carbon fixation.

5
A Proposed Role for Calcification in *Achromatium*: Calcite Precipitation Linked to Sulfide Dissolution

A key question with regard to calcification in *Achromatium* is: What ecological challenge necessitates this unique intracellular process? The answer to this question may lie in the geochemical constraints imposed on *Achro-*

matium spp. by their sedimentary environment, namely the very low levels of free sulfide and, therefore, the necessity for the active dissolution of iron sulfides by these organisms. In sediment pore waters the solubility of iron sulfide is dependent on the concentration of both dissolved Fe^{2+} and pore water pH (Eq. 3). From this equilibrium consideration, it can be deduced that high concentrations of dissolved Fe^{2+} and high pH favour sulfide precipitation, whereas low pH and low concentrations of Fe^{2+} favour sulfide dissolution (Eq. 4):

$$FeS + H^+ \Longleftrightarrow Fe^{2+} + HS^- \quad (3)$$

$$[HS^-] = K[H^+]/[Fe^{2+}]\,. \quad (4)$$

Given the role that *Achromatium* spp. play in the precipitation of carbonate and their likely dissolution and oxidation of iron sulfides, these two processes may be linked, whereby intracellular calcification provides a sink for hydroxyl ions generated as a result of proton export (Fig. 3). In this suggested mechanism the purpose of proton export by *Achromatium* spp. would be to alter extracellular pH sufficiently to mobilise iron sulfides (Eq. 3), with the subsequent oxidation of the liberated HS^- either by oxygen or by nitrate reduction (Eqs. 5–7). Critically, the dissolution of iron sulfide by acidification releases

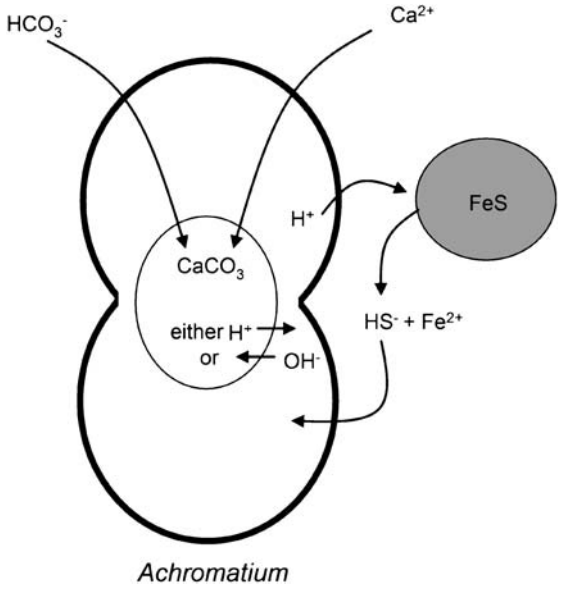

Fig. 3 Putative mechanism for intracellular calcification linked to sulfide mineral dissolution in *Achromatium*. A dividing *Achromatium* cell is shown with just a single calcite inclusion for illustrative purposes. Calcite precipitation occurs as a result of either proton export from or hydroxyl import into the inclusion to facilitate carbonate formation

dissolved Fe^{2+} into solution which would further decrease sulfide solubility (Eq. 4). To maintain the balance of Fe^{2+} concentraton and H^+ concentration at the iron sulfide mineral surface and, therefore, sustain continuous dissolution, an additional proton would be required for each sulfide dissolved. In this proposed mechanism, the export of protons could be achieved either by proton extrusion as a result of chemiosmotic energy generation or by proton-pumping ATPases; however, the exported protons, owing to their reaction with metal sulfides or through their role in maintaining pore water H^+ concentrations, would be unavailable for transport across the cell membrane by ATP synthase. An inevitable consequence of this proton consumption would be an increased cytoplasmic pH. As with calcification in the coccolithophorid, a potential sink for excess hydroxyl ions would be the buffering reaction with bicarbonate, the formation of carbonate and precipitation of calcite. In this putative process of calcification coupled to FeS mobilisation and oxidation (Fig. 3), the complete oxidation of the liberated HS^- to sulfate by consumption of oxygen would regenerate a proton (Eq. 5). This reaction would serve to neutralise half the excess hydroxyl ions. Consequently, for each mole of sulfide liberated and oxidised to sulfate by oxygen, a minimum of 1 mol of precipitated carbonate would be required. The requirement for calcite precipitation would be quantitatively greater if, as is highly likely, *Achromatium* spp. use nitrate (Gray et al. 2004) as their terminal electron acceptor to produce N_2 or NH_3 (Eqs. 6, 7).

$$HS^- + 2O_2 \longrightarrow SO_4^{2-} + H^+ \quad (5)$$

$$5HS^- + 8NO_3^- + 3H^+ \longrightarrow 5SO_4^{2-} + 4N_2 + 4H_2O \quad (6)$$

$$HS^- + NO_3^- + H^+ \longrightarrow SO_4^{2-} + NH_4^+ \quad (7)$$

A key feature of this proposed role for calcification in *Achromatium* is that calcite deposition, with its associated energetic cost, is linked to the energy-generating process of sulfide oxidation. So it remains to be seen from a proper evaluation of the energetics and enzymology of calcification and sulfur oxidation whether this process is feasible. Certainly in environments with high dissolved sulfide concentrations, the additional energy costs of calcification are likely to make *Achromatium* uncompetitive compared with non-calcifying sulfur oxidisers, so it is interesting to note that the only *Achromatium* species known to inhabit sulfidic marine sediments (*Achromatium volutans*) does not precipitate calcite.

6 Conclusion

The unique and enigmatic process of intracellular calcification in *Achromatium* has interested microbiologists for well over 100 years. However, it has

only been through recent in situ physiological studies and through detailed characterisation of the environments inhabited by these organisms that we have gained an understanding of the relationship between their ecology and the geochemical constraints imposed on them. With the advent of genomic technologies it should soon be possible to gain a greater understanding of the genetic basis of calcification in *Achromatium*, an adaptation which is surely critical to this environmental relationship.

Acknowledgements I would like to thank all those who have been involved in work on *Achromatium* over the last decade. In particular I would like to thank Ian Head, Arlene Rowan, Richard Howarth, Ken Clarke, Roger Pickup and Daria Comaskey, who have all played their part. I would also like to thank the Natural Environment Research Council and the Leverhulme Trust for their financial support.

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Metal Inclusions in Bacteria

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Abstract A variety of bacterial metal and metalloid inclusions have recently been described. These include particles of iron, manganese, cobalt, chromium and selenium. Iron and manganese inclusions may provide a mechanism for the storage of excess electron acceptors. Cobalt- and chromium-containing inclusions appear to function like classical magnetosomes, and may also provide protection against metal and oxygen toxicity. Selenium inclusions may also provide a defense mechanism against selenate and selenite toxicity, and (for at least one species) may represent a storage form of reducible selenium. The production of these particles may have important implications for research in biogeochemical cycling, bioremediation, biotechnology and even in the search for extraterrestrial life.

1 Introduction

Bacteria can produce a wide variety of intracellular granules, including polyphosphate (Chavez et al. 2004), polyhydroxybutyrate (James et al. 1999) and carboxysomes (Cannon et al. 2001). However, the production of metal (or metalloid) intracellular granules is remarkably rare. Until recently, the phenomenon was exemplified by the magnetite and greigite mineral inclusions formed by magnetotactic genera (Blakemore 1975; Farina et al. 1990; Mann et al. 1990), and by the intracellular sulfur granules formed by such organisms as *Beggiatoa* spp. and *Chromatium* spp. (Ehrlich 2002). Polyphosphate granules (formed by a very large and diverse group of bacteria) have also

been shown to serve as intracellular sinks for a variety of metals (van Veen et al. 1994; Lechaire et al. 2002). All of these inclusions have been studied for decades, and a large volume of work has been generated on their formation. Consequently, they are beyond the scope of this cameo and are discussed, in detail, in previous chapters of this series. The interested reader is here referred to those sections. In this section we will focus, instead, on some of the cytoplasmic metal and metalloid inclusions that have recently been described. While the volume of available literature on these novel particles is correspondingly small, their discovery suggests that the phenomenon may not be as rare as once believed.

2 Iron Inclusions

Following the discovery of magnetosomes by Blakemore (1975) the production of intracellular iron minerals was thought to be peculiar to magnetotactic genera. However, in 2002, a novel iron-rich particle was discovered inside cells of the nonmagnetotactic bacterium, *Shewanella putrefaciens* CN32. When growing anaerobically, this organism can couple oxidation of an organic substrate to the reduction of a variety of terminal electron acceptors, including ferric iron. Glasauer et al. (2002) followed growth of the cells during anaerobic reduction of a solid-phase ferric iron mineral, two-line ferrihydrite. At about the same time that ferrous iron began to accumulate within the

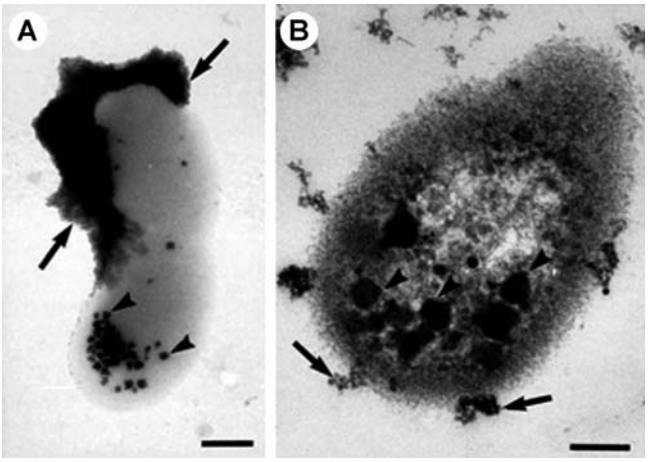

Fig. 1 Transmission electron micrograph of a *Shewanella putrefaciens* CN32 cell in whole mount (**a**)and in thin section (**b**)following anaerobic respiration using extracellular ferrihydrite (*arrows*). *Arrowheads* point to intracellular Fe granules. *Scale bars* **a** 250 nm, **b** 100 nm. (Reproduced from Glasauer et al. 2002, with permission)

culture supernatant, electron microscopy revealed the appearance of small (30–50-nm-wide), electron-dense particles located at the poles of the cells (Fig. 1). The particles were never observed prior to the onset of iron reduction and were never observed in cultures where fumarate replaced the ferrihydrite as an electron acceptor, suggesting that the production of the particles was linked to the metabolic reduction of ferric iron.

As the culture aged and iron reduction progressed, the number of particles per cell increased, and the number of cells containing particles approached 100%. Images of thin-sectioned cells revealed that the particles resided within the cytoplasm and were not simply ferrihydrite adsorbed onto the cell surface. Elemental analysis of the particles using energy-dispersive X-ray spectroscopy (EDS) confirmed that they were composed primarily of iron. Thin sections also revealed the presence of putative membranes surrounding the iron granules, similar to those found surrounding magnetosomes. Selected-area electron diffraction (SAED) of the granules confirmed that the intracellular mineral was more crystalline than the ferrihydrite supplied to the cells (i.e., the intracellular mineral was being formed de novo, during growth). SAED also confirmed that the intracellular mineral was distinct from both the extracellular magnetite, which is commonly formed during anaerobic iron reduction (e.g., by *Geobacter metallireducens*; Lovley et al. 1986), and from the magnetite found in magnetosomes.

More recently, Glasauer et al. (S. Glasauer, S. Langley, M. Boyanov, B. Lai, K. Kemner, and T.J. Beveridge, unpublished results) have established that the granules are also formed during reduction of soluble ferric iron (i.e., ferric citrate) and, to a lesser extent, in response to exposure of the cells to ferrous iron during growth on fumarate. Analysis of the granules using high-resolution synchrotron X-ray fluorescence and X-ray absorption near-edge spectroscopy allowed a comparison of the granule mineral with various iron mineral standards of known valence and structure. The results indicated that the granules are composed of a mixed-valence iron mineral that is more oxidized than green rust, but more reduced than magnetite. Exposure of anaerobic cells to air did not result in a change in the valence state of the granules, suggesting that the iron is stabilized, perhaps by counterions or organic complexing. Stabilization of polyphosphate by complexation with iron has been reported (Lechaire et al. 2002); however *Shewanella putrefaciens* has not been observed to produce polyphosphate under either aerobic or anaerobic conditions.

Glasauer et al. (unpublished results) have proposed that ferrous iron is taken up by the cells during anaerobic respiration and is compartmentalized near the plasma membrane. The membranes surrounding the granules may moderate an electron flow, leading to the mixed oxidation state of the iron and, possibly, allowing the granules to serve as an energy source for the cell. Presumably granule formation would take place while sources of external ferric iron remain high and, upon depletion of the external electron acceptor, the

granules could then be utilized to maintain viability. In support of this possibility, Glasauer et al. point out that the granules disappear when the external ferrihydrite is exhausted.

3 Manganese Inclusions

Manganese oxides, like iron oxides, are common components of soils and sediments, and manganese can serve as an important electron acceptor during anaerobic respiration. In the process, insoluble Mn(IV) is reduced to soluble Mn^{2+}. *Shewanella putrefaciens* CN32 can also use Mn(IV) minerals as terminal electron acceptors. After 5 days of growth on either birnessite or pyrolusite (solid-phase Mn oxides), about 85% of the cells were observed to contain electron-dense particles within the cytoplasm (Glasauer et al. 2004). These particles were very similar to those described, earlier, for iron in the same organism (Fig. 2). They were 30–40 nm across, clustered toward the poles of the cells, and may have been surrounded by a membrane. EDS confirmed that the granules were enriched in Mn, P and O. However, unlike the

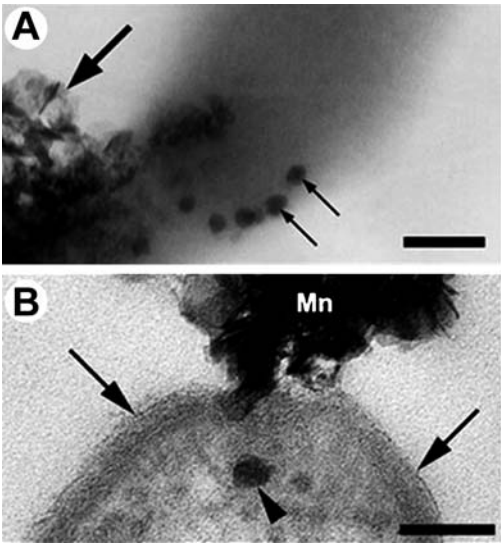

Fig. 2 **a** Transmission electron micrograph of a *S. putrefaciens* CN32 cell in whole mount following anaerobic respiration using extracellular birnessite (*large arrow*). Intracellular Mn granules are indicated by the *small arrows*. **b** A similar cell in thin section with the *arrowhead* pointing to an intracellular Mn granule. The *large arrows* delineate the cell surface. *Mn* extracellular birnessite. *Scale bars* 100 nm. (Reproduced from Glasauer et al. 2004, with permission)

iron granules formed by the same organism, the Mn granules had an amorphous structure, as revealed by electron diffraction.

The granules appeared to break down as the cultures aged, so it is possible that the cells might use the granules as a storage form of reducible Mn for those times when external electron acceptors become limiting. The Mn may be deposited in a reoxidized form, or possibly in a partially oxidized state (i.e., Mn(III)) or a mixed-valence state. Interestingly, and in contrast to iron reduction, precipitation of Mn was also observed in the periplasm of the cells. This suggests the possibility that Mn(IV) may be incompletely reduced in the periplasm (Glasauer et al. 2004).

When grown anaerobically in the presence of both iron and manganese minerals, *Shewanella putrefaciens* CN32 will produce mixed Fe/Mn granules (Langley et al. 2002; Glasauer et al., unpublished results). If the membrane surrounding the granules is indeed responsible for controlling the precipitation of the metal in this organism, then it remains to be determined whether a separate enzyme system exists which is capable of processing both metals, or if each metal is precipitated via its own pathway. Regardless of the pathway(s) involved, the process appears to be unique to this organism, as similar granules have not been reported in other well-studied dissimilatory metal reducers such as *Geobacter* spp., or even in other *Shewanella* spp.

4 Selenium Inclusions

Selenium is a metalloid element of the same periodic group as sulfur. Consequently, it shares many of the same characteristics as sulfur, and can serve as a sulfur analog in some amino acid and enzyme structures. It exists in aqueous environments as the soluble forms selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), both of which can serve as electron acceptors for a variety of bacteria during anaerobic respiration. *Bacillus selenitireducens*, for example, reduces selenite to elemental selenium (Se^0), which it deposits as small, extracellular spheres (Switzer Blum et al. 1998). More recently, it was discovered that the same organism also deposits Se^0 "nanospheres" within its cytoplasm during selenite respiration (Fig. 3; Oremland et al. 2004). Two other organisms, *Sulfurospirillum barnesii* and *Selenihalanaerobacter shriftii* also produced internal selenium nanospheres when respiring selenate. EDS of the nanospheres confirmed their elemental composition; however, owing to the electron density of the nanospheres, the presence of a surrounding membrane could not be determined. The production of Se^0 nanospheres by cells from such a physiologically and phylogenetically broad range of organisms suggests that it may be a widespread phenomenon (Oremland et al. 2004). Indeed, cells of *Ralstonia metallidurans* also reduce selenite to Se^0, which is incorporated as intracellular granules (Roux et al. 2001; Sarret et al. 2005). In-

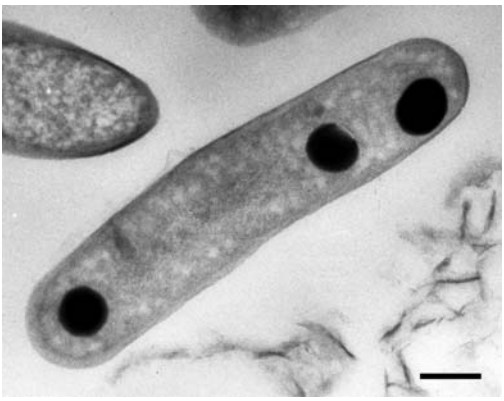

Fig. 3 Transmission electron micrograph of a *Bacillus selenitireducens* MLS-10 cell in thin section showing the presence of three electron-dense, intracellular spheres of elemental selenium. *Scale bar* 500 nm

tracellular selenium accumulation has also been observed in the purple sulfur bacterium *Chromatium vinosum* (Nelson et al. 1996).

Oremland et al. (2004) speculate that the internal Se^0 nanospheres may function as receptacles for the storage of reduced selenate (or selenite) that somehow bypassed the respiratory enzymes in the cell envelope. In this sense, the formation of nanospheres may serve as a detoxification mechanism. In the specific case of *B. selenitireducens*, storage of Se^0 may also be advantageous because elemental selenium can be reduced (to H_2Se) by this organism (Herbel et al. 2003) in times of external electron acceptor depletion.

5 Other Metal-Rich Inclusions

Vainshtein et al. (2002) have suggested that the production of magnetic intracellular particles may not be unique to the "classic" magnetotactic genera. So-called magnet-sensitive inclusions (MsI) were observed to form in a wide variety of cells, including representatives of both domains *Bacteria* and *Archaea*. The MsI ranged in size from 10 to 150 nm, and displayed amorphous structure with variable electron density. In some specimens, the inclusions showed "classic" magnetosome traits (i.e., "... a thread of beads along the long axis of the cell"; Vainshtein et al. 2002), but in others the particles were randomly dispersed within the cell. They were surrounded by a "non-unit membrane", and could be separated from disrupted biomass. Both intact cells and isolated MsI were attracted to a static magnet placed in proximity to the culture vessel (Vainshtein et al. 2002). The authors suggest that the MsI are not mineral inclusions like magnetosomes, but rather some sort of or-

ganic matter enriched with iron (however, the rationale and evidence for this suggestion are not entirely clear).

What makes the MsI intriguing is that while some are enriched in iron, others are not. More recent data from the same group have indicated that MsI will form in the absence of iron, in this case when the cells are supplied with cobalt or chromium in the growth medium (Ariskina et al. 2004). The particles formed are globular, or spherical, with diameters of 20–250 nm, with an electron-translucent central core and an electron-dense periphery. In at least one instance, the particles appeared to be localized in the cell poles (similar to the Fe and Mn inclusions of *Shewanella putrefaciens* described earlier). Elemental analysis by EDS revealed that the particles were enriched in Co or Cr, but only in the outer, electron-dense periphery. The lack of significant peaks for sulfur or phosphorus in the same spectra suggest that the particles are not simply organic inclusions which might be serving as metal sinks (e.g., polyphosphate granules). The authors suggest that metal-containing particles serve as a defense mechanism against the toxic effects of high concentrations of the metal, or may serve to mediate oxygen toxicity by acting as a redox buffer (Ariskina et al. 2004).

6 Summary

Bacterial production of intracellular metal-rich particles may serve a variety of physiological functions depending on the organism, including (1) magnetotaxis, (2) storage of excess electron acceptors, (3) precipitation of excess toxic metal ions and, possibly, (4) mediation of oxygen toxicity. The fact that many of the particles described to date appear to be enclosed within a membrane suggests that their formation may be strictly regulated by the host organism (e.g., several enzymes have been localized to the magnetosome membrane and have been shown to play a role in the formation of the magnetite particle; Nakamura et al. 1995; Komeili et al. 2004). Not very long ago, the production of intracellular metal inclusions by bacteria was thought to be the sole purview of the magnetotactic bacteria. However, recent studies, highlighted here, show that this is no longer the case. Clearly the phenomenon may be more widespread than previously believed. The production of intracellular metal particles has important implications for our understanding of biogeochemical cycling, and may even lead to novel applications and advancements in areas such as bioremediation, biotechnology and the search for extraterrestrial life. Despite the discovery of magnetosomes some 30 years ago, it seems likely that research into the field of bacterial metal inclusions may still be in its infancy.

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Inclusion Proteins from other Insecticidal Bacteria

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Abstract The toxicity of *Bacillus thuringiensis* and *B. sphaericus*, insecticidal bacteria that have been used widely for insect pest and vector control, is due to insecticidal protein crystals produced during sporulation. Whereas the insecticidal crystals of these two species have been studied extensively owing to their practical value, there are several other lesser known entomopathogenic bacteria that also produce either crystalline or noncrystalline protein inclusions, some of which are insecticidal. For example, *Paenibacillus popilliae*, a biological control agent of grubs, the immature stages of beetle larvae, forms parasporal crystals toxic to scarab larvae. A mosquitocidal strain of *Clostridium bifermentans* produces parasporal inclusion bodies lacking a crystalline structure. Mosquitocidal strains of *Brevibacillus laterosporus* produce crystalline inclusions of various shapes and sizes. In addition, two nematode-symbiotic bacteria, *Photorhabdus luminescens* and *Xenorhabdus nematophilus* produce large amounts of crystalline proteins that are not toxic to insects. In this paper, the characteristics of the protein inclusions produced by these entomopathogenic bacteria are described.

1 Inclusions Proteins of *Paenibacillus popilliae*

The Gram-positive, spore-forming bacterium *Paenibacillus popilliae*, formerly known as *Bacillus popilliae*, is a pathogenic agent of milky disease in at least two species of the insect family Scarabaeidae (Coleoptera), the Japanese beetle, *Popillia japonica*, and the European chafer, *Amphimallon*

majalis (Valyasevi et al. 1990; Petterson et al. 1999). Because *P. popilliae* is highly fastidious and cannot be mass-produced in vitro, cultures are grown in grubs and then formulated as insecticidal preparations. These have been registered for use as insecticides in the USA since the 1950s to suppress Japanese beetle populations (Klein 1992). Beetle larvae ingest bacterial spores during feeding on the roots of grasses, where they breed. Spore germination occurs in the larval gut, followed by penetration of the hemocoel by vegetative cells. After a period of growth in larval hemolymph, the bacteria sporulate and spores are liberated into the soil following death and disintegration of the cadaver (Splittstoesser et al. 1978; Harrison et al. 2000). In larvae just prior to death, *P. popilliae* spores reach concentrations of about 2×10^9/ml of hemolymph. Despite considerable research aimed at obtaining efficient in vitro production of spores, only very low levels have been achieved in the laboratory. The lack of knowledge about the requirements of this bacterium for growth and sporulation has been the major factor limiting the utilization of *P. popilliae* as a biological control agent for a wider range of scarab species (Stahly and Klein 1992).

Upon sporulation, *P. popilliae* forms parasporal crystals in a manner similar to that of *Bacillus thuringiensis* (Fig. 1); however, the role of the parasporal crystals in *P. popilliae* pathology remains unclear. Significant toxicity was detected when the parasporal crystals were injected into hemolymph, but not when they were fed to larvae (Weiner 1978).

The first *cry* gene from *P. popilliae* subsp. *melolonthae* H1, which encodes a 79-kDa parasporal crystal protein, was cloned and sequenced by Zhang et al. (1997). They detected two open reading frames that encode polypeptides composed of 175 and 706 amino acids with estimated molecular masses of 19.6 and 79 kDa, respectively. The smaller open reading frame, *orf1*, has substantial similarity to *orf1* of the *cry2Aa* operon (Widner and Whiteley 1989) and *p*19 of the *cry11Aa* operon (Dervyn et al. 1995) of *B. thuringiensis*. The polypeptide encoded by the longer open reading frame, designated *cry18Aa1*,

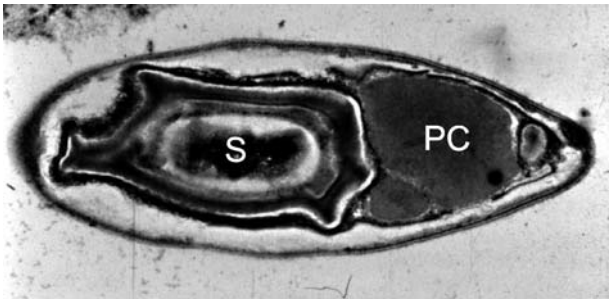

Fig. 1 Transmission electron micrograph of a sporulating *Paenibacillus popilliae* cell. *S* spore, *PC* parasporal crystal. (Source: Color slide atlas of microbial control, Society for Invertebrate Pathology)

has significant sequence similarity to other Cry proteins of *B. thuringiensis* (Schnepf et al. 1998; Crickmore et al. 1998, 2005). The distribution of hydrophobicity of Cry18Aa1 seems to be quite similar to that of Cry3A and Cry1Aa (Li et al. 1991; Grochulski et al. 1995), suggesting it consists of three domains. Transcriptional analysis of *cry18Aa1* of *P. popilliae* in *B. thuringiensis* shows that the transcriptional start site is located 29 base pairs upstream of the translational start of *orf1*, indicating *orf1* and *cry18Aa1* are transcribed as an operon. The transcription of *cry18Aa1* can be driven by σ^E and σ^K types of RNA polymerase in *B. thuringiensis* (Zhang et al. 1998).

2 Inclusion Proteins of *Clostridium bifermentans*

About 15years ago, a new mosquitocidal bacterium was recovered from mangrove swamp soil in Malaysia and identified as *Clostridium bifermentans* subsp. *malaysia* subsp. *malaysia* (de Barjac et al. 1990). This was the first discovery of a strictly anaerobic bacterium with a high toxicity to mosquito and blackfly larvae. When sporulation of *C. bifermentans* subsp. *malaysia* was examined, Charles et al. (1990) observed parasporal inclusions that appeared in the sporulated cell at sporulation stage IV, i.e., 5 h after the end of exponential growth (Fig. 2). At this stage they were visible with phase-contrast microscopy. These inclusions neither have a crystalline structure nor does an envelope surround them. In addition, from this stage, brushlike or featherlike appendages appeared in the sporulated cell attached to the spores and exosporium after cell lysis. Spores may have up to 12 appendages attached to each end. Their function is unknown.

To identify the toxins produced by *C. bifermentans* subsp. *malaysia*, two genes, encoding the 67- and 66-kDa proteins, were cloned (Nicholas et al. 1993; Barloy et al. 1996). These genes were originally named *cbm71* and

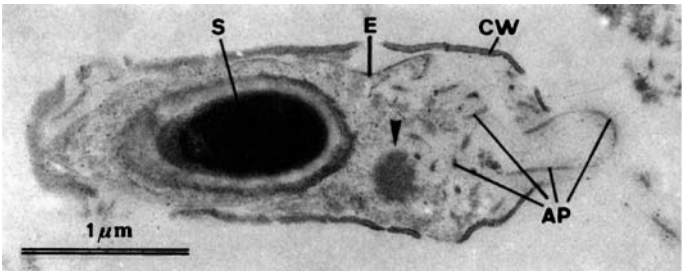

Fig. 2 Transmission electron micrograph of a sporulated cell of *Clostridium bifermentans* subsp. *malaysia* during sporangium lysis. An inclusion body (*arrowhead*) and some featherlike spore appendages (*AP*) are visible. *CW* sporangium cell wall, *E* exosporium, *S* mature spore. (Courtesy of Jean-François Charles, Institut Pasteur, France)

cbm72. However, because their deduced amino acid sequences are similar to those of δ-endotoxins of *B. thuringiensis* (Barloy et al. 1996), they were renamed *cry16Aa1* and *cry17Aa1*, respectively (Barloy et al. 1998a, b; Crickmore et al. 1998, 2005). The amino acid sequence of Cry16Aa1 is about 30% similar to that of Cry proteins of *B. thuringiensis*, with the similarity limited to the first four conserved blocks of Cry proteins. Among these blocks, I and IV are the most similar, where, for example, the similarity with these blocks in Cry1 is 88 and 58%, respectively. Furthermore, Cry16Aa1 does not contain block V, although Cry17Aa1 does (Barloy et al. 1996; Schnepf et al. 1998). Like Cry16Aa1, the amino acid sequence similarities of Cry17Aa1 with other Cry toxins are restricted to the conserved blocks, especially to blocks I and V (Barloy et al. 1998a). When *cry16Aa1* was expressed alone in a crystal-negative strain of *B. thuringiensis*, transformants produced aggregates rather than crystalline inclusions during sporulation (Barloy et al. 1996). However, purification of these aggregates was not possible with conventional techniques such as on sucrose gradients, and thus the toxicity of these aggregates could not be tested. Nevertheless, bioassays performed using trichloroacetic acid precipitated supernatants of the Cry16Aa1-producing transformant against larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex pipiens* showed the same activity spectrum as *C. bifermentans* subsp. *malaysia*, with the highest toxicity against *An. stephensi* and lowest toxicity against *Ae. aegypti*. The toxicity values obtained against the three different mosquito species were similar, suggesting that the activity is nonspecific. Because Cry17Aa1 could not be synthesized in *B. thuringiensis*, it was not possible to determine whether it played a role in the toxicity of *C. bifermentans* subsp. *malaysia* (Barloy et al. 1996, 1998a). More recently, Juárez-Pérez and Delécluse (2001) successfully expressed *cry16Aa1* and *cry17Aa1* genes in *B. thuringiensis* and *Escherichia coli*, and concluded that these proteins are not involved in mosquitocidal activity. Therefore, further studies are required to determine the role of these proteins in mosquitocidal activity.

C. bifermentans subsp. *malaysia* does not contain plasmids and, thus, its toxin genes are on the chromosome (Seleena and Lee 1994). Such chromosomal localization of mosquitocidal toxin genes has been reported for *B. sphaericus* (Aquino de Muro et al. 1992; Federici et al. 2003). The *cry* genes cloned from *C. bifermentans* subsp. *malaysia* are also present in other subspecies of *C. bifermentans*, as well as other *Clostridium* and *B. thuringiensis* strains (Barloy et al. 1998b).

3 Inclusion Proteins of *Photorhabdus luminescens*

The bacterium, *Photorhabdus luminescens* is a Gram-negative, rod-shaped member of the family *Enterobacteriaceae*. This species is a bioluminescent

symbiont of entomopathogenic nematodes found commonly in soil, and is pathogenic to a wide range of insect species (Poinar et al. 1977; Bowen et al. 1998; ffrench-Constant and Bowen 1999; Chattopadhyay et al. 2004). Recently, the complete genome sequence of *P. luminescens* subsp. *laumondii* strain TT01 was determined (Duchaud et al. 2003). Cells of *P. luminescens* growing in vivo and in vitro produce inclusion proteins within the cytoplasm (Khan and Brooks 1977).

During growth, *P. luminescens* exists in two forms, designated phase I and phase II variants, which differ in many phenotypic traits. One of the characteristic phenotypes of phase I, but not phase II, cells is the presence of two types of intracellular protein inclusions that each have a molecular mass of approximately 10 kDa (Fig. 3), and can account for up to 40% of the total protein content of cells (Bowen and Ensign 2001). The function of the inclusion proteins of *P. luminescens* is unknown, although production of unusually

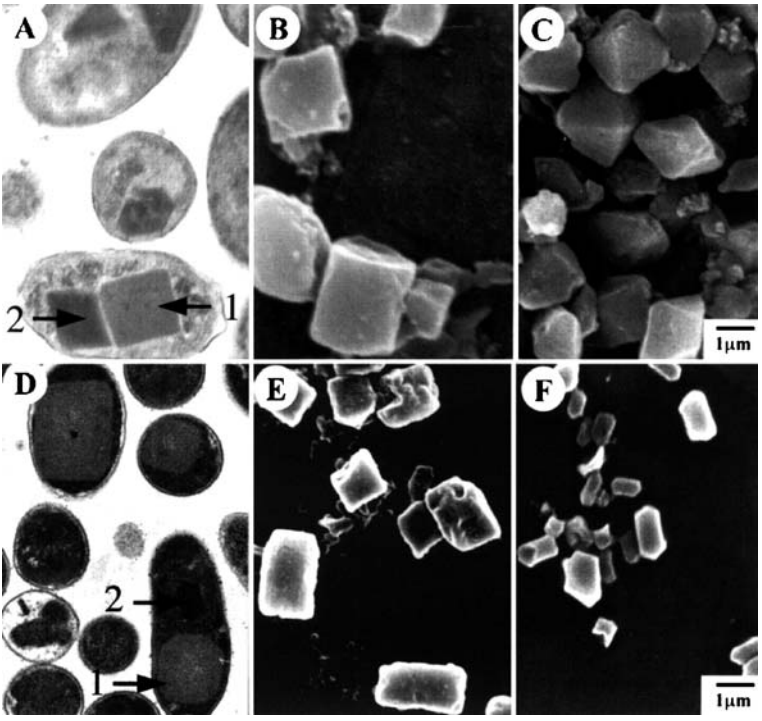

Fig. 3 Transmission electron micrographs of thin sections of cells of *Photorhabdus luminescens* NC1 (**a**) and Hm (**d**) showing that cells contain inclusions with two distinct shapes. Scanning electron micrographs of protein inclusions separated by density gradient centrifugation. **b**, **c** Type 1 and type 2 inclusions of NC1, respectively; **e**, **f** Type 1 and type 2 inclusions of Hm, respectively. (Bowen and Ensign 2001)

large amounts of these proteins suggests that the proteins serve an important function for the bacteria.

The genes encoding two inclusion proteins, *cipA* and *cipB*, of *P. luminescens* strain NC1 have been cloned and characterized (Bintrim and Ensigh 1998). The genes are located at different loci and show little sequence similarity to each other. In addition, *cipA* and *cipB* do not share homology with any known genes, including the insecticidal crystal protein genes of *B. thuringiensis* and *B. sphaericus*.

4 Inclusion Proteins of *Xenorhabdus nematophilus*

Like *P. luminescens*, *Xenorhabdus nematophilus* is an entomopathogenic Gram-negative bacterium belonging to the family *Enterobacteriaceae*. This species is also symbiotically associated with insect parasitic nematodes (Akhurst and Dunphy 1993; Chattopadhyay et al. 2004).

When cultured in vitro, *X. nematophilus* occurs in two forms designated phase I and phase II, which can be distinguished according to colony morphology and pigmentation on various bacterial media, or on the basis of antibiotic production (Akhurst 1980, 1982). Both forms are pathogenic to insects, but only the phase I form is isolated from infective nematodes (Akhurst 1980). This species produces one or two phase-bright large inclusions in cells only from phase I form cultures (Couche and Gregson 1987). A large cigar-shaped inclusion (type 1) occupying up to one third of the cytoplasm occurs

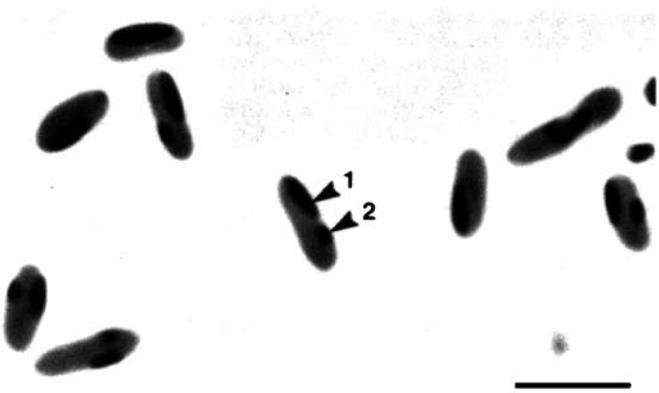

Fig. 4 Stationary-phase cells of *Xenorhabdus nematophilus* subsp. *nematophilus*. The loss of refractivity of normally phase bright inclusions is caused by the glycerol-mounting medium. Two morphological forms of inclusions are present, type 1 (*1*) and type 2 (*2*), *Bar* 5 μm. (Couche and Gregson 1987)

in all cells. When a cell contained two inclusions (Fig. 4), the second was always a smaller ovoid form (type 2). The role of these inclusion proteins has not yet been determined.

5
Inclusion Proteins of *Brevibacillus laterosporus*

Recently, two strains of *Brevibacillus laterosporus*, a spore-forming entomopathogenic bacterium, were identified that produce crystalline inclusions (Fig. 5) of various shapes and sizes (Smirnova et al. 1996; Orlova et al. 1998). Both strains are highly toxic to mosquito larvae and particularly one of two strains (615) shows toxicity comparable to that of *B. thuringiensis* subsp. *israelensis* against *Ae. aegypti* and *An. stephensi*. These two strains of *B. laterosporus* are more toxic to two species of mosquitoes, *Ae. aegypti* and *An. stephensi*, and are less toxic to *Cx. pipiens*. The crystalline inclusion of *B. laterosporus* responsible for mosquitocidal activity has not yet been characterized. These recent findings are interesting because this species had previously not been known to produce parasporal inclusions (Favret and Yousten 1985; Rivers et al. 1991).

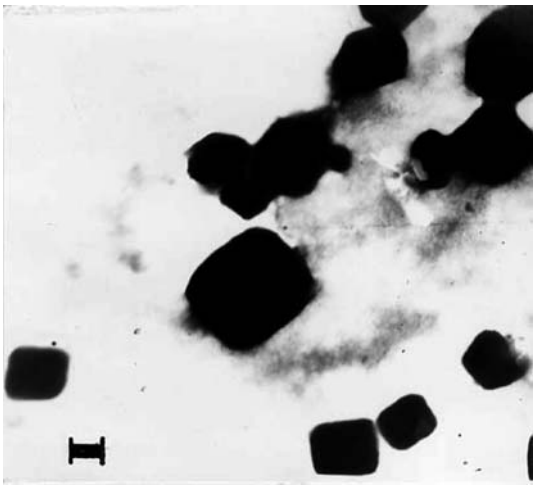

Fig. 5 Electron micrograph of purified crystals of *Brevibacillus laterosporus* 615 in a negatively stained preparation. Flat rectangular crystals lacking clear corners are predominant. Staining was with 1% uranyl acetate. *Bar* 100 nm. (Orlova et al. 1998)

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R-bodies

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Abstract R-bodies are insoluble protein ribbons coiled in the cell into hollow cylindrical structures. They are synthesized by a few bacterial strains, including paramecia endosymbionts of the genus *Caedibacter* and some free-living nonsymbiotic bacteria. In *C. taeniospiralis* R-body synthesis is coded by plasmid pKAP, which probably evolved from a bacteriophage. In other bacteria apart from *C. taeniospiralis*, the synthesis of R-bodies appears to be also related to defective prophages, but cloning of the genes coding for these structures has not been reported. *Caedibacter* confers to the hosting paramecia the killer phenotype, a characteristic associated with the synthesis of the R-body. No physiological role has been demonstrated for R-bodies produced by nonsymbiotic bacteria.

Keywords Extrusive apparatus · Prophages · R-bodies · Rhapidosomes

1 Introduction

R-bodies are cytoplasmic inclusion bodies synthesized by only a few species of bacteria that were described for the first time in obligate endosymbionts of paramecia or kappa particles (Preer and Preer 1967). They are highly insoluble proteins ribbons coiled in the cell into cylindrical structures. The denomination R-body stands for “refractile inclusion body”, referring to the fact

that this structure is observed under phase-contrast microscopy as a bright particle (Preer and Stark 1953).

It was observed lately that R-bodies are synthesized by some free-living Gram-negative bacteria belonging to the genus *Pseudomonas* (Lalucat et al. 1979; Wells and Horne 1983; Fusté et al. 1986; Espuny et al. 1991), by the anoxigenic photosynthetic *Rhodospirillum centenum* (Favinger et al. 1989) and by the melanogenic marine bacterium *Marinomonas mediterranea* (Hernández-Romero et al. 2003).

In this review, R-bodies synthesized by different organisms will be analyzed with regard to their structural features, as well as the genetic determinants involved in their synthesis. Other bacterial structures related to R-bodies in terms of structural similarity, such as the extrusive apparatus of epixenosomes (Petroni et al. 2000), or in terms of a possible phage origin, such as the rhapidosomes (Lewin 1963), will also be considered. Finally, some issues to be addressed in future research on R-bodies will be discussed.

2
Ultrastructure of R-bodies

R-bodies synthesized by different organisms show a similar ultrastructure; however, depending on the microorganism considered, some differences that will be discussed in the following sections can be appreciated.

2.1
Endosymbiontic Bacteria of Ciliates

The large size of ciliate cells offers habitats for a wide diversity of bacteria colonizing different cellular compartments (Görtz 2001). The genus *Caedibacter* comprises the intracellular bacteria characterized by their capacity to produce R-bodies (Preer and Preer 1984). However, in spite of these common characteristics, they are phylogenetically diverse, belonging to at least two different classes of *Proteobacteria*. *C. taeniospiralis* is a γ-proteobacterium, while *C. caryophylus* is an α-proteobacterium (Beier et al. 2002). Bacteria synthesizing R-bodies confer to the hosting paramecium the "killer phenotype", that is, the capacity to kill other sensitive strains of paramecia that do not bear such symbionts (Preer et al. 1974).

Inside the bacterial cell, R-bodies appear as a multilayered tightly rolled hollow cylindrical structure with a diameter of approximately 0.5 μm (Fig. 1). Under certain conditions R-bodies unroll. The stimulus causing this event can vary depending on the R-body; some of them are induced by a lowering in the pH, and others by a thermic treatment. The mode of unrolling and the dimensions and morphology of the ribbon have been used to classify R-bodies in different types, 51, 7, Cc, Pt and Pa, named according

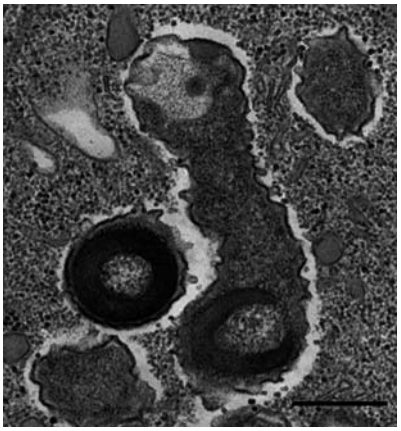

Fig. 1 Ultrastructure of *Caedibacter taeniospiralis* harboring type 51 R-bodies within the cytoplasm of its host, *Paramecium tetraurelia*. *Bar* 0.5 μm. (Reprinted with permission from Beier et al. 2002)

to the bacterial strain from which they were first described (Pond et al. 1989). Three different classes of R-bodies have been described in endosymbiotic bacteria. Each *Caedibacter* species produces only one type of R-body. *C. taeniospiralis* synthesizes type 51 R-body, which is the most thoroughly studied. *C. caryophilus* produces type Cc, and type 7 is produced by several other species.

2.2 Nonsymbiotic Bacteria

Pseudomonas taenospiralis was the first nonsymbiotic bacterium in which R-body synthesis was observed (Lalucat and Mayer 1978). In this microorganism the diameter of the coiled ribbon is approximately 0.25 μm (Lalucat et al. 1979). R-bodies have also been described in other *Pseudomonas* strains, such as *P. avenae*, which produces a large R-body with a length of 30 μm once unrolled (Wells and Horne 1983), *Pseudomonas* sp. strain EPS-5028 (Fusté et al. 1986) and *P. aeruginosa* (Espuny et al. 1991). However, it is important to bear in mind that the genus *Pseudomonas* included classically many polarly flagellated, Gram-negative, rod-shaped and aerobic bacteria, which were later transferred to other genera. For example, *P. avenae* is now named *Acidovorax avenae* subsp. *avenae* (Willems et al. 1992). The mode of unrolling of the R-bodies produced by *P. taeniospiralis* and *A. avenae* and the dimensions of the ribbon revealed that they constitute new types of these structures (Pt and Pa types, respectively) (Pond et al. 1989). The purple non-sulfur photosynthetic bacterium *R. centenum* synthesizes an R-body with a size of 0.3 μm (Favinger et al. 1989).

R-bodies produced by the aforementioned nonsymbiotic bacteria and the R-bodies produced by the endosymbionts of paramecia show a very similar ultrastructure. In contrast, the R-bodies produced by *Marinomonas mediterranea* show a more complex structure. The main difference is that they show an additional heavily stained envelope that consists of several concentric layers with a thickness of about 4 nm each (Fig. 2). This structural difference might explain why *M. mediterranea* R-bodies are not refractile under light microscopy (Hernández-Romero et al. 2003).

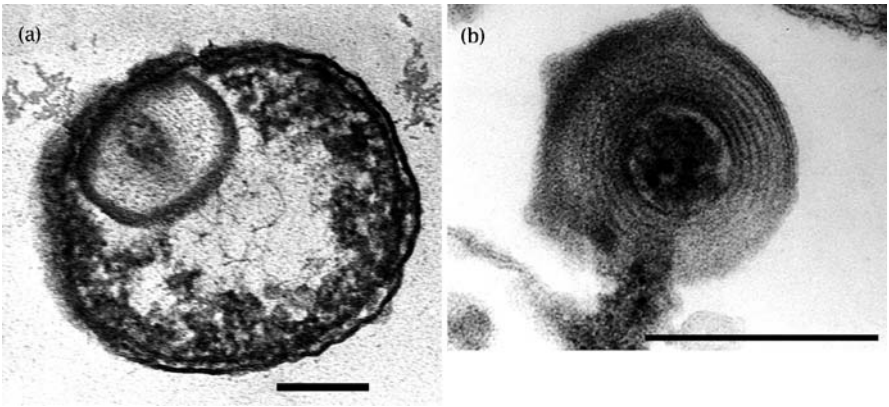

Fig. 2 Electron micrographs of *Marinomonas mediterranea*. **a** Thin section of an R-body producing cell. **b** Thin sections of cytoplasmic structures released from lysed cells. *Bar* 0.2 μm. (Reproduced with permission from Hernández-Romero et al. 2003)

3 Genetics of R-bodies Synthesis

One of the most intriguing aspects of the R-bodies is the origin of the genetic elements determining their expression. The comparison of these elements in different bacterial strains could help to clarify this subject.

3.1 Endosymbiotic Bacteria of Ciliates

In *Caedibacter* species the production of R-bodies has been related to extrachromosomal elements. In four species it is thought that they are coded by defective phages (Preer et al. 1974). This interpretation is supported by the observation of phagelike particles associated with R-bodies and by the isolation of phages from bacteria synthesizing this structure (Preer et al. 1971).

C. taeniospiralis strains contain plasmids coding for type 51 R-bodies. These plasmids are denominated with the prefix pKAP followed by the strain

number. The size of the plasmid in each strain varies depending on the insertion of transposons (Quackenbush et al. 1986). The plasmid genes coding for R-bodies were cloned and expressed in *Escherichia coli*. The synthesis of R-bodies with the same appearance as those synthesized by *C. taeniospiralis* was observed, but no toxicity towards sensitive strains of paramecia was detected. This result suggests that the compound responsible for the toxic effect is not a structural component of the R-body (Quackenbush and Burchar 1983). Three species of polypeptides of 18, 13 and 10 kDa are necessary for R-body assembly (Kanabrocki et al. 1986a). They are coded by three independently transcribed genes (*reb*). These genes have been sequenced in different strains, showing a high level of conservation (Heruth et al. 1994; Jeblick and Kusch 2005). So far this kind of R-body is the most thoroughly studied, but even in this case it is not possible to propose a model for R-body polymerization (Heruth et al. 1994). Sequencing and transcriptional analysis of the whole plasmid pKAP289 revealed that other genes apart from those involved in R-body synthesis are expressed. It has been suggested that the toxic effects on paramecia might be related to a protein with homology to ATPases (Jeblick and Kusch 2005).

3.2 Nonsymbiotic Bacteria

The synthesis of R-bodies in nonsymbiotic bacteria has been related to phages. This hypothesis is mainly based on the detection of phage particles after prophage induction under different conditions. For example, in *P. taeniospiralis*, the induction with mitomycin C determined the appearance of tail-like defective phage particles. Moreover, although in low numbers, structures resembling bacteriophage heads were observed in suspensions of R-bodies (Lalucat et al. 1979).

Pseudomonas sp. strain EPS-5028 also showed structures resembling phage or phage heads after UV induction, but not after mitomycin treatment (Fusté et al. 1986). In the case of *P. aeruginosa* strain 44T1, irradiation experiments allowed the detection of structures similar to R-type bacteriocins (Espuny et al. 1991). Similarly, UV or mitomycin induction of *M. mediterranea* allowed the detection of different bacteriophage-like particles, some of them also similar to R-type bacteriocins (Hernández-Romero et al. 2003). The genes coding for R-bodies in *M. mediterranea* have not yet been identified, although it has been shown that their expression is under the same genetic control as polyphenol oxidase expression and melanin synthesis (Lucas-Elío et al. 2002; Hernández-Romero et al. 2003). In spite of this common regulation and the striking similarity of this bacterial structure to melanosomes from higher organisms, the direct involvement of the *M. mediterranea* R-body in melanin synthesis has not been proven (Hernández-Romero et al. 2003).

Finally, in *A. avenae* the presence of bacteriophage-like particles or plasmids has not been detected and hence it was postulated that the genes involved in R-body synthesis are chromosomal (Pond et al. 1989). No studies have been reported regarding the possible origin or function of the R-bodies synthesized by *R. centenum* (Favinger et al. 1989).

4
Other Bacterial Structures Related to R-bodies

R-bodies have been described in a few bacterial strains; however, some other unusual bacterial structures bear some similarities to R-bodies in terms of structural organization or possible viral origin.

4.1
Extrusive Apparatus of Epixenosomes

The structure most similar to R-bodies described in other bacteria is the extrusive apparatus synthesized by bacteria of the division *Verrumicrobia* that live as ectosymbionts ("epixenosomes") of ciliates of the genus *Euplotium*. During the life cycle, these bacteria pass through two different stages. In stage I they show a typical prokaryotic cell organization. During stage II they possess a complex structure showing, among other features, a ribbon rolled around a central core, the extrusive apparatus, with a high similarity to R-bodies (Petroni et al. 2000). Certain stimuli can induce the ejection of the extrusive apparatus in a process that involves its unrolling from the inside and the formation of a hollow tube. This process is a mechanism of defense against predators (Rosati et al. 1999). As far as we know, no report has been published on the genetic determinants of this extrusive apparatus synthesis.

The extrusomes are a diverse group of membrane-bound eukaryotic organelles that includes the ejectisomes synthesized by the families *Cryptophyceae* and *Prasinophyceae*, which have been compared to R-bodies and the extrusive apparatus of the *Verrumicrobia* (Pond et al. 1989; Petroni et al. 2000). Ejectisomes are also coiled ribbons that unroll to form a hollow tube (Morrall and Greenwood 1980). It has been speculated whether some extrusomes, such as the trichocysts synthesized by ciliates and dinoflagellates, which are also involved in defense or capture of prey, originated from internalized epixenosome-like bacteria (Petroni et al. 2000).

4.2
Rhapidosomes

Rhapidosomes are tubular structures composed of proteins that were described for the first time in the gliding bacterium *Saprospira* (Lewin 1963).

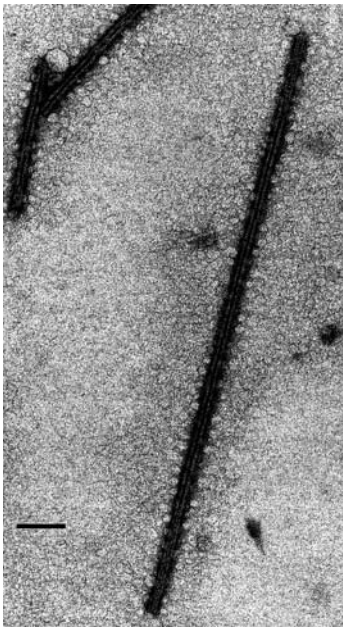

Fig. 3 Transmission electron microscopy of rhapidosomes isolated from *Aquaspirillum itersonii*. *Bar* 0.1 μm. (Reproduced with permission from Pazirandeh and Campbell 1993)

They have also been described in several bacteria such as *Aquaspirillum itersonii* (Evers and Murray 1980), *Cytophaga* (*Chondrococcus*) *columnaris* (Pate et al. 1967), *P. fluorescens* (Baechler and Berk 1972) and *Clostridium botulinum* (Ueda and Takeya 1972). Morphologically, rhapidosomes show a sheath of approximately 25–30 nm with an inner core of 8 nm and a variable length between 50 nm and 1.5 μm (Fig. 3). Once polymerized, rhapidosomes are highly stable to a variety of extreme conditions and hence they are considered as a model for studying protein self-assembly (Pazirandeh and Campbell 1993).

The origin of rhapidosomes is controversial. Some authors consider them as normal constituents of cells (Pate et al. 1967), but very early in the study of these structures some authors suggested that they are related to phages (Bradley 1965), or even that they are the result of the polymerization of sheaths of defective phages (Clark-Walker 1969). The possible relationship between rhapidosomes and defective phages was studied in *Aquaspirillum itersonii*. It was observed that the molecular weight of the proteins forming part of those structures was different, and that defective bacteriophages were not recognized by antibodies against rhapidosomal proteins (Evers and Murray 1980).

5
Future Perspectives in the Study of R-bodies

One important issue in the study of R-bodies is the determination of the genetic elements involved in their synthesis. As previously discussed, in many cases they have been related to extrachromosomal elements, and particularly phages. The strongest evidence of the relationship between prophages and R-bodies is the case of *C. taeniospiralis*, in which they are coded by a plasmid. The sequencing of plasmid pKAP indicated that it may have derived from a phage, since many genes in this plasmid show similarity to viral genes. However it does not have all the genes required to synthesize a complete infective virus (Jeblick and Kusch 2005). In the other endosymbiotic and nonsymbiotic bacteria, they are also considered to be coded by prophages, but conclusive evidence is lacking.

The cloning and sequencing of genes coding for R-body synthesis in additional bacteria, and particularly in nonsymbiotic bacteria, is an important issue that will also help to clarify the degree of the relationship between R-bodies produced by different microorganisms. Taking into consideration serological studies and the similarities at the DNA level available at that time, Pond et al. (1989) proposed that R-bodies synthesized by different bacteria are distinct from one another. For instance, Southern blot analysis was performed using as a probe a region of plasmid pKAP47 from *C. taeniospiralis*, but no positive signal was obtained with total DNA from *P. taeniospiralis*, suggesting a different evolutive origin for both types of structures (Kanabrocki et al. 1986b). In addition, no sequence similarity between the *C. caryophila* DNA and the coding sequence for the type 51 R-body was detected (Schmidt et al. 1988). New molecular data will shed light on the important question of whether the capacity to synthesize R-bodies appeared independently in different bacteria, or alternatively, genes coding for this unusual structure are related in the different bacterial groups. This last possibility would be in agreement with the hypothesis that they have been acquired by horizontal transfer through extrachromosomal elements. The availability of the sequence of the proteins involved in R-body polymerization would also help to clarify the process that creates a macrostructure with high stability and resistance to standard methods used to dissociate proteins, and hence of potential interest in the field of nanomaterials.

Another important question is whether R-bodies harbor some physiological role in cells expressing them. In the case of bacterial endosymbionts of paramecia the synthesis of the R-body confers the killer phenotype, which gives competitive advantages to the *Paramecium* (Kusch et al. 2002). In turn, the multiplication of the infected *Paramecium* favors *Caedibacter* spreading. However, it is important to bear in mind that the advantage for the *Caedibacter* is at the population level, since cells in which an R-body has been synthesized are no longer able to multiply. In favor of the consider-

ation of the R-body as a physiologically relevant structure is also the high conservation of the pKAP plasmid sequence, and particularly the *reb* genes, cloned from several *C. taeniospiralis* strains isolated from different regions. This strongly indicates that R-body structure and functionality requires proteins selected in an adaptative process (Heruth et al. 1994; Jeblick and Kusch 2005). These observations suggest that R-bodies could be an additional example of defective prophages that still harbor functional genes, and that have evolved to play an important role in the bacterial cell cycle (Casjens 2003). Another example is some of the *Pseudomonas* bacteriocins similar to phage tails: F bacteriocins resemble flexible and noncontractile tails, while R-type bacteriocins are similar to inflexible and contractile tails of T-even bacteriophages. The sequencing of the genes coding for these bacteriocins has clearly demonstrated that they are in fact related to phages (Nakayama et al. 2000). The same case applies to the gene transfer agent (GTA) of *Rhodobacter capsulatus*, a phagelike particle that mediates genetic exchange (Marrs 1974). The cloning and analysis of the genes coding for the GTA has shown that it is indeed related to phages from different bacteria (Lang and Beatty 2000).

So far it is unknown whether R-bodies play any physiological role in nonsymbiotic bacteria. In fact, at least in *M. mediterranea* the production of R-bodies seems to be, similarly to that in *Caedibacter*, associated with cell death (unpublished observations). Until now, R-bodies have been described in a few nonsymbiotic bacteria, belonging to different genera and occupying different environments. The study and characterization of R-body synthesis in additional nonsymbiotic bacteria would help to clarify whether R-body synthesis is associated with any particular taxonomic group or physiological condition.

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