John A. Fuerst *Editor*

Planctomycetes: Cell Structure, Origins and Biology

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Preface

The planctomycetes have been from the moment of their discovery organisms on the edge of our understanding, on the frontier of our knowledge of what microorganisms might be like, forming new models for microbial life and for biology. They have from this time on posed many fascinating and stimulating problems regarding their true identity, their evolutionary relationships, and their cell structure and biology. Even their name reflects this intriguing ambiguity—encapsulating the idea as actinomycetes once did that a eukaryote affinity might exist. The basis for that early decision—morphological similarity with fungi—is now not supportable since the pioneer work of Jean Schmidt and Mortimer Starr revealed the non-cellular nature of the stalks mistaken for mycelia in those early aquatic rosettes of *Planctomyces bekefii* seen originally only via light microscopy (Chapter 1). Perhaps though the time is not yet ripe to unreservedly apply the name 'planctobacteria' to these organisms as some have done informally. Planctomycetes have since Gimesi's time emerged as organisms with an internal organizational plan which appears to be one of the most complex known in bacteria or archaea—the cell contents are divided by internal membranes into two or even three distinct compartments, the nucleoid DNA is tightly folded, and in some cases membrane vesicles can form, imparting the ability to incorporate macromolecules from the environment analogous to eukaryote endocytosis and probably via similar molecular mechanisms (Chapters 2 and 3). They are indeed new models for cell structure, stretching our imagination of what a bacterial cell can look like and challenging our concept of a 'prokaryote' at the purely organizational level (without even considering the impact of phylogenetics and Archaea on this concept). The pure culture models for planctomycete cell biology and genetics *Gemmata obscuriglobus*, *Planctomyces limnophilus* and *Rhodopirellula baltica* have been central to our progress in these areas. Planctomycetes are of central significance to evolutionary microbiology and cell biology and must be taken into account in any future theories of eukaryote and eukaryote nucleus origins (Chapter 11). Thus, planctomycetes are of wide significance not only to microbiology but also to the biology of most organisms visible to the naked eye and must be taken into account if we are to solve major problems of biology concerning the marked transitions in life's evolution involving cellular

complexity. Changing perspective to our contemporary global problems, planctomycetes are also ready to help. There are now immense bioreactors at industrial scale, from the Netherlands city of Rotterdam to a monosodium glutamate factory in China, where anaerobic ammonium-oxidizing planctomycetes (Chapter 4) help us clean up environmental ammonia-rich waste while saving energy and reducing our CO_2 footprint. In addition, marine versions of these planctomycetes are central to the global nitrogen cycle, responsible for at least 50 % of nitrogen removal from marine ecosystems, and substantial amounts of the nitrogen we breathe may be produced by marine anammox species growing in oxygen-minimum zones of the world's oceans; regions thought to inevitably increase with increasing global warming. This anammox process is dependent on the internal membrane-bounded compartment known simply as the anammoxosome, a body which may be a true energy-generating organelle, one unique within the bacteria, but bearing comparison with eukaryotic mitochondria in some ways (Chapter 4). New unusual habitats such as acid peat bogs have excitingly revealed new taxonomic diversity among the planctomycetes and thrown light on the potential breadth of their ecological roles and importance (e.g. in microbial communities of ecosystems under threat with global climate change) (Chapter 5). If this were not enough microbiological stimulation from one bacterial group, it turns out that they harbour enzymes known previously from C1 transfer pathways involving methane generation and oxidation, and which may be significant for our understanding of how such major geomicrobiological processes for the global carbon cycle may have originated (Chapter 8). Of course, the answers to many of our questions regarding planctomycete cell biology and biochemistry may await the development of genetic systems so powerful for analysing the functions of genes in other bacteria—promising progress is being made to give planctomycetologists these essential molecular tools, and proteomics has already made progress in the understanding of unique features such as the protein cell wall of the model marine planctomycete *Rhodopirellula baltica* (Chapter 6). In the meantime, genomics and bioinformatics are revealing important features for our understanding and will provide a solid necessary basis for any future experimental genetics (Chapter 7). One of the remarkable features of planctomycetes revealed by genomics complemented is their possession of enzymes for pathways manipulating C1 compounds (Chapter 8), but in the apparent absence of methane oxidizing or generating abilities. Whatever their contemporary function, these enzymes are of great evolutionary interest, since they seem to be quite divergent from those known in other bacteria and in archaea, and perhaps go back to the very beginnings of methane biogeochemistry on Earth.

Beyond planctomycetes, we now know that planctomycetes have relatives within the bacteria, in the PVC superphylum, and comparative cell biology and genomics between members of this superphylum may form one of the keys to understanding their evolution. New extremophile PVC verrucomicrobia in the genus *Methyloacidiphilum* (Chapter 9) which, in contrast with planctomycetes, possess both C1 transfer pathways and methane oxidation metabolism, have added to our understanding of the immense physiological diversity within the PVC superphylum, encompassing as it does not only this thermoacidophilic methane oxidizer but also mesophilic aerobic chemoheterotrophs such as *Gemmata obscuriglobus*, the

moderately thermophilic phototactic planctomycete *Isosphaera pallida*, obligately anaerobic chemolithoautotrophs like the anammox planctomycetes, anaerobic human intestinal microbiome organisms like *Akkermansia muciniphila* in the verrucomicrobia and *Victivallis vadensis* in the Lentisphaerae, as well as the obligate intracellular pathogens in the phylum *Chlamydiae* (Chapter 10). Of necessity, this book discusses only some of the many significant PVC species beyond the planctomycetes.

Perhaps one of the reasons that planctomycetes and their relatives are frontier microorganisms is that they indeed include some very ancient bacteria representing some features of the pioneer habitats first available on the early earth (in the case of the anaerobic anammox ‘ammonium eaters’) and perhaps some features of the very earliest eukaryotes or even a eukaryote-like last common ancestor of the 3 Domains (Chapter 11). Analyses of the likely nitrogen cycle on the early Earth, for example, suggest that anammox planctomycetes were the first producers of nitrate on the planet and that anammox was the only process which could have closed the nitrogen cycle returning fixed nitrogen to the dinitrogen pool in the anaerobic biosphere. If alternatively planctomycetes or their ancestors did later on contribute by gene transfer or more direct vertical inheritance to the molecular basis of eukaryality, those events must have been ancient also. The phylogenetic and bioinformatic analyses are still controversial on how ancient planctomycetes and their closest relatives may be and on how homologous their eukaryote-like features to eukaryotes might be. Whatever the case, due to their widespread presence and activities they are one of the central microbial keys to understanding natural aquatic, terrestrial and perhaps even human microbiome microbial communities, and are a key to understanding the possible mechanisms of origin of the type of cell organization our very own human cells have inherited from the first eukaryote. They may thus form a model for origins of the biology of the modern cell and a key to truly understanding our own biology at the deep evolutionary level. As the late Carl Woese, the great discoverer of the Archaea and the three Domains of life emphasized, without such an evolutionary understanding there is no truly deep understanding of any life, that essentially historical entity.

Planctomycetes and their relatives are an excellent example of how understanding the true extent of microbial diversity can yield insights for science unimaginable if our focus was trained exclusively on *E. coli*. I would like this book also to widen your microbial, biological and scientific horizons to include the planctomycetes, new models for cell structure, origins and biology.

I would like to express my sincere thanks to all our authors—their great contributions have made this first book on planctomycetes focused on their cell biology possible. We would hope that in the future there will be another volume wholly devoted to the significant ecology and environmental significance of the planctomycetes. I also extend my thanks to Springer for publishing this book, one which will be immensely valuable for those in the field of planctomycetology and those entering it for the first time (of whom we hope there will be many more!).

St. Lucia, QLD, Australia John A. Fuerst

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Chapter 1
History, Classification and Cultivation of the Planctomycetes

Cheryl Jenkins and James T. Staley

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1.1 History and Classification of the Planctomycetes

1.1.1 The History of the Planctomycetes

The first report of the *Planctomycetes* phylum came from Nándor Gimesi, a Hungarian biologist who observed and photographed an unusual microcolonial form he found in Lake Lagymanyos in Budapest (Gimesi 1924). At this time, this lake although relatively wild was apparently eutrophic with a high organic and also high sulphate content possibly due to pollution from nearby farms (Langó 2005). Because he thought they were planktonic fungi, he named the type species of the

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genus, *Planctomyces bekefii* (from Gr. adj. *planktos*, wandering, floating; Gr. n. *mûkes*, fungus; n. *planctomyces*, floating fungus). The species was named to honour a Hungarian abbot, Remigius Békefi (1858–1924), cultural historian, university professor and abbot of the Hungarian Cistercian Order. The organism, as observed in samples from its aquatic habitat, has a very distinctive morphology with several spherical cells each with its own stalk with a holdfast at its tip that holds the cells together to form a microcolonial rosette (Fig. 1.1a, b). Much later, in the 1970s, by which time the lake had been filled in so that its extent had been reduced to a pond several hundred square metres in extent close to a railway bridge across the Danube(Langó 2005), the *Pl. bekefii* morphotype was still able to be documented in this type locality (Schmidt and Starr 1980a—see below).

Arthur T. Henrici's laboratory in the USA was the first bacteriological group to observe members of the *Planctomycetes* (Henrici and Johnson 1935). In the 1930s they incubated microscope slides in Midwestern lakes and removed and photographed them after several days' incubation. In their investigations, Johnson and Henrici reported budding bacteria that they called *Blastocaulis sphaerica* for the stalked forms (Fig. 1.2a–c) and *Blastobacter* for the non-stalked forms. They were unaware of the previous work by Gimesi. Although they did not isolate any of these organisms, it is clear that they are members of the *Planctomycetes* based upon their distinctive morphology and evidence of budding cell division. Indeed, Peter Hirsch, who later carefully compared *Pl. bekefii* to *Blastocaulis sphaerica*, concluded that they were members of the same genus (Hirsch 1972).

When the Approved List of Bacterial Names was prepared by V. B. D. Skerman in 1980, *Pl. bekefii* was included as one of the few bacteria that had a type species that was not in pure culture. And so it remains to this day (Ward 2010). *Pl. bekefii* has been reported elsewhere in Europe, Asia, Australia and North America where it is found in ponds and lakes. However, it is noteworthy that there are differences in the observed morphology of *Pl. bekefii*-like organisms depending on the locations where it has been reported. For this reason, it is likely that these geographically separated types may comprise different species (Schmidt and Starr 1980a).

A number of limnologists reported observing *Planctomyces* spp.. in freshwater lakes, and many different species names were ascribed to them based on their morphological traits alone (Hirsch and Skuja 1974). Undoubtedly the most morphologically striking species is *Planctomyces guttaeformis* (Hortobágyi 1965) in which the cells are not spherical, but are club shaped, and the mature cells in the rosette bear a long, tapering apical appendage that extends over 20 μm in length (Fig. 1.3). The species name means 'drop shaped'. Club-shaped buds are produced beneath the long apical appendage. The buds lack the apical appendage indicating that it is formed later in the organism's life cycle. Interestingly, the cells are joined together by a holdfast at the narrow pole of the club, so they do not have a stalk like *Pl. bekefii*. The narrow part of the club with its holdfast, which connects the cells together, appears to be cellular and not the acellular filamentous stalk found in *Pl. bekefii*. The long apical appendage of *Pl. guttaeformis* consists of multiple fibrils structurally analogous to the *Pl. bekefii* stalk (Fig. 1.3); however, it does not have a holdfast at its tip (Starr and Schmidt 1984).

Another named but uncultivated species, *Planctomyces stranskae* (named after the discoverer F. Wawrik's biology teacher W.L. Stransky), produces club-shaped cells

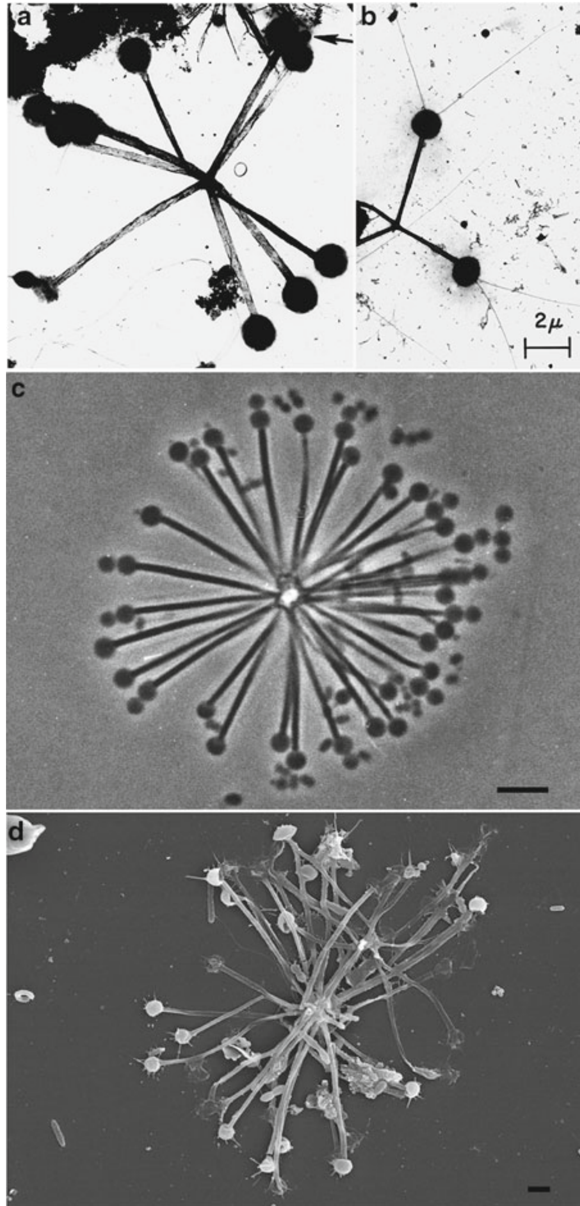

Fig. 1.1 (**a**) Electron micrograph of a microcolony of *Planctomyces bekefii* cells from University Lake near Chapel Hill, North Carolina. Note the single bud forming on one cell (see *arrow*). (**b**) Electron micrograph of another *Pl. bekefii* rosette from University Lake showing the two long apical spines that emanate from each cell. Cell diameters are approximately 1.5–2.0 μm (supplied courtesy of J.T. Staley). (**c**) Phase contrast micrograph of a *Pl. bekefii* rosette from Australian lake water at the University of Queensland (Bar = 5 μm) (from Fuerst (1995). Micrograph by J.A Fuerst and J.T. Staley). (**d**) Scanning electron micrograph of a *Pl. bekefii* rosette from The University of Queensland lake (Bar = 1 μm) (from Margaret K Butler (2006) PhD thesis (The University of Queensland). Supplied courtesy of J.A. Fuerst.)

like those of *Pl. guttaeformis*; however, this species lacks the distinctive long apical appendage produced by *Pl. guttaeformis* (Starr and Schmidt 1984). On the basis of such variable morphology, it seems incongruous that *Pl. guttaeformis* and *Pl. stranskae* are placed in the same genus as *Pl. bekefii*. However, the true taxonomic status of these organisms will remain unclear until pure cultures can be obtained and studied (Schmidt et al. 1981). A number of other morphospecies of *Planctomyces* have been described, but these are regarded as species incertae sedis (Ward 2010).

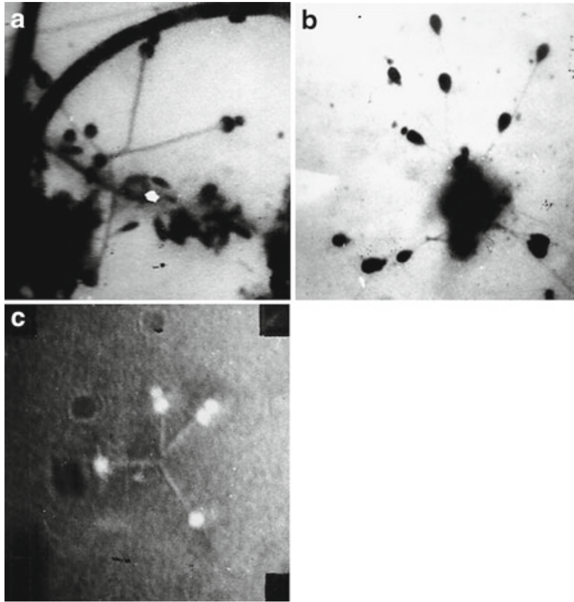

Fig. 1.2 (**a**) Light photomicrograph of the *Blastocaulis sphaerica* organism that Henrici and Johnson published in 1935[1]. (**b**) Light photomicrograph of another *Blastocaulis* rosette from A. T. Henrici's unpublished photos[1]. Note that the cells of this unidentified *Blastocaulis* species are pear shaped. (**c**) Light photomicrograph of what we regard as a *Planctomyces bekefii* rosette (negatively stained) with some budding cells from Henrici's unpublished photographs[1]. While the photo quality is poor, the resemblance to *Pl. bekefii* is clear. Note evidence of the black tape that Henrici used to adhere the photos to folio paper in C.

Jean Schmidt and Mortimer Starr in the USA studied these and other microcolonial forms of planctomycetes from freshwater habitats (e.g. from Arizona but also from the type habitat of *Pl. bekefii* in Hungary) and developed a morphotype system of classification of the genus *Planctomyces* (largely based on cell and stalk morphology) to avoid premature commitment to nomenclature (Schmidt and Starr 1978, 1979a, b, 1980b, 1982). However, the reliance of the morphotype system on stalk dimensions and appearance was problematic in that the acellular stalks can become encrusted with iron and manganese oxides, obscuring their fine structure (Schmidt et al. 1981, 1982). Furthermore, following the isolation of the first few planctomycete representatives in axenic culture, it was evident that culture conditions can affect the appearance of the stalk in *Planctomyces* spp., while in other planctomycete genera, acellular stalks are not formed (Schmidt 1978; Staley 1973; Schlesner 1986).

[1] A. T. Henrici's photomicrographs shown here along with another from his 1935 publication were left with Professor Erling Ordal in the Deparment of Microbiology at the University of Washington after Henrici's death and were given to JTS at the time of Professor Ordal's retirement. They have been returned to Professor Marty Dworkin in the Department of Microbiology at the University of Minnesota

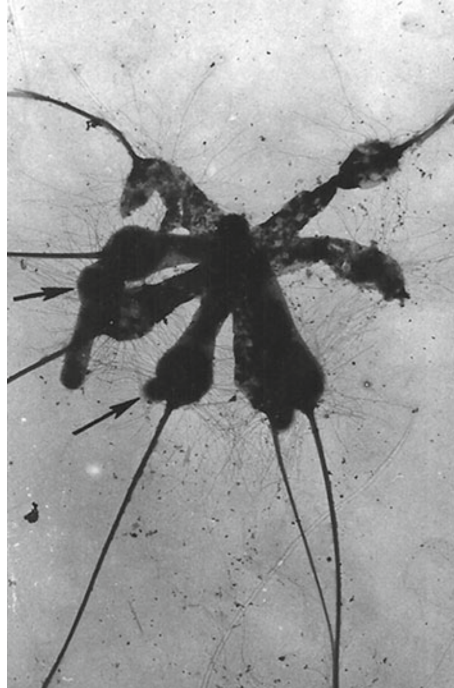

Fig. 1.3 Electron micrograph of *Planctomyces guttaeformis* from University Lake near Chapel Hill, North Carolina. Note that two of the club-shaped cells have buds (*arrows*). The bud from one cell is smaller and spherical and therefore younger than the club-shaped bud. Neither bud has the apical appendage indicating that it is formed later in the life cycle. The upper three cells are lysed and the apical appendage of the one on the upper right appears to be frayed indicating that it consists of multiple fibrils. Cell diameters are approximately 1.5–2.0 μm (supplied courtesy of J.T. Staley)

1.1.2 Classification of the Planctomycetes

1.1.2.1 Morphological Features of the Phylum Planctomycetes

While the morphotype system has been superseded by molecular taxonomy, morphological features can be useful for presumptive identification of planctomycetes. As mentioned previously, most species divide by budding and may or may not possess acellular stalks (Fig. 1.4). Stalked species often have a holdfast at the exposed tip of the stalk which allows cells to connect to one another to form a rosette (but not by the mechanism of *Caulobacter* spp. where cells attach directly to each other) or to attach to other organisms or detritus. In addition, cells are usually larger in diameter than other bacteria (up to 3.5 μm and occasionally larger), can be spherical, pear shaped, ellipsoid or club shaped and may exhibit both sessile and swarmer phases (Staley 1973; Schlesner 1994; Tekniepe et al. 1981).

Electron microscopy often reveals the presence of the hair-like surface appendages known as fimbriae. The planctomycetes are amongst the most hirsute bacteria known. Most recognised species have fimbriae, and the fimbriae may have particular locations on the cell surface depending on the genus and species. Some are perifimbrial (Fig. 1.5), where the fimbriae are located completely around the cell (Bauld and Staley

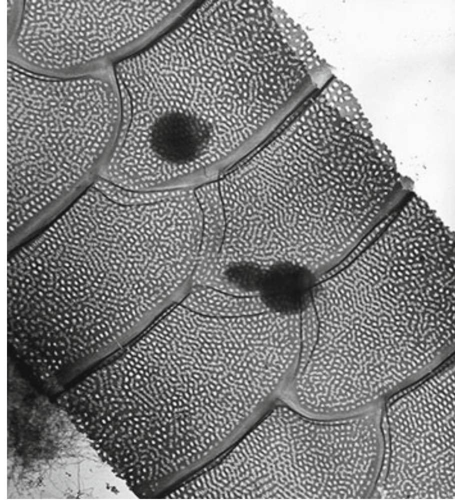

Fig. 1.4 An electron micrograph showing two non-stalked planctomycete cells attached to a diatom frustule (*Asterionella*) from Lake Washington. Note that one cell has a distinctive bud emerging from the broader reproductive pole of its cell. Note also how their cells are almost transparent to the electron beam (supplied courtesy of J.T. Staley)

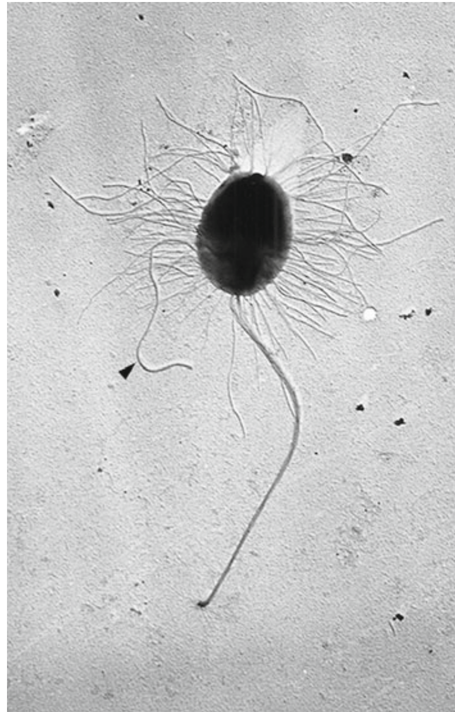

Fig. 1.5 *Planctomyces maris* as shown in a whole cell electron micrograph. Note that the cell is perifimbrial and that the stalk consists of several fimbriae-like fibrils bundled together. Also, note that the stalk is bent indicating its flexibility and that the cell has a flagellum (*arrow*) (supplied courtesy of J.T. Staley)

1976; Schmidt and Starr 1978). Others have polar fimbriae that are found at one pole and not the other (Staley 1973; Schlesner 1986). The fimbriae can be bundled together to form stalks (Hirsch and Müller 1985) and other related appendages such as the apical appendages of *Pl. guttaeformis* (Starr and Schmidt 1984). Fimbriae may be associated with crateriform structures, which are distinctive recessed areas in the cell wall;

however, a specific relationship between these structures is yet to be identified (Schmidt and Starr 1979a; Fuerst 1995). Nearly all members of the planctomycetes studied to date possess these structures.

In thin section, a unique proteinaceous cell wall that lacks a layer of peptidoglycan (a component of nearly all bacterial cell walls) is evident in all members of the planctomycetes (König et al. 1984). In addition, all planctomycetes have a compartmentalised cell plan consisting of a diverse range of membrane-bound internal cell structures (Lindsay et al. 2001; Fukunaga et al. 2009; see Chap. 2 in this volume). All species that have been examined to date possess distinctive internal membranes; the precise nature of which depends on the genus, but with some shared organisational features (Chap. 2 in this volume). As would be predicted, the separation of the cellular components by membranes may provide special organisational advantages for these organisms, including metabolic compartmentalisation (Damsté et al. 2002; van Niftrik et al. 2004), but they also pose barriers to transport between different sections of the cell presumably making intracellular communication more complex. This may in part explain the relatively large genomes of many of the planctomycetes (4–9 Mb).

1.1.2.2 Molecular Taxonomy

Despite the fact that Gimesi first recorded the presence of planctomycetes in water samples in 1924, for many years the planctomycetes were considered 'unculturable', and the first isolates of the phylum were not obtained until the 1970s. *Pirellula staleyi* was the first species isolated in pure culture in 1973, initially as the neotype strain of 'Pasteuria ramosa' (Staley 1973) but which was later renamed *Pirellula staleyi* (Schlesner and Hirsch 1984, 1987). The first species isolated from the genus *Planctomyces* was the stalked marine species, *Planctomyces maris* (Bauld and Staley 1976). Soon after, Schmidt (1978) reported the isolation of a number of members of the genus *Planctomyces* from freshwater habitats. Since then, the numbers of strains, species and genera have increased dramatically (Table 1.1). A description of isolation methods and of the various taxa is provided in greater detail in Sects. 1.2–1.4.

In 1986, the genera *Planctomyces* and *Pirellula* were assigned to a new family (*Planctomycetaceae*) and order (*Planctomycetales*) (Schlesner and Stackebrandt 1986) based on 16S rRNA cataloguing and phenotypic features. This order later expanded to include the genera *Gemmata* and *Isosphaera* (Ward et al. 1995). The diversity within these genera as well as the discovery of new strains has since resulted in the division of some of these clades into multiple genera as well as the addition of several new genera. Based on 16S rRNA analyses, the *Planctomycetes* are now considered their own phylum (Ward 2010) and this currently comprises 11 genera and 6 Candidatus genera, many of which are monospecific (Table 1.1). Some of these taxa still contain organisms with considerable genetic diversity and are likely to be split into further genera in the future.

The phylum *Planctomycetes* is considered to contain three distinct classes, the *Planctomycetia* (Ward 2010), the *Phycisphaerae* (Fukunaga et al. 2009) and the deep-branching anammox planctomycetes of the order *Candidatus* 'Brocadiales'

Table 1.1 Currently recognised genera and species within the phylum Planctomycetes

Class	Order	Family	Genus	Species	Taxonomic comments	Notable features
Planctomycetia	Planctomycetales	Planctomycetaceae	*Planctomyces*	*Pl. bekefii*	Type species. Named based on morphological features only. Not isolated in pure culture	Forms rosettes via thick tube-like stalks
				Pl. guttaeformis	Named based on morphological features only. Not isolated in pure culture	Large club-like cells. Long apical appendage. Buds form under the appendage
				Pl. stranskae	Named based on morphological features only. Not isolated in pure culture	Large club-shaped cells. No apical appendage observed
				Pl. maris	First species of the genus isolated in pure culture isolate	Marine species. Forms thin, flexible stalks. Colonies are non-pigmented. Genome sequence available
				Pl. limnophilus		Freshwater species. Forms a stalk. Colonies are red pigmented. Genome sequence and genetic tools available

		Pl. brasiliensis		Isolated from a hypersaline lagoon. Forms a stalk and possesses a single unicorn-like prostheca. Colonies are orange pigmented. Genome sequence available
	Schlesneria	*Sc. paludicola*	Distinct genus within the Planctomyces clade	Isolated from a peat bog. Moderately acidophilic. Forms short stalk-like structures. Polar distribution of crateriform structures
	Pirellula	*Pi. staleyi*	Type species of the genus. First planctomycete isolated in pure culture. Formerly *Pasteuria ramosa* (Staley 1973) and *Pirella staleyi* (Schlesner and Hirsch 1984)	Freshwater species. Lacks stalks but occasionally a 'fascicle' is observed. Crateriforms are distributed over the upper half of the cell. Genome sequence available
	Blastopirellula	*B. marina*	Type species. Formerly *Pirellula marina* (Schlesner 1986; Schlesner and Hirsch 1987)	Marine species. Crateriform structures distributed over the upper half of the cell. Genome sequence available

(continued)

Table 1.1 (continued)

Class	Order	Family	Genus	Species	Taxonomic comments	Notable features
			Rhodopirellula	*R. baltica*	Type species. Formerly '*Pirellula* sp. Strain 1'	Marine species. Forms pink colonies. Crateriform structures distributed over upper fifth of cell. Genome sequence available. Contains a large number of sulphatases
			Gemmata	*G. obscuriglobus*		Freshwater species. Possesses a double-membrane-bounded nuclear body and an endocytosis-like system. Draft genome sequence available
			Zavarzinella	*Z. formosa*	Distinct genus within the Gemmata clade	Isolated from an acidic peat bog. Forms rosettes. Possesses thick stalks that appear to be involved in the budding process

Isosphaera	*I. pallida*		Thermophilic species isolated from hot springs. Forms filaments via intercalary budding. Exhibits gliding motility and phototaxis. Contains gas vesicles. Oligotrophic. Genome sequence available
Singulisphaera	*Si. acidiphila*	Distinct genus within the Isosphaera clade. Displays 95 % similarity to 'Nostocoida limicola III'	Isolated from acidic wetlands. Colonies are non-pigmented. Spherical cells that do not form shapeless aggregates rather than filaments. Moderately acidophilic
	Si. rosea	Distinct genus within the Isosphaera clade. Displays 95 % similarity to *Si. rosea*	Isolated from acidic sphagnum peat. Forms pink colonies. Spherical cells found in pairs or short chains. Do not form long filaments. Moderately acidophilic

(continued)

Table 1.1 (continued)

Class	Order	Family	Genus	Species	Taxonomic comments	Notable features
			Candidatus 'Nostocoida limicola III'	'Nostocoida limicola III'	Originally named based on morphology but since recognised as a member of the Planctomycetes within the Isosphaera clade	Present in activated sludge. Cells are spherical to discoid. Forms filaments
			Aquisphaera	*A. giovannonii*	Distinct genus within the Isosphaera clade. Displays 92 % similarity to *Si. acidiphila*	Freshwater species. Forms pink-pigmented colonies. Non-motile
Phycisphaerae	Phycisphaerales	Phycisphaeraceae	*Phycisphaera*	*Ph. mikurensis*	Only species isolated within the Class Phycisphaerae	Isolated from a marine alga. Divides by binary fission rather than budding. Very high %GC content in genome (>73 %). Facultative anaerobe that can ferment D-xylose
Candidatus 'Brocadiae'	'Brocadiales'	'Brocadiaceae'	*Candidatus* 'Brocadia'	'Brocadia anammoxidans'	*Candidatus* species not yet isolated in pure culture	First anammox organism discovered. Contains an anammoxosome
				'Brocadia fulgida'	*Candidatus* species not yet isolated in pure culture	Species found in wastewater. Oxidises acetate. Autofluorescent

Candidatus 'Kuenenia'	'Kuenenia stuttgartiensis'	*Candidatus* species not yet isolated in pure culture	Species found in wastewater. Genome sequence available
Candidatus 'Anammoxoglobus'	'Anammoxoglobus propionicus'	*Candidatus* species not yet isolated in pure culture	Species found in wastewater. Mixotrophic metabolism. Can oxidise propionate
Candidatus 'Scalindua'	'Scalindua brodae'	*Candidatus* species not yet isolated in pure culture	Species found in wastewater
	'Scalindua wagneri'	*Candidatus* species not yet isolated in pure culture	Species found in wastewater
	'Scalindua sorokinii'	*Candidatus* species not yet isolated in pure culture	Marine species found in low-oxygen regions of the Black Sea
	'Scalindua arabica'	*Candidatus* species not yet isolated in pure culture	Marine and freshwater species. Found in low-oxygen regions
	'Scalindua richardsii'	*Candidatus* species not yet isolated in pure culture	Marine species found in low-oxygen regions of the Black Sea
Candidatus 'Jettenia'	'Jettenia asiatica'	*Candidatus* species not yet isolated in pure culture	Species found in wastewater

(Jetten et al. 2010). The latter group we will term here class *Candidatus* 'Brocadiae'. Although this classification differs from the recent classification of the *Planctomycetes* in the second edition of Bergey's Manual of Systematic Bacteriology (Ward 2010), in which the phylum is separated into two groups at the ordinal level, recent evidence from new strains that have been isolated and phylogenetic analyses of as yet uncultivated strains support the three classes (Nogales et al. 2001; Fukunaga et al. 2009; Jetten et al. 2010). Thus, we agree with several other researchers that the divergence between the groups is sufficiently great phylogenetically for them to be considered three separate classes (Janssen 2006; Elshahed et al. 2007; Fukunaga et al. 2009; Fuchsman et al. 2012). Some phenotypic features of members of the three classes currently support this view also. For example, the members of the *Planctomycetia* and *Candidatus* 'Brocadiae' all reproduce by budding and possess crateriform structures, while the known strains of *Phycisphaerae* reproduce by binary transverse fission and appear to lack crateriform structures. Furthermore, all known members of the *Planctomycetia* and *Phycisphaerae* are chemoheterotrophs, while members of *Candidatus* 'Brocadiae' are autotrophic (or mixotrophic) in their metabolism. In addition, there is evidence that C1 transfer genes are found only in the *Planctomycetia* and not in the anammox planctomycetes (Woebken et al. 2007).

The position of phylum Planctomycetes within the domain Bacteria has been the subject of some debate. They are generally believed to form a 'superphylum' with the *Verrucomicrobia*, *Lentisphaerae* and *Chlamydiae* phyla (PVC superphylum) based on analysis of ribosomal proteins and RNA polymerase subunits (Hou et al. 2008) and rDNA analyses (Wagner and Horn 2006), although some early sequence analyses did not support this relationship (Jenkins and Fuerst 2001; Ward et al. 2000). Some shared phenotypic traits are also used as evidence to link these phyla. For example, all known planctomycetes lack the cell wall component peptidoglycan and the cell division protein FtsZ features shared with members of the *Chlamydiae* phylum. Both *Planctomycetes* and *Verrucomicrobia* possess a compartmentalised cell plan and share unusual membrane coat proteins (Santarella-Mellwig et al. 2010). These features, which are shared with the Eukarya and some Archaea, have also been used to argue for a deep phylogenetic origin for the PVC superphylum within the Bacteria, or that the last universal common ancestor was a relative of the PVC group (Brochier and Philippe 2002; Reynaud and Devos 2011; Fuerst and Sagulenko 2012). Indeed planctomycetes possess additional eukaryote-like features including endocytosis-like processes, condensed DNA, sterols, integrin genes and membrane-bound DNA, as well as archaeal features such as ether- and ester-linked lipids, and genes for C1 transfer reactions (Chistoserdova et al. 2004; Damsté et al. 2002; Devos and Reynaud 2010; Fuerst and Webb 1991; Fuerst and Sagulenko 2010; Jenkins et al. 2002; Lindsay et al. 2001; Lonhienne et al. 2010; Pearson et al. 2003). Many of these features are described in detail in later chapters. Some molecular analyses also support the view that the planctomycetes, rather than hyperthermophilic organisms as has been traditionally posited, form an ancestral bacterial lineage (Brochier and Philippe 2002).

In contrast, other researchers have argued that many of the features of the planctomycetes and/or PVC superphylum are merely analogous to eukaryotic

or archaeal features, rather than homologous (membrane-bound DNA), have been derived by horizontal gene transfer (C1 transfer genes), or represent degenerative evolution (loss of FtsZ and peptidoglycan) rather than ancestral traits (McInerney et al. 2011). Regarding the lack of peptidoglycan in planctomycete cell walls, possible evidence for degenerative or reductive evolution is implied by the presence of some or even most of the genes required for peptidoglycan biosynthesis in the genomes of some species (Glöckner et al. 2003; Strous et al. 2006).

Whether the presence of these remarkable features represent a case of analogy or homology, the planctomycetes are increasingly playing a major role in understanding the evolution of cellular complexity and organisation.

1.2 Methods for the Enrichment, Isolation and Cultivation of the Planctomycetes

Low nutrient or oligotrophic enrichments have been used as a primary step in the isolation of particular organisms from aquatic environments. The use of low nutrient enrichments came from the method of Houwink (1951) who added low concentrations of peptone, 0.01 % to aquatic samples, for the enrichment and isolation of *Caulobacter* spp. This same approach, using 0.01 % peptone or 0.005 % peptone and 0.005 % yeast extract combined, was subsequently used to enrich and isolate prosthecate Proteobacteria as well as strains of planctomycetes (Staley 1968, 1973; Bauld and Staley 1976). These liquid enrichments are incubated at room temperature and examined microscopically after 1–2 weeks or more for evidence of budding bacteria that appear to be members of phylum Planctomycetes. Inoculum from these enrichments can then be streaked onto similar solid oligotrophic media for their isolation. Extended incubation of water samples without the addition of nutrients, in the dark to prevent cyanobacterial and algal growth, has also been used successfully in the enrichment of planctomycete strains (Hirsch and Müller 1986).

Most planctomycetes are not strictly oligotrophic, and while many do reside in relatively low-nutrient aquatic habitats, they are also found in eutrophic water, soils, wastewater and other nutrient-rich environments. In particular, in a survey of one mesotrophic and several oligotrophic Australian lakes as well as eutrophic ponds, the viable concentrations of the *Planctomycetes* group were found to be highest in the eutrophic ponds where their numbers were as high as 240 per ml. However, their numbers relative to total viable heterotrophs remained similar regardless of trophic state (Staley et al. 1980). Nonetheless, all planctomycetes have relatively long generation times and as such, enrichment in dilute solutions favours planctomycete growth, where the use of nutrient-rich media can result in the overgrowth of more rapidly dividing bacteria. Low nutrient enrichment has been successfully applied to the isolation of a number of freshwater planctomycete strains (Staley 1973; Schmidt 1978) and has proven to be a very effective technique for isolation of planctomycetes from soil (Yee et al. 2008) where overgrowth of fungi can otherwise prove problematic. Marine

strains have been successfully isolated using dilute peptone enrichment medium made with artificial seawater and on commercially available marine agar at half or lower strength (Bauld and Staley 1976; Fuerst et al. 1997; Fukunaga et al. 2009). Remarkably, the type strain of the species *Isosphaera pallida*, a true oligotroph, was initially isolated on a solid medium suitable for autotrophs, without the addition of any specific organic carbon sources. This organism is chemoheterotrophic rather than autotrophic in its metabolism and was apparently able to survive and multiply using only the organic contaminants within the agar itself (Giovannoni et al. 1987). Specific testing of *I. pallida* strains on a variety of carbon sources indicated that growth of this organism is inhibited by glucose concentrations of just 0.05 %. Some strains are also inhibited by low concentrations of ribose, fructose, maltose or glycolate (Giovannoni et al. 1987).

The ability of some planctomycete genera to attach to surfaces via a holdfast structure has also been exploited in some isolation techniques such as the 'petri dish method' of Hirsch and Müller (1986). In this method, sterile glass coverslips are placed upright in a petri dish containing the water sample of interest and incubated for several days to allow planctomycete organisms to attach to the glass. The coverslip is then placed face down on a nutrient-containing agar to allow colonies to develop. More recently, this technique has been combined with molecular detection methods for the isolation of planctomycete strains from acidic peat. In that study, planctomycetes were enriched on coverslips immersed in peat water and fluorescent in situ hybridisation, employing planctomycete-specific probes, was used to monitor enrichment of planctomycetes within the coverslip biofilms (Kulichevskaya et al. 2006). The petri dish method has been used successfully for the isolation of planctomycete strains possessing holdfasts such as *Planctomyces, Pirellula* spp. (Hirsch and Müller 1986) and *Zavarzinella formosa* (Kulichevskaya et al. 2009) as well as strains exhibiting glycocalyx formation such as *Singulisphaera* (Kulichevskaya et al. 2006, 2008).

The distinctive morphology of many planctomycetes, their relatively large cell size and/or tendency to form rosettes, enables their presumptive identification within a complex sample. The distinctive appearance of these organisms can then be exploited in their isolation through the use of microtools. Micromanipulation, a technique employing a fine glass tool attached to a low-powered microscope lens, and the forces of surface tension (Skerman 1968) enabled the capture and subsequent cultivation of cells of *Gemmata obscuriglobus* (Franzmann and Skerman 1984) and *Candidatus* 'Nostocoida limicola III' (Liu et al. 2001). More modern but equivalent methods, such as the use optical (laser) tweezers (Fröhlich and König 2000) or gel microdroplet encapsulation (Zengler et al. 2002), are potentially promising techniques for the isolation of uncultivated planctomycete strains.

Increased knowledge regarding the ecology and physiology of planctomycetes facilitated the development of selective media for their isolation, and a comprehensive study of selective chemoheterotrophic enrichment and isolation techniques by Schlesner (1994) resulted in the isolation of a large collection of planctomycetes from diverse aquatic habitats varying in salinity, pH and nutrient levels. A notable finding from this study was the ability of many of these strains to utilise *N*-acetyl-*D*-glucosamine as a sole source of carbon and nitrogen. *N*-acetyl-

D-glucosamine is the basic subunit of chitin, which may explain the observed association of some planctomycetes with chitinous organisms such as *Daphnia* sp. ('water flea'), the giant tiger prawn, *Penaeus monodon*, diatoms, termites (*Cubitermes* spp.) as well as carbon-rich marine detrital aggregates known as 'marine snow' (Staley 1973; Fuerst et al. 1991, 1997; Morris et al. 2006; Köhler et al. 2008; DeLong et al. 1993). The discovery that planctomycetes possess a unique proteinaceous cell wall structure lacking in the (almost universal) bacterial feature, peptidoglycan (König et al. 1984), led Schlesner to incorporate ampicillin into his selective media. Indeed, the inherent resistance of planctomycetes to β-lactam antibiotics is highlighted by the fortuitous isolation of a new strain from a laboratory ampicillin solution that had not been filter-sterilised (Wang et al. 2002). By employing media containing *N*-acetyl-D-glucosamine or peptone, yeast extract and glucose combined with ampicillin and cycloheximide to minimise bacterial and fungal growth, Schlesner was able to isolate a total of 256 new planctomycete strains from diverse habitats including oligotrophic eutrophic and saline ponds, marine aquaria, manure, sewage, chalk and gypsum mine water, an Antarctic lake and the pitcher of a *Nepenthes* spp. (Schlesner 1994). Subsequently, selective media containing combinations of antibiotic agents such as D-cycloserine, penicillin or ampicillin and streptomycin or chloramphenicol, to which planctomycetes are also inherently resistant, and antifungal agents such as cycloheximide, amphotericin B or benomyl, were successfully employed in isolation of planctomycetes from freshwater, soil and an acidic peat bog (Wang et al. 2002; Yee et al. 2008; Kulichevskaya et al. 2009; Lage and Bondoso 2011). An improved cultivation method specifically designed for the isolation of *Rhodopirellula* strains has also been developed, whereby particulate matter including sediment granules, plankton or biofilm scrapings are plated onto selective agar (Winkelmann and Harder 2009; Lage and Bondoso 2011). This method exploits the ability of these planctomycetes to attach to particles and was found to greatly improve the yield of new strains (Winkelmann and Harder 2009).

The remarkable diversity within the *Planctomycetes* and their ability to occupy disparate ecological niches has become ever more apparent with each new pure culture isolate. In some cases, isolation of a single species has necessitated the creation of an entirely new genus (or class) to accommodate it. Molecular surveys of different ecosystems across the globe indicate that substantial uncultivated diversity still exists within the *Planctomycetes*; however, most cultivated strains isolated so far comprise aerobic chemoheterotrophs of the class *Planctomycetia*. Nonetheless, there are hints that even the *Planctomycetia* may be more physiologically diverse than present isolation methods presume. The fact that members of the *Planctomycetia* were detected via molecular methods in the anaerobic and sulphur-rich Zodletone Spring raises the question of whether these organisms might be capable of elemental sulphur respiration (Elshahed et al. 2007). It is clear that the cultured strains reflect the methods used to isolate them, and if a broader range of planctomycete representatives are to be grown axenically, then culture methods must be designed that take into account the metabolic and physiological diversity within this group.

1.3 Pure Culture Representatives of the Phylum Planctomycetes

1.3.1 Class Planctomycetia

1.3.1.1 The Pirellula Clade: Genera *Pirellula, Rhodopirellula* and *Blastopirellula*

Pirellula staleyi (ATCC 27377 = DSM 6068) was the first characterised member of the Planctomycetes to be grown in axenic culture (Staley 1973); however, the taxonomic affiliation of this organism was not clarified until sometime later (Starr et al. 1983). *Pi. staleyi*, a pear-shaped organism, was isolated from Lake Lansing, Michigan, and was also found in association with the carapace of the cladoceran invertebrate *Daphnia pulex* ('water flea') collected from Lake Erie. A morphologically similar organism *Pasteuria ramosa*, which is parasitic on *Daphnia* spp., had been observed nearly a century earlier by Metchnikoff (1888). *Pa. ramosa* was described as an endospore-forming bacterium; however, no evidence of endospores or binary fission was noted in the organism associated with *D. pulex*, which instead reproduced by budding. This led to the conclusion that the Lake Lansing strain, ATCC 27377, was a rediscovery of *Pasteuria ramosa,* and that Metchnikoff had mistaken the immature buds for endospores. As such, this organism was initially assigned as the type strain of *Pa. ramosa* (Staley 1973). Later studies revealed that the endospore-forming cladoceran parasite of Metchnikoff did indeed exist and was demonstrably different from the Lake Lansing strain (Sayre et al. 1983). ATCC 27377 was initially reclassified as *Planctomyces staleyi* based on certain morphological similarities to other members of the *Planctomyces–Blastocaulis* group (Starr et al. 1983). In that study, Starr and colleagues noted that considerable morphological diversity existed within the *Planctomyces–Blastocaulis* group, and that ATCC 27377 may form its own genus within the group. Indeed, *Planctomyces staleyi* was later reassigned to *Pirella staleyi* and finally to *Pirellula staleyi* (due to the prior use of '*Pirella*' for a fungal genus) (Schlesner and Hirsch 1987), based on morphological, physiological and molecular data (Schlesner and Hirsch 1984).

Pi. staleyi remains the only named species of the genus *Pirellula*, although a number of related strains have since been cultivated from freshwater sources (Schlesner 1994; Schlesner et al. 2004). Two strains isolated from the brackish Baltic Sea were initially assigned to the genus *Pirellula* based on general morphological similarities to *Pi. staleyi*: a non-pigmented strain classified as *Pirellula* ('*Pirella*') *marina* (Schlesner 1986) and a pink-pigmented strain simply described as *Pirellula* sp. SH 1 (Schlesner 1994). Numerous additional strains from saline and hypersaline habitats, the tissues of the giant tiger prawn, *Penaeus monodon* (Fig. 1.6a) (Fuerst et al. 1991, 1997) and from the marine sponge *Aplysina aerophoba* (Gade et al. 2004) have also been described as *Pirellula*-like (Schlesner 1994). The considerable phenotypic and genetic diversity within the group was noted

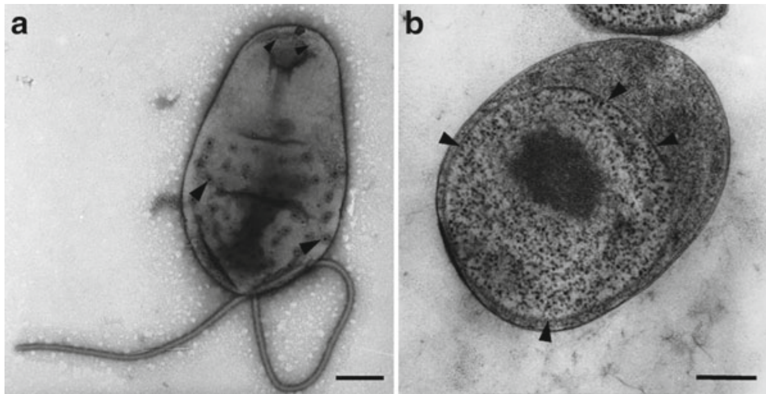

Fig 1.6 (**a**) Electron micrograph of a negatively stained cell of a *Pirellula*-like planctomycete isolated from tissues of the giant tiger prawn, *Penaeus monodon*. The cell is pear shaped and possesses a single polar sheathed flagellum. *Large arrowheads* indicate large crateriform structures that are distributed over half of the cell surface. Small crateriform structures are also present at the opposite cell pole (indicated by the small *arrowheads*) (prawn isolate PRPL-2 from Fuerst et al. (1991). Micrograph by J.A Fuerst). (**b**) Transmission electron micrograph of an ultrathin section of a cell of Pirellula group planctomycete. The *arrowheads* indicate the single membrane that forms the internal compartment known as the 'pirellulosome' (Bars = 0.2 μm) (from Lindsay et al. (1997). Micrograph by M.R. Lindsay.)

(Fuerst et al. 1997) and later confirmed by 16S rRNA gene sequencing, DNA–DNA hybridisation and chemotaxonomic studies (Schlesner et al. 2004). These data resulted in the reclassification of *Pirellula marina* as the type strain of a new genus and species *Blastopirellula marina*^{T}, while *Pirellula* sp. SH 1 was assigned the type strain of the genus *Rhodopirellula* as *Rhodopirellula baltica*^{T}. *Pirellula*, *Blastopirellula* and *Rhodopirellula* (referred to here as the Pirellula clade) do nonetheless have shared morphological features and are genetically more closely related to each other than to other planctomycetes. Interestingly, pigmentation and other phenotypic features of the Pirellula clade appear to have some phylogenetic relevance within the group. Thus, *Rhodopirellula* spp. are all pink pigmented and along with *Blastopirellula*, inhabit saline environments, while freshwater strains fall within the *Pirellula* genus. Clone libraries constructed from bacterial 16S rRNA genes indicate that members of the Pirellula clade are amongst the most common planctomycetes in flooded rice microcosms and municipal wastewater basins (Derakshani et al. 2001; Chouari et al. 2003) and are also one of the dominant lineages associated with diatom blooms (Morris et al. 2006) (Fig. 1.4).

Rhodopirellula baltica is perhaps the best studied of all planctomycetes, having been subjected to genome sequencing as well as proteomic and transcriptomic analyses (Glöckner et al. 2003; Rabus et al. 2002; Gade et al. 2005; Hieu et al. 2008; Wecker et al. 2009). A notable finding from genome sequencing studies is that there

are over 100 genes within *R. baltica* and a similar number within *B. marina* encoding sulphatases. These enzymes may well play a role in degradation of sulphated polysaccharides such as fucoidan, heparan sulphate and chondroitin sulphate. Sulphated polysaccharides are abundant in the oceanic environment within seaweed and other marine organisms (Fuerst and Sagulenko 2011). Indeed chondroitin sulphate, which is common in crustaceans, molluscs and other invertebrates (Yamada et al. 2011), has been shown to be an excellent substrate for both *R. baltica* and *B. marina* (Schlesner et al. 2004). Interestingly, *Rhodopirellula* spp. comprise over 50 % of organisms within biofilms on sea kelp *Laminaria hyperborea* (Bengtsson and Øvreås 2010) and are also found associated with other marine macroalgae (Lage and Bondoso 2011), therefore these associations may be directly related to the metabolic preferences of *Rhodopirellula* spp. These findings, combined with the recent discovery that a relative of *B. marina* is capable of anaerobically reducing sulphur to sulphide (Elshahed et al. 2007), suggest that members of the Pirellula clade are emerging as potentially important players in the sulphur cycle.

Members of the Pirellula clade form rosettes with cells attaching directly to each other via a holdfast secreted from the narrow cell pole. True multifibrillar stalks are absent in all studied members of the Pirellula clade, but a less rigid and well-defined 'fascicle' consisting of twisted fibrils was initially reported in *Pi. staleyi* strain ICPB 4232 (= Schmidt CLPM = clone BT2) isolated from a freshwater lake in Baton Rouge, Louisiana, USA. Subsequent ultrastructural studies of a subclone of this strain (ATCC 35122) did not record the presence of a fascicle, suggesting that this structure is rarely formed, and perhaps only under certain culture conditions (Butler et al. 2002). *Pi. staleyi* also possesses prosthecate appendages that appear as horn-like protrusions (2 per cell) on either side of the narrow pole of the cell. The function of these appendages is unknown, although their positioning suggests that they may play a role in attachment (Butler et al. 2002), perhaps during rosette formation. These prosthecae have not been observed in *B. marina* or *R. baltica*.

Crateriform structures, which appear as small indentations in the cell wall, are visible by electron microscopy in negatively stained preparations and are yet another unusual ultrastructural feature of members of the Pirellula clade and indeed all Planctomycetia species described to date. In most planctomycete species these structures are evenly distributed over the cell surface, while in members of the Pirellula clade they are polarly distributed with large crateriform structures (12 nm diameter) occurring at the broader (proximal) cell pole in association with fimbriae, and in some cases at least, small crateriform structures (5–7 nm) occurring at the narrow cell pole (Tekniepe et al. 1981; Fuerst et al. 1991) (Fig. 1.6a). In *Pi. staleyi* and *B. marina,* large crateriform structures are distributed over the upper half of the cell, while in *R. baltica*, they are confined to the upper fifth. The polar nature of members of the Pirellula clade is further evidenced by the presence of the internal membrane-bounded compartment termed the 'pirellulosome' (Fig. 1.6b) (Lindsay et al. 1997), which is positioned towards the wider cell pole (*R. baltica* also contains several smaller internal compartments in addition to the pirellulosome (Schlesner et al. 2004)).

Consistent with this intracellular organisation, the reproductive cell cycle is also polar in nature. The life cycle of *Pi. staleyi* has been studied in some detail and involves the formation of a bud from the reproductive (broader, non-holdfast) pole of the mother cell, followed by development of a single polar to subpolar sheathed flagellum on the daughter cell which subsequently detaches to form a motile swarmer. The swarmer cell matures over approximately a 30 h period during which it enlarges, forms pili at the reproductive pole and loses its flagellum, at which point it becomes the mother cell for another generation of cell division. Buds form as a mirror image of the mother cell with the broader cell pole of the bud emanating from the equivalent pole of the mother cell; however, crateriform structures are initially distributed evenly across the surface of the bud before taking on a polar distribution in enlarged buds (Tekniepe et al. 1981). The life cycles of *R. baltica* and *B. marina* are similar to that of *Pi. staleyi*; however, in the case of the latter species, the buds are bean shaped, rather than a mirror image of the mother cell (Schlesner 1986; Schlesner et al. 2004).

1.3.1.2 The Planctomyces Clade: Genera *Planctomyces* and *Schlesneria*

Planctomyces bekefii, the type species of the genus *Planctomyces*, was first characterised by Gimesi in 1924 based on morphological observations; however, it was not until 1976 that a member of this genus, *Pl. maris*, was isolated in axenic culture (Bauld and Staley 1976). Up until that time, most observations of *Planctomyces* sp. had been in freshwater habitats; although Hirsch and Rheinheimer (1968) noted their presence in brackish water and seawater sediments. The genus *Planctomyces* currently encompasses only three named species that have been isolated in pure culture, all from aquatic environments varying in salinity. *Pl. maris* was isolated from the neritic waters of Puget Sound (Washington, USA), *Pl. limnophilus* from freshwater Lake Plußsee (Holstein, Germany) and *Pl. brasiliensis* from Lagoa Vermelha, a hypersaline lagoon (near Rio de Janeiro, Brazil) (Bauld and Staley 1973; Hirsch and Müller 1985; Schlesner 1989). A second strain of *Pl. brasiliensis* was later isolated from post-larvae of the giant tiger prawn, *Penaeus monodon* (Fuerst et al. 1997). Reflecting their different habitats, these *Planctomyces* species vary in their tolerance for salt, with *Pl. limnophilus* being stenohaline, while *Pl. maris* and *Pl. brasiliensis* are moderately euryhaline. *Pl. brasiliensis* exhibits the widest range of salt tolerance (Schlesner 1989), which may explain its association with *Penaeus monodon* itself a euryhaline species. Members of the *Planctomyces* genus have also been isolated from a meromictic lake in Antarctica, an alkaline chalk mine, a gypsum mine, compost, sewage and soil (Schlesner 1994). A few strains have also been isolated from soil (Yee et al. 2008); however, like members of the Pirellula clade, the vast majority of pure culture isolates have been derived from aquatic rather than terrestrial habitats Nonetheless, culture-independent studies of microbial soil populations have revealed that members of the Planctomyces and Pirellula clades are relatively abundant in these habitats (Lee et al. 1996, Buckley et al. 2006).

A number of moderately acidophilic strains belonging to the Planctomyces lineage were isolated from sphagnum-dominated boreal wetlands and were found to be sufficiently divergent from characterised members of the genus *Planctomyces,* based on 16S rRNA gene sequence similarity, to form their own genus and species, *Schlesneria paludicola* (Kulichevskaya et al. 2007 and Chap. 5 of this volume). *Sc. paludicola* appears to be most closely related to two strains, *Planctomyces* sp. strain 638 and 642, isolated from compost water (Schlesner 1994). All three species are unpigmented. The closest relative of *Sc. paludicola* amongst the named strains is the red-pigmented *Pl. limnophilus*; however, *Sc. paludicola* only exhibits 28 % DNA–DNA hybridisation with this species (Kulichevskaya et al. 2007). Phylogenetic diversity within the Planctomyces clade is significant, and it is likely that it encompasses yet more genera.

Members of the Planctomyces clade are ovoid to ellipsoid in morphology and crateriform structures are distributed over the entire cell surface. The one notable exception to this is *Sc. paludicola* which has a polar distribution of crateriform structures, similar to members of the Pirellula clade, over the upper third of the cell. *Sc. paludicola* also possesses a single prosthecate spur-like projection on the reproductive pole of the cell. A very similar structure has been noted in strains of *Pl. brasiliensis* (Fuerst et al. 1997) but not in other members of the genus *Planctomyces.*

All cultivated species from the *Planctomyces* genus form rosettes and possess stalks consisting of bundles of twisted fibrils. These are in contrast to the rigid stalks of the type strain, *Pl. bekefii,* which have been described as tube or ribbon-like. *Pl. maris* forms particularly fine stalks that are not visible with phase contrast microscopy and can only be observed under the electron microscope (Bauld and Staley 1976). The stalk is flexible and would be expected to serve as a tether that allows the cells to move around with the currents in an aquatic environment (Fig. 1.5). Stalk morphology may not be a particularly useful taxonomic marker; however, as stalk width and appearance can vary with culture conditions, and in the uncultivated species, with levels of iron encrustation. *Sc. paludicola* also forms rosettes but cells attach via much shorter stalk-like structures composed of loose arrangements of fimbriae. These structures are often observed coated in electron-transparent, vesicle-like bodies of unknown function (Kulichevskaya et al. 2007).

Like all planctomycetes, members of the Planctomyces clade have a relatively complex intracellular structure composed of membrane-bounded compartments. The intracellular organisation of *Pl. limnophilus* is similar to that seen in the Pirellula clade, while the internal structure of *Pl. maris* is more akin to that of *Isosphaera pallida* (Lindsay et al. 2001). Our understanding of the functioning of these compartments, as well as physiological, metabolic and regulatory processes within this clade, is likely to expand considerably in the future, with the recent sequencing of the genomes of *Pl. limnophilus*, *Pl. maris* and *Pl. brasiliensis* (LaButti et al. 2010) and the development of genetic tools for the manipulation of *Pl. limnophilus* (Jogler et al. 2011).

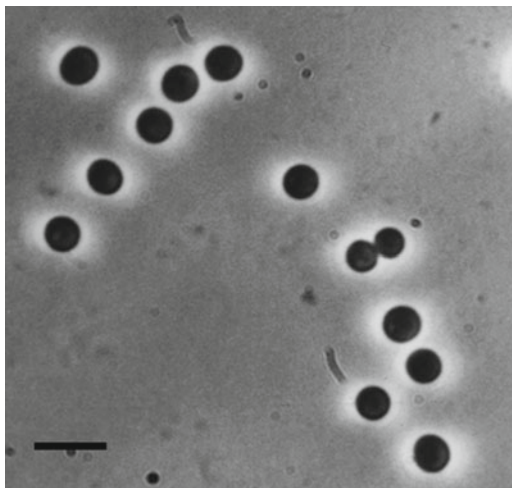

Fig 1.7 Phase contrast micrograph of a planctomycete isolated from Iowa soil that is related to *Gemmata obscuriglobus*. Cells are spherical, lack stalks and do not form rosettes. One cell is in the process of budding (Bar = 5 μm) (micrograph by C. Jenkins)

1.3.1.3 The Gemmata Clade: Genera *Gemmata* and *Zavarzinella*

Gemmata obscuriglobus, was isolated from the freshwater Maroon Dam (Queensland, Australia), via micromanipulation (Franzmann and Skerman 1984) and has since become one of the most studied planctomycete strains in relation to its complex and unusual cell biology. Upon cultivation, *G. obscuriglobus* was recognisable as a planctomycete but was sufficiently different from other strains that it was immediately assigned to its own genus and species. *G. obscuriglobus* differs from members of the Pirellula and Planctomyces clades in that cells are spherical, do not possess discrete holdfasts and do not form rosettes (Fig. 1.7). Furthermore, *G. obscuriglobus* does not form stalks, short stalk-like structures or fascicles, although fimbriae are present, and crateriform structures are uniformly distributed over the cell surface (Franzmann and Skerman 1984).

While *G. obscuriglobus* may seem to lack a polar cell organisation, studies on the life cycle of this organism have revealed that budding division occurs repeatedly from the same cell surface location on the mother cell, suggesting that a reproductive pole exists (Lee et al. 2009). *G. obscuriglobus* cells are motile via multitrichous flagella, and like *Pi. staleyi* (ICPB 4232), are sessile during budding; however, the length of each life cycle stage appears to vary between these strains, with the period from the initiation of bud formation to bud detachment taking much longer in *G. obscuriglobus* (Lee et al. 2009). Remarkably, the almost universal bacterial cell division protein, FtsZ, has been found to be absent from the draft genome of this species and indeed all planctomycete strains sequenced thus far, leaving open the question of how cell division is mediated in these organisms. Genes encoding proteins very distantly homologous to the FtsZ family have been subsequently identified in planctomycetes and have been proposed as possible candidates for cell division proteins (Makarova and Koonin 2010).

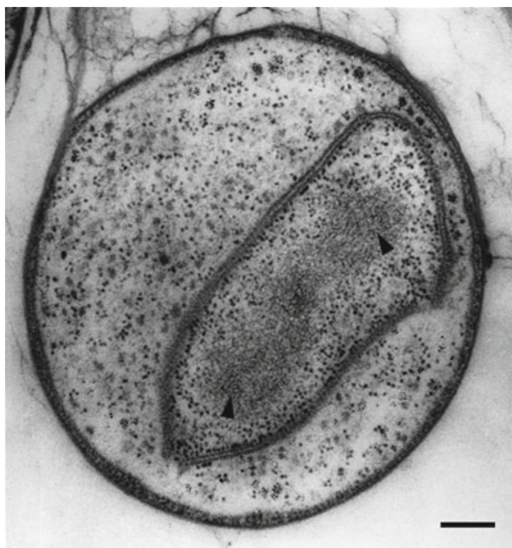

Fig 1.8 Transmission electron micrograph of a *Gemmata obscuriglobus* cell in ultrathin section. The chromosomal material, indicated by the *arrowheads*, is condensed and is enclosed by two distinct membranes (Bar = 0.2 μm) (from Lindsay et al. (2001). Micrograph by M.R. Lindsay.)

The presence of spherical phase-dark inclusions, noted upon microscopic examination of *G. obscuriglobus* cells, was one feature used to distinguish this organism from *Pi. staleyi*. This phase-dark inclusion was initially described by Franzmann and Skerman as 'packaged' genomic material, although it now seems likely that it is the phase-light region of the cell that comprises the nuclear material (Lee et al. 2009). While extensive internal membranes were noted in ultrathin sections of *G. obscuriglobus* in the initial Franzmann and Skerman study, the structural analogy to the eukaryotic nucleus, namely, that the DNA is fully enclosed within a double membrane, was not recognised until sometime later (Fuerst and Webb 1991) (Fig. 1.8). The ultrastructure of the nuclear body has been extensively studied and is covered in greater detail in Chaps. 2 and 3. It has been suggested that the presence of sterols, another feature typical of eukaryotes (Pearson et al. 2003) but also found in *Gemmata*, is of potential significance to the functioning of these internal membranes (Fuerst 2005). Yet another eukaryote-like feature of *G. obscuriglobus* related to its cell biology is the ability of cells to take up proteins in an energy-dependent process analogous to endocytosis (Lonhienne et al. 2010). *G. obscuriglobus* even encodes structural homologs of eukaryotic membrane coat proteins that are involved in vesicle formation (see Chap. 4). These membrane coat proteins have been found in a number of planctomycete genomes implying that endocytosis-like processes may be widespread within the phylum (Santarella-Mellwig et al. 2010). *G. obscuriglobus* also shares some features of the methanogenic Archaea, with the discovery of genes for C1 transfer reactions within this species and subsequently in other planctomycetes (Chistoserdova et al. 2004). The ongoing discovery of such features combined with intense speculation regarding the possible deep-branching nature of the lineage (Brochier and Philippe 2002), with potential links to the Eucarya (Fuerst 2005; Reynaud and Devos 2011; Fuerst and Sagulenko 2012) is the major reason

that the planctomycetes are considered an emerging model for the evolution of cell biology (Fuerst 2004, 2005).

Despite the fascinating cell biological features of *Gemmata* spp., relatively few strains have been isolated in axenic culture. *G. obscuriglobus* remains the only fully characterised species within the genus although a number of other species have since been isolated from freshwater, compost water and especially from soil (Schlesner 1994; Wang et al. 2002; Yee et al. 2008). All six *Gemmata*-like strains isolated in one of these studies (soil and lake water isolates) exhibited the double-membrane-bounded nuclear body characteristic of *G. obscuriglobus* (Wang et al. 2002). Sequence data from 16S rDNA clone libraries suggests that *Gemmata* spp. are present in reasonable numbers in wastewater (Chouari et al. 2003) and river biofilms (Brümmer et al. 2004).

Zavarzinella formosa is a moderately acidophilic strain from the Gemmata clade isolated from a Siberian peat bog (Kulichevskaya et al. 2009 and Chap. 5 of this volume). This organism is most closely related to freshwater strain JW10-3f1 in the Gemmata group (Wang et al. 2002) but displays only 90 % 16S rDNA sequence similarity to *G. obscuriglobus,* and therefore warranted classification in its own genus. *Z. formosa* is also morphologically very distinct from *Gemmata* spp., possessing ellipsoid cells and striking stalks that are both thick (up to 0.7 μm diameter) and unique in their bead-like structure. Rosettes are formed by mature cells attaching via holdfast material excreted from one cell pole, while young cells are motile via a monotrichous flagellum. Stalks of mature *Z. formosa* cells are fibrillar but contain numerous phase-dark vesicular inclusions of unknown function. It has been suggested that these unusual stalks may act as a fibrillar sheath for dividing cells and may derive their unique shape from a repetitive budding process (Kulichevskaya et al. 2009). Indeed, unlike other stalked planctomycetes, budding appears to occur from the stalked cell pole.

1.3.1.4 The Isosphaera Clade: Genera *Isosphaera, Singulisphaera* and *Aquisphaera*

The type species of the genus *Isosphaera, Isosphaera pallida*, was initially isolated from Kah-Nee-Ta Hot Springs (Oregon, USA), but additional strains of this species have also been isolated from a number of other North American hot springs. *I. pallida* is a moderate thermophile growing at temperatures up to 55 °C (optimum 42 °C) and forms filaments in association with cyanobacterial mats (Giovannoni et al. 1987). *I. pallida* appears to have a specific relationship with the photosynthetic bacterium *Heliothrix oregonensis*, with which it forms a stable co-culture. While *I. pallida* has been isolated in axenic culture, *H. oregonensis* appears to be dependent on *I. pallida* for growth (Pierson et al. 1985).

I. pallida is pink pigmented (due to the presence of carotenoids) and is the only plantomycete species that is motile by gliding. It is also phototactic and forms 'comets' on semi-solid media, but curiously this organism is chemoheterotrophic rather than photosynthetic in its metabolism. Indeed, *I. pallida* is the only known

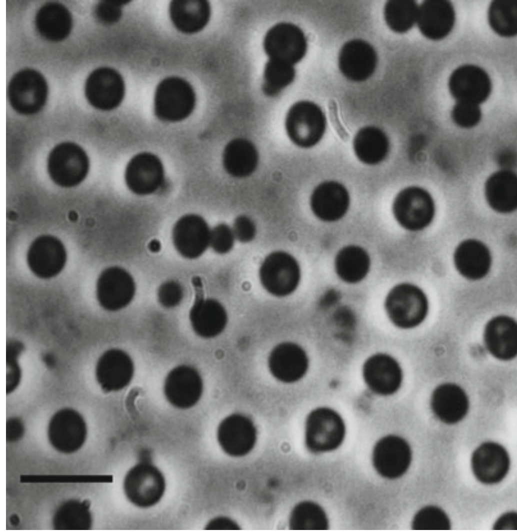

Fig 1.9 Phase contrast micrograph of a planctomycete strain isolated from Texas soil. This organism is related to the *Singulisphaera* genus. Cells are spherical, lack stalks and form short chains (see image) or amorphous clusters (Bar = 5 μm) (micrograph by C. Jenkins)

chemoheterotroph to exhibit phototaxis, a feature that may be important for aligning itself in the water column of hot springs, which tend to be stratified in terms of both temperature and nutrients. Gas vesicles, which are present in *I. pallida* but eventually become lost through laboratory passage, may serve a similar purpose (Fuerst 1995).

I. pallida is the only taxonomically characterised species within the genus; however, a number of related strains have been cultivated from freshwater and compost water, and a large number have been isolated from various North American soils (Wang et al. 2002, Schlesner 1994, Yee et al. 2008). Organisms originally described as 'Nostocoida limicola III' based on morphotype were isolated from activated sludge and were found to belong to the Isosphaera clade (Liu et al. 2001). Additional strains recently isolated from freshwater aquarium sediment (Bondoso et al. 2011), an acidic wetland (Kulichevskaya et al. 2008) and an acidic peat bog (Kulichevskaya et al. 2012) have been assigned to new genera and species within the greater Isosphaera clade (see also Chap. 5 of this volume). The two latter species are moderately acidophilic and have been assigned to the genus *Singulisphaera* as *Singulisphaera acidiphila* and *Singulisphaera rosea* respectively, while the aquarium strain has been assigned to the genus and species *Aquisphaera giovannonii* (Kulichevskaya et al. 2008; Kulichevskaya et al. 2012; Bondoso et al. 2011). All strains belonging to the Isosphaera clade are spherical in morphology (some 'Nostocoida limicola III' strains are discoid), have uniformly distributed crateriform structures, lack stalks and do not form rosettes (Fig. 1.9). Instead, large shapeless aggregations of cells held together by a moderate to thick glycocalyx-like layer are reportedly formed by some species (Bondoso et al. 2011; Kulichevskaya et al. 2008; Kulichevskaya et al. 2012), while *I. pallida* and some members of the 'Nostocoida limicola III' group form filaments or short chains of cells, resulting

from intercalary budding along a single axis (Giovannoni et al. 1987; Liu et al. 2001). All members of the Isosphaera clade exhibit a compartmentalised cell plan (described in detail in Chap. 2), but perhaps the most interesting amongst these strains are the ‘Nostocoida limicola III’ group that display both internal membranes and extensive intracytoplasmic tubules (Liu et al. 2001).

There are several notable differences between *I. pallida* and all other cultivated representatives within the Isosphaera clade. *I. pallida* is the only thermophilic species so far described (all other strains are mesophilic) and is the only strain containing gas vesicles and exhibiting phototaxis. All strains are aerobic; however, *I. pallida* requires a CO_2-enriched atmosphere and utilises a much narrower range of carbon sources for growth (Giovannoni et al. 1987). *Isosphaera*, *Singulisphaera* and *Aquisphaera* spp. are all non-flagellate and therefore lack a swarmer phase; *I. pallida* is the only motile member of the clade thus far described but exhibits gliding rather than swimming motility. Many of the unusual features of *I. pallida* appear to be adaptations to its hot spring habitat. Indeed, this species serves as a reminder that planctomycetes occupy a myriad of niches, and that substantial cultivatable diversity is still likely to exist within this remarkable phylum. The genome sequence of *I. pallida* has recently been released and will certainly provide new insight into the physiology of this organism (Göker et al. 2011).

1.3.2 The Class Phycisphaerae

The *Phycisphaerae* is a class within the *Planctomycetes* phylum that has been very recently described and consists of only three isolated and characterised strains belonging to a single species, *Phycisphaera mikurensis* (Fukunaga et al. 2009). The *Phycisphaerae* largely comprise a group of uncultured planctomycetes (formerly referred to as group WPS-1) that were initially identified in soil polluted with polychlorinated biphenyl using culture-independent techniques (Nogales et al. 2001). Sequences from 16S rDNA clone libraries also revealed that *Phycisphaerae* are relatively prevalent in anoxic sulphur- and sulphide-rich Zodletone Spring (Oklahoma, USA) (Elshahed et al. 2007), in Black Sea suboxic zone particulates (Fuchsman et al. 2011), and in various soils and in sulphate–methane transition zones of continental margin sediments (Harrison et al. 2009).

Ph. mikurenesis was isolated from a marine alga (*Porphyra* sp.) collected from Mikura Island in the Pacific Ocean off Japan. The organism was initially cultivated under aerobic conditions but subsequent testing revealed that this organism is a facultative anaerobe exhibiting fermentation of D-xylose. Xylose fermentation has not been reported amongst other axenically cultured planctomycetes (Fukunaga et al. 2009), although *B. marina* is fermentative on glucose (Schlesner 1986), and *Pl. limnophilus* ferments glucose, saccharose, maltose and galactose (Hirsch and Müller 1985). Strangely, all genes required for heterolactic acid fermentation have been identified in the *Rhodopirellula baltica* genome despite the fact that fermentative metabolism has not yet been established to occur in this

organism (Glöckner et al. 2003). Detection of members of the *Phycisphaerae* as well as other planctomycetes in low oxygen and anoxic habitats suggests that anaerobic fermentation of carbohydrates and other forms of anaerobic metabolism may be widespread within this group (Elshahed et al. 2007).

Like many other planctomycetes, *Ph. mikurensis* contains carotenoids conferring a pink–red pigmentation upon colonies and possesses resistance to β-lactam antibiotics. Amino acid analysis of whole cell lysates suggests that *Ph. mikurensis* also lacks peptidoglycan in its cell wall. *Ph. mikurensis* cells are spherical and are motile via a single polar flagellum, but stalks and crateriform structures were not observed. Further investigation of *Ph. mikurensis* using control planctomycete strains and validated electron microscopic methods may be warranted, given that crateriform structures have been reported in all members of the class *Planctomycetia* and the *Candidatus* class 'Brocadiae' studied thus far. Intracellular membranes were noted in *Ph. mikurensis* but the nature of these structures has not yet been characterised. The mol %GC of the *Ph. mikurensis* is the highest of any cultivated planctomycete at 73 % (other species exhibit 50–69 mol %GC) and is also among the highest mol %GC of all bacteria. Probably the most notable feature distinguishing *Ph. mikurensis* from all other cultured planctomycetes is that it divides by binary fission (Fukunaga et al. 2009). Isolation of further species is required to determine whether this feature is widespread within the *Phycisphaerae*; however, this finding raises interesting questions regarding the evolution of budding division within the *Planctomycetes* and indeed other bacterial groups. It would be of interest to determine whether *Ph. mikurensis* contains the gene for the cell division protein FtsZ, which is absent in all other planctomycete representatives studied thus far. It has been suggested that FtsZ was likely present in planctomycetes and was later lost (Erickson and Osawa 2010); discovery of an FtsZ homolog in *Ph. mikurensis* may lend weight to that hypothesis.

1.4 The Class *Candidatus* 'Brocadiae': Uncultured Planctomycetes of the Deep-Branching Anammox Clade

While all cultured representatives of the planctomycetes are chemoheterotrophs, worthy of note is the divergent anammox clade, first identified in wastewater, but since detected in diverse habitats such as oceanic surface sediments, the Black Sea suboxic zone, marshes, paddies, permafrost, agricultural soils (Kuypers et al. 2003; Humbert et al. 2010; Hong et al. 2011; Strous et al. 1999; Zhu et al. 2011) and high-temperature oil reservoirs (Li et al. 2010). Interestingly the existence of a microbial group that carries out the anaerobic oxidation of ammonium was predicted initially by a chemical oceanographer, Francis A. Richards (Richards 1965) and subsequently by Engelbert Broda based upon the energetics of the reaction (Broda 1977). Dutch microbiologists working in a wastewater treatment facility found evidence that the anaerobic oxidation of ammonium was

occurring in a fluidised bed reactor, coined the term anammox, and patented the process as a means of removing nitrogen from wastewater (Mulder et al. 1995). Subsequently this group demonstrated that the process was biologically driven (van de Graaf et al. 1995; Kartal et al. 2012).

The anammox group currently comprises five *Candidatus* genera 'Brocadia', 'Kuenenia', 'Anammoxoglobus', 'Jettenia' and 'Scalindua', all of which derive from wastewater with the exception of some 'Scalindua' spp. which are widespread in the marine environment (Schmid et al. 2003; van de Vossenberg et al. 2008; Fuchsman et al. 2012). The anammox organisms branch deeply within the *Planctomycetes* suggesting that they may represent the most ancient of the three classes within the phylum (Strous et al. 2006).

Planctomycetes belonging to the anammox group are autolithotrophs notable for their ability to oxidise ammonium and reduce nitrite to produce dinitrogen gas under strictly anaerobic conditions via a unique pathway. Remarkably, in this reaction one atom of nitrogen derives from each substrate producing the highly toxic and volatile molecule hydrazine, a compound used in rocket fuels, as a metabolic intermediate (Strous et al. 1999). As a likely consequence of this, the anammox process is confined to an organelle, the 'anammoxosome' (Lindsay et al. 2001; van Niftrik et al. 2004; van Niftrik and Jetten 2012), bound by a membrane composed of concatenated cyclobutane (ladderane) lipids that form a tight barrier against diffusion (Damsté et al. 2002). Ladderane lipids as well as hydrazine oxidoreductase, the enzyme involved in the catalysis of the anammox reaction, are unique to this group of planctomycetes and can be used as molecular signatures for their culture-independent detection (Schmid et al. 2005).

More recent work has demonstrated that in addition to the anammox process, these planctomycetes mediate dissimilatory nitrate reduction to ammonium via nitrite and therefore are not limited by the availability of ammonium in the environment (Kartal et al. 2007a). Thus, the anammox planctomycetes fulfil an important role in the global nitrogen cycle; indeed recent studies reveal that the anammox process may account for up to 50 % of nitrogen loss from the world's oceans (Kuenen 2008; Kuypers et al. 2005). Some members of the anammox clade have been shown to possess a mixotrophic metabolism assimilating carbon from carbon dioxide but also oxidising (but not assimilating) carbon compounds such as propionate (*Candidatus* 'Anammoxoglobus propionicus') and acetate (*Candidatus* 'Brocadia fulgida') (Güven et al. 2005; Kartal et al. 2007b, 2008). Marine 'Scalindua' spp. closely related to *Candidatus* 'Scalindua brodae' and 'Scalindua sorokinii' are able to oxidise formate, acetate and propionate (van de Vossenberg et al. 2008).

Despite a growing body of knowledge regarding this metabolically versatile group, anammox planctomycetes have not yet been cultured axenically, although they can be enriched to ~80 % of the population under controlled conditions in strictly anaerobic sequential batch reactors (Strous et al. 1998; Dapena-Mora et al. 2004; Egli et al. 2001) and in marine sediments using media based on Red Sea salt (van de Vossenberg et al. 2008). The extremely slow growth rate of these organisms hampers their enrichment, taking several months even under favourable bioreactor

conditions. There is currently no evidence that the 'satellite' populations of bacteria associated with anammox enrichments are essential for the anammox process; however, they may supply essential nutrients or scavenge inhibitory metabolic by-products thereby precluding (using present methods) the isolation of anammox planctomycetes in axenic culture (Strous et al. 1999).

Despite the lack of pure cultures of these most interesting and exciting planctomycetes, they have nonetheless been widely studied using alternative techniques. Data derived from enriched cultures, the metagenome of 'Kuenenia stuttgartiensis' (Strous et al. 2006), predictive proteomics (Medema et al. 2010) and ultrastructural studies (Lindsay et al. 2001; van Niftrik et al. 2004; see Chap. 4 of this volume) have all contributed substantially to our understanding of the metabolic, evolutionary and cell biological properties of these fascinating organisms.

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Chapter 2
Cell Compartmentalization and Endocytosis in Planctomycetes: Structure and Function in Complex Bacteria

John A. Fuerst, Richard I. Webb, and Evgeny Sagulenko

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Abbreviations

Anammox	Anaerobic ammonium oxidation
PVC	*Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae*
TEM	Transmission electron microscopy
CM	Cytoplasmic membrane
CW	Cell wall
N	Nucleoid
MC	Membrane-coating

2.1 Introduction

When planctomycetes were first investigated, electron microscopy was not available and cell compartments were not suggested, though since organisms such as *Planctomyces bekefii* were mistaken as fungi (see Chap. 1 and (Fuerst 1995)), this might have been so. However, the main reasoning for fungal relationship was based on what we now know are noncellular stalks of these freshwater bacteria, not mycelia.

In the 1970s and 1980s planctomycetes were first examined via electron microscopy by Jean Schmidt and Mortimer Starr, often from environmental water samples or enrichments, but even when pure cultures were examined (of a *Pirellula*-like "morphotype IV" strain) chemical fixation methods applied did not clearly reveal internal cell compartments (Schmidt and Starr 1980), though various "parallel stacked structures," amorphous regions, and inclusions were observed (Schmidt and Starr 1982). This was one of the reasons for placing these organisms with the bacteria and prokaryotes, though there was no molecular data at that time (Schmidt and Starr 1982). At the same time when *Pirellula staleyi* was first described as "*Pasteuria ramosa*" (Staley 1973) and *Planctomyces maris* first described, the chemical fixation used for preparation of cells for TEM of thin sections was not sufficient to reveal compartments in cells. The first indication that there may be more complex organization of the cell in planctomycetes was the original description by Peter Franzmann and VBD Skerman (1984) of the freshwater planctomycete *Gemmata obscuriglobus* where a "packaged DNA" was noted from electron micrographs and variation in phase-darkness of different regions of the cell observed from phase contrast light microscopy. They noted "considerable intracellular membrane development that may give an unwarranted appearance of membrane involvement in the DNA package," but a membrane-bounded nucleoid was not clearly indicated from their chemically fixed cells (all grown on low-nutrient media such as lake water agar or soil extract agar or "Staley's maintenance medium"). In 1991 Fuerst and Webb (Fuerst and Webb 1991) applied cryosubstitution involving freeze-fixation to *Gemmata obscuriglobus* and found that much less distortion of cell morphology and shape resulted than in the case of chemical fixation of the same strain.

It was later found that if the chemical fixative was too high in osmolarity (e.g., 3 mM cacodylate buffer), cells would shrink and distort asymmetrically giving rise to a crescent morphology not observed in cryosubstituted cells (Lindsay et al. 1995).

This also is relevant to the question of the cytoplasmic membrane, since absence of plasmolysis under high osmolar conditions suggests shrinkage of a cell wall closely apposed to a cytoplasmic membrane in this organism. Of interest are early reports of autolysis in at least some freshwater planctomycetes under conditions poor in divalent cations (Schmidt 1978); this does not seem to apply to all such freshwater planctomycetes however, e.g., *Gemmata obscuriglobus*.

In 1997, the first ultrastructure study of *Pirellula* group species *Pirellula staleyi* and *Blastopirellula marina* was published, and a simple single intracytoplasmic membrane discovered separating a ribosome-free outer region from a major compartment containing the ribosomes and nucleoid (Lindsay et al. 1997). The major ribosome-containing compartment was termed a pirellulosome.

In 2001, a study of several available pure cultured species representing genera *Gemmata*, *Pirellula*, *Blastopirellula*, *Planctomyces*, and *Isosphaera* as well as the non-pure cultured anammox "*Candidatus* Kuenenia stuttgartiensis"—a shared cell plan (described below)—was discovered to be common to all planctomycetes examined (Lindsay et al. 2001). Variation occurred as further membrane-bounded structures within the pirellulosome major compartment, as in *Gemmata* with its membrane-bounded nuclear body and anammox planctomycete species with their anammoxosomes. Recent advances include confirmation of extensive internal membranes and the condensed nature of the nucleoid in *Gemmata obscuriglobus* (Lieber et al. 2009b) and its correlation with radiation resistance in this species.

Tomography of cryosubstituted cells of anammox species demonstrated an intriguing cell division ring (van Niftrik et al. 2009) and also the occurrence of ATP synthase in all the membranes of "*Candidatus* Kuenenia stuttgartiensis" including those of the membrane underlying the cell wall (consistent with its identification as cytoplasmic membrane) and the anammoxosome (consistent with biochemical models for anammox physiology postulating a pmf generation across the anammoxosome membrane) (van Niftrik et al. 2010) (see Chap. 4 in this volume).

Functionally, one of the most dramatic findings has been that of protein uptake by *Gemmata obscuriglobus* (Lonhienne et al. 2010), a process similar in many features to receptor-mediated endocytosis of eukaryotes, involving internal vesicle formation and association of ligand protein with such vesicles. These functional results are consistent with the bioinformatic prediction in planctomycetes (and other members of the PVC superphylum) of proteins in a family of membrane-coating (MC) proteins of eukaryotes, some of which are necessary for receptor-mediated (clathrin-mediated) endocytosis (Santarella-Mellwig et al. 2010). Significantly in relation to the unique occurrence of the protein endocytosis only in planctomycetes within the domain Bacteria, bioinformatics analysis initially revealed MC protein homologs only within the PVC superphylum members among the bacteria (recent analysis indicates that there may also be MC proteins in at least one other phylum, Bacteroidetes—see Chap. 3 and (McInerney et al. 2011)).

2.2 Shared Compartments and Components of the Planctomycete Cell

2.2.1 Introduction

All planctomycetes share a common cell plan involving internal compartments separated from other regions of the cell via membranes (Fuerst 2005; Fuerst and Sagulenko 2011). Within this plan, there are then more complex variations involving further compartments and membranes (Fig. 2.1). The simplest, shared plan is one

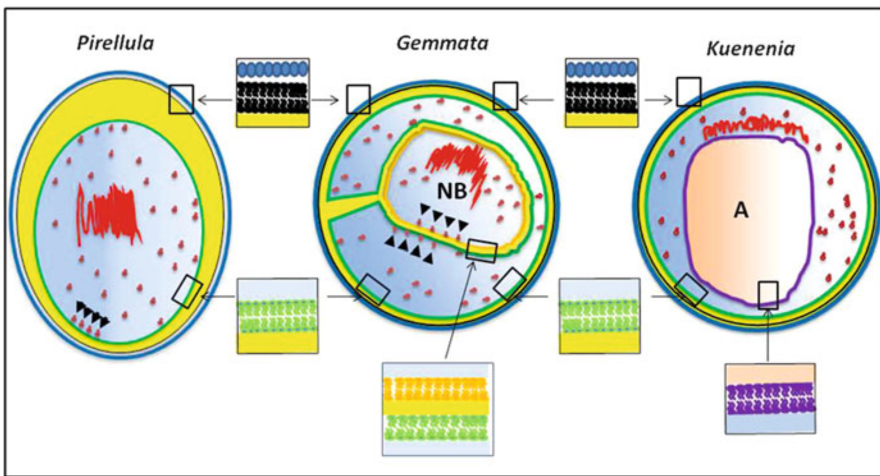

Fig. 2.1 Schematic diagram of planctomycete cell plans for members of genera *Pirellula*, *Gemmata*, and *Kuenenia*, displaying the shared features of the planctomycete cell plans as well as the modifications beyond this shared cell plan in *Gemmata* and *Kuenenia*. The cell compartments characteristic for these plans are defined by internal membranes. All internal cytoplasm is surrounded by a cytoplasmic membrane—a single bilayer membrane (*black*)—which is closely apposed to the cell wall (*dark blue*). A single bilayer intracytoplasmic membrane (ICM) (*green*) is present in all the example cells shown, separates the ribosome-free paryphoplasm (*yellow*) from the ribosome-containing pirellulosome (*light shaded blue*), and thus surrounds the pirellulosome. Pirellulosome can also be termed riboplasm due to its content of ribosomes. These ribosomes in some places can form linear arrays along internal membranes, in *Pirellula* along the inner side of the ICM, and in *Gemmata* along both membranes and thus both sides of the nuclear envelope. In *Gemmata* the ICM is continuous with the outer membrane of a double-membrane envelope composed of this outer membrane and a closely apposed inner membrane (*orange*). The “pericisternal” space between the two membranes of the nuclear envelope is continuous with the paryphoplasm. This envelope surrounds the nucleoid DNA (*red*) and some riboplasm, forming the nuclear body (NB). In Kuenenia and other anammox planctomycetes, a single bilayer membrane (*purple*) surrounds an internal organelle situated within the pirellulosome, the anammoxosome (*light shaded brown*). In summary, all three plans have two compartments in common, the paryphoplasm and pirellulosome, and in *Gemmata* and *Kuenenia* there is a third compartment situated within the pirellulosome. *Insets* show enlarged views of particular (*boxed*) regions of membrane significant for understanding the nature of the membranes in relation to associated compartments

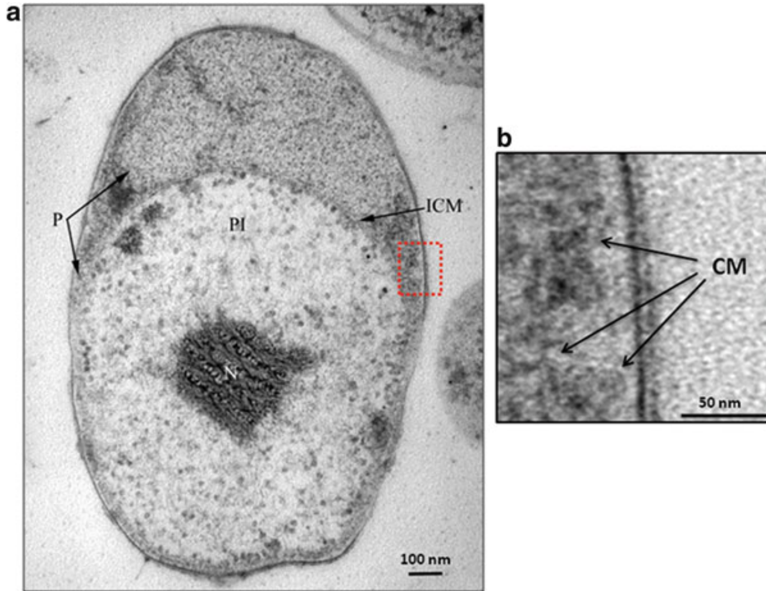

Fig. 2.2 (**a**) Transmission electron micrograph of thin-sectioned cell of *Blastopirellula marina*, processed by cryosubstitution. Two cell compartments are seen within the cell, a ribosome-free paryphoplasm (P) forming a polar cap region at one pole of the cell, and a major pirellulosome region (PI) containing a condensed fibrillar nucleoid (N) and ribosomes. These compartments are separated by an intracytoplasmic membrane (ICM). Bar marker, 100 nm. Modified from, (**b**) *(enlargement of red box in (a))* shows enlarged view of the paryphoplasm area. *Arrows* indicate invagination of cytoplasmic membrane (CM). Bar marker, 50 nm

illustrated by organisms such as *Pirellula staleyi*, *Blastopirellula marina* (Lindsay et al. 1997), *Planctomyces limnophilus* (Jogler et al. 2011), and *Schlesneria paludicola* (Kulichevskaya et al. 2007). In this plan (Fig. 2.2) the cell is divided into two compartments, a ribosome- and nucleoid-containing major compartment termed the "pirellulosome" and, surrounding this pirellulosome, a ribosome-free region, the paryphoplasm (Lindsay et al. 1997). This plan has also been found to apply to *Planctomyces limnophilus* (Jogler et al. 2011). There are other planctomycetes such as *Isosphaera* which seem to preserve the basic topology of this plan even though they appear superficially distinct (thus, the paryphoplasm may invade even the center of the cell yet still connect with its outer origin so that the cell is still divided into just two regions) (Lindsay et al. 2001). More complexity is observed however in the case of *Gemmata* species and the anammox planctomycetes, where there appears to be a third membrane-bounded cell compartment, the effectively double-membrane-bounded nuclear body in the case of *Gemmata* (Figs. 2.3 and 2.4) and the single-membrane-bounded anammoxosome in the case of anammox planctomycetes such as *Kuenenia* (Lindsay et al. 2001).

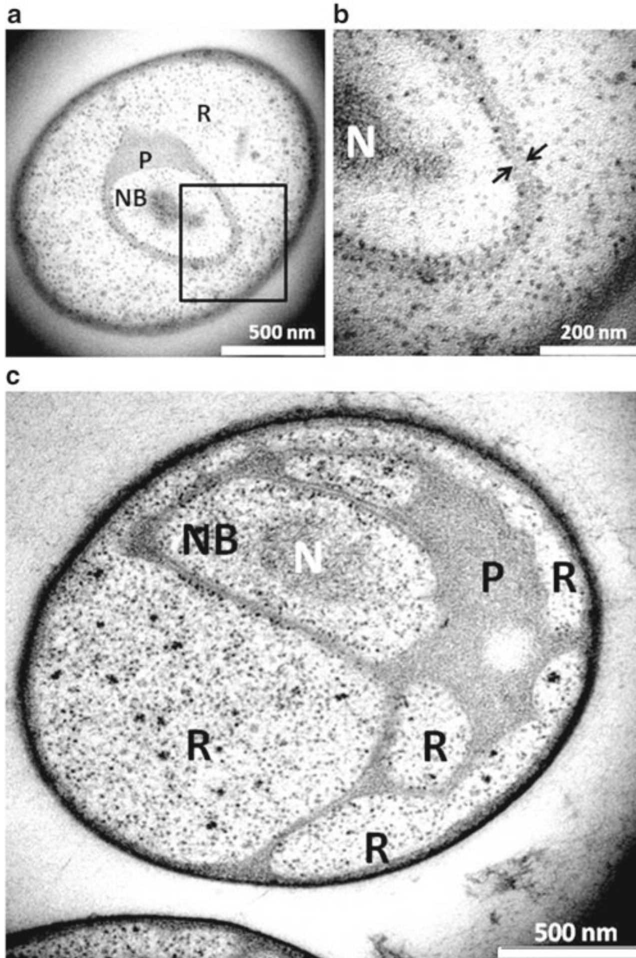

Fig. 2.3 Transmission electron micrographs of thin-sectioned cells of *Gemmata obscuriglobus* prepared by cryosubstitution (**a**) section of whole cell showing a single nuclear body (NB) surrounded by a nuclear envelope and connected to a portion of paryphoplasm (P) continuous with the pericisternal space between the membranes of the nuclear envelope. Riboplasm (R) of the pirellulosome surrounds the nuclear body, which also contains ribosomes. Bar marker, 500 nm. *Inset box* is enlarged in (**b**) where the nuclear envelope can be seen to consist of two membranes (*arrows*), and the fibrillar structure of the condensed nucleoid (N) is displayed. Ribosomes can be seen to be bound to both inner and outer membranes of the nuclear body. Bar marker, 200 nm. (**c**) An example of a more complex internal cell structure in *G. obscuriglobus*. As in (**a**) and (**b**) the nuclear body possesses an envelope of two membranes, but the space between the nuclear membranes is continuous with a complex paryphoplasm (P) connecting both to paryphoplasm at the cell rim and with other regions, appearing to divide the pirellulosome riboplasm (R) into distinct vesicle-like regions, each surrounded by ICM. The appearance of a complex endomembrane system is given in such cells. Ribosomes are bound in linear array only to the nuclear envelope membranes surrounding the nucleoid. Bar marker, 500 nm

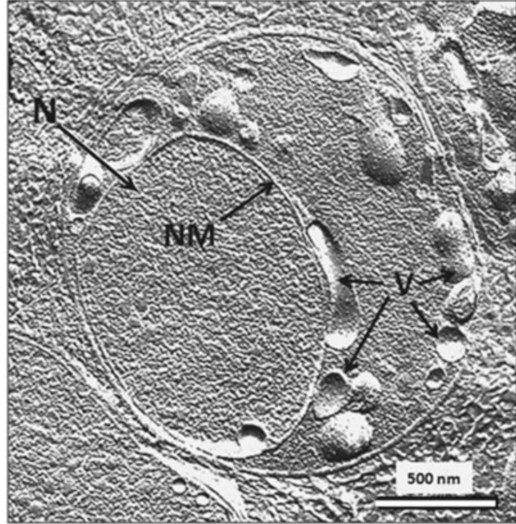

Fig. 2.4 Transmission electron micrograph of freeze-fracture replica of a cross-fractured *Gemmata obscuriglobus* cell showing the separate compartment nature of the nuclear body containing the nucleoid (N) and completely surrounded by a nuclear envelope (NM). The region of the cell cytoplasm surrounding the nuclear body contains numerous vesicles (V), while the nuclear body is mostly devoid of vesicles. Bar marker, 500 nm

Following some perspectives used in synthetic biology, the structure and nature of the unique components and compartments of the planctomycete cell as now understood will be approached first as a "parts list." These can then be integrated after appreciating the nature of each part, for discussion of their potential interrelations and inter-part communication mechanisms.

2.2.2 The Parts of the Planctomycete Cell: Cell Wall and Cytoplasmic Membrane

In a synthetic biology project, how would we go about constructing a planctomycete? One of the first components we would need would be a cell wall composed largely of protein, in contrast to all other known bacterial walls. The unusual indeed unique nature of planctomycetes was really indicated firstly by chemical analysis of cell wall composition (König et al. 1984; Liesack et al. 1986), which revealed no evidence of either muramic acid or meso-diaminopimelic acid, components characteristic of peptidoglycan, the polymer essential for cell wall structural strength and integrity in all the known bacteria with cell walls with the exception of chlamydia (which in light of suggestions of a planctomycete relationship to chlamydia may be an evolutionarily expected exception). Consistent with the absence of peptidoglycan analytically, early reports of planctomycete resistance to penicillin G and cycloserine, antibiotics targeting peptidoglycan synthesis (Schmidt 1978) were followed by confirmation of the resistance of many planctomycetes to peptidoglycan synthesis-targeting antibiotics such as beta-lactams and cycloserine, antibiotics inhibiting peptidoglycan synthesis at different stages in that synthesis (König et al. 1984). Such

resistance has been confirmed for wider selections of planctomycete species (Cayrou et al. 2010; Jogler et al. 2012). Although some *dcw* operon genes (e.g., *murD*) needed for peptidoglycan synthesis as well as cell division in other bacteria are present in planctomycetes (Pilhofer et al. 2008), they are distributed among different operons in different species or are not organized at all (Jogler et al. 2012). It has been suggested that an ancestral planctomycete or even a PVC superphylum ancestor may have contained all *dcw* operon genes including all for peptidoglycan synthesis, that an FtsZ-dependent divisome may have been present, and that these have been lost differentially in different members (Pilhofer et al. 2008). Even accepting that, planctomycetes seem to have replaced this divisome with one not depending on FtsZ or connected with peptidoglycan synthesis, so the function of the *dcw* operon genes in planctomycetes is not clear, as is the case to a lesser degree with chlamydiae (which have most of the peptidoglycan synthesis genes but no FtsZ).

The dominance of protein in the wall is indicated by the chemical analysis of several planctomycetes including *Gemmata obscuriglobus* (Stackebrandt et al. 1986), *Isosphaera pallida* (Giovannoni et al. 1987a), and a number of organisms in the *Pirellula* and *Planctomyces* groups such as *Planctomyces maris* and *Pirellula staleyi* (studied as "*Pasteuria ramosa*") and strains related to *Pirellula staleyi* or the *Pirellula* group (König et al. 1984; Liesack et al. 1986). In *I. pallida*, 99 % of the dry weight of the rigid detergent-resistant wall layer was recovered as amino acids. In one study, percentages of protein versus total cell dry weight range from 51 % for *Gemmata obscuriglobus* to 80 % for *Planctomyces maris* and 82 % for *Pirellula staleyi* (Stackebrandt et al. 1986) while in another in 7/8 strains studied the protein of the wall comprised 75–82 % of the 10 % SDS-resistant envelope dry weight, with only *Pl. maris* having a significantly lower amount at 62.6 % (Liesack et al. 1986). The amino acid composition is also of interest since cystine was a significant component in walls of *Pirellula* and *Planctomyces* strains indicating that significant cross-linking via disulfide bonds might occur in intact walls (Liesack et al. 1986). However this may not be universal as *G. obscuriglobus* displayed low cystine content relative to that in *P. staleyi* and *Pl. maris* walls (Stackebrandt et al. 1986).

Proteinaceous walls of planctomycetes have the remarkable property of resisting prolonged (e.g., 30 min) boiling in 10 % SDS detergent (Liesack et al. 1986; Stackebrandt et al. 1986), an approach which would normally be used to isolate peptidoglycan from Gram-negative bacteria, e.g., from *Neisseria* (Dougherty 1985), and eliminate any typical Gram-negative outer membrane. This has been explained for some species on the basis of a high content of cystine in the wall protein suggesting disulfide bridge stabilization (Liesack et al. 1986). The resulting rigid sacculi seem to retain the cell shape, as do isolated peptidoglycan wall sacculi of other bacteria, so they represent structurally functional walls (Giovannoni et al. 1987a). A characteristic property of planctomycete cell surfaces is the presence of pit-like structures called crateriform structures (Fig. 2.5), and these seem also to be preserved in the wall when these are isolated after SDS treatment (Giovannoni et al. 1987a; Liesack et al. 1986; Stackebrandt et al. 1986). The nature of these structures is not clear but they do not seem to be simple holes or pits as judged by negative stained preparations of whole cells (but regularly spaced perforations can be seen in isolated

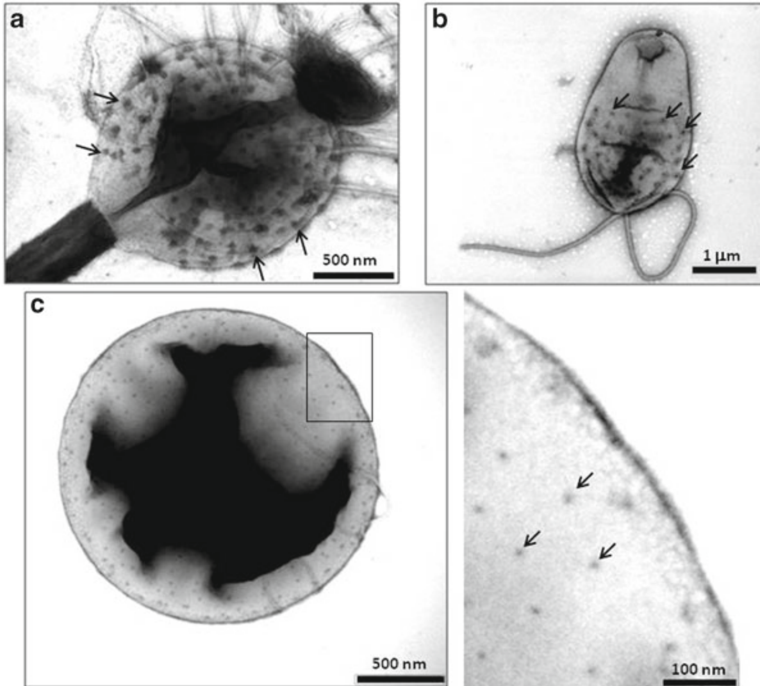

Fig. 2.5 Transmission electron micrograph of negatively stained planctomycete cells showing crateriform structures (**a**) *Planctomyces bekefii* morphotype cell from a freshwater lake showing numerous uniformly distributed large crateriform structures (*arrows*). A stalk consisting of many closely packed fibrils can be seen extending from one side of the cell—this stalk connected the cell to the center of a rosette of several radiating stalks with cells at their tips. Bar marker, 500 nm. (**b**) Transmission electron micrograph of negatively stained cell *Blastopirellula* group planctomycete isolated from giant tiger prawn *Penaeus monodon* showing large crateriform structures (*arrows*) only at the broad lower pole (much smaller crateriform structures are clustered at the upper pole). Bar marker, 1 µm. (**c**) Transmission electron micrograph of a negatively stained *Gemmata obscuriglobus* cell showing uniformly distributed crateriform structures. Bar marker, 500 nm. *Inset* shows an enlarged view of the *boxed* region on the whole cell illustrating more detailed structure and uniform spacing of these crateriform structures (*arrows*) on the cell surface. Bar marker, 100 nm

walls—e.g., see Giovannoni et al. (1987a)). Their distribution varies with the taxonomic position of the planctomycete—members of the *Pirellula* group have polar distribution while *Gemmata* species have uniform distribution of these structures over the cell surface. Different classes of crateriform structures based on size can be distinguished—in a freshwater *Pirellula staleyi* strain 12 and 5–7 nm classes can be discerned in more mature buds (Tekniepe et al. 1981), with the former's large form at the pole of the bud attached to the mother cell, while the small form clustered at the opposite pole distal to the mother cell. In the mature mother cell, the large forms can enlarge to a maximum diameter of 30 nm, while the smaller structures become less visible. *Isosphaera pallida* also possesses large (27 nm) diameter ringlike

crateriform structures (Giovannoni et al. 1987b). In *Planctomyces limnophilus* swarmer (swimmer) cells, two size classes of crateriform structures also occur—a large form is uniformly distributed while the small form is again limited to a flagellum-containing pole (Jogler et al. 2011). Such observations strongly suggest that polar localization of proteins is likely to be correlated with the cell cycle in planctomycetes, as extensively documented in other bacteria with sessile cell-dispersal cell life cycles and cell differentiation (Ebersbach and Jacobs-Wagner 2007; Iniesta and Shapiro 2008).

Via electron microscopy, cell walls of planctomycetes appear similar overall to those of a *Pirellula staleyi* strain where they appear to consist in chemically fixed cells of inner and outer electron-dense layers separated by a narrow electron-transparent layer, with the inner layer observed as wider and more electron dense than the outer layer (Schmidt and Starr 1982) but without any indication of a clear dense peptidoglycan layer. Similar studies on *Planctomyces* and Pirellula group strains also indicated no peptidoglycan layer but identified the other structures as an "outer membrane" (König et al. 1984). In *Isosphaera pallida* the width of the wall "trilaminar structure" resembling a unit membrane was at 11 nm noted as thicker than the width of classical unit membranes of 7–8 nm (Giovannoni et al. 1987a). Planctomycete walls of several genera when isolated after harsh detergent treatment retain the cell shape (Giovannoni et al. 1987a; Liesack et al. 1986), suggesting that they are not typical lipid unit membranes, but also that they are covalently bounded rigid macromolecules. No peptidoglycan-consistent layer similar to one seen in sectioned Gram-negative bacterial cells is seen. There are some suggestions or impressions that planctomycete walls are Gram-negative in structure (Cavalier-Smith 2010), but the nature of the rigid protein wall and absence of peptidoglycan typical of Gram-negative bacteria walls are not consistent with this perspective. Some planctomycetes may contain hydroxy fatty acids consistent with lipid A of Gram-negative bacterial LPS, and genes for lipid A synthesis and modification via dephosphorylation have also been annotated (Jogler et al. 2012; Sutcliffe 2010), as well as markers for outer membrane biogenesis and homologs to outer membrane proteins of Gram-negative bacteria. It is not clear yet where these molecules are localized within the cell envelope and whether they are localized in the wall or the membranes internal to the wall (see below). It is possible that planctomycetes could be described as "diderm" but without lipid A or LPS in the wall, as occurs in some other bacterial phyla such as *Spirochaetes*, *Deinococcus-Thermus*, and *Thermotogae* (Sutcliffe 2010), but data are insufficient to decide this at present. However, all of those phyla lack lipid A synthesis genes, unlike planctomycetes. The walls of some planctomycetes seem to have prostheca-like projections positioned in a distinct polar orientation, as in the "humps" of *Pirellula staleyi* (Butler et al. 2002) and the "spurs" of *Schlesneria paludicola* (Kulichevskaya et al. 2007), *Planctomyces brasiliensis*, and *Planctomyces* sp. AGA/M18 (Fuerst et al. 1997), and other prosthecate planctomycetes have been observed among environmental isolates (Schlesner 1994). This suggests some structural differences within either the wall or the internal cytoskeletal proteins maintaining a polar projection of wall.

Homologs to some genes normally needed for peptidoglycan synthesis seem to occur in planctomycetes (e.g., *Rhodopirellula* (Glockner et al. 2003)) as they do in chlamydiae where peptidoglycan also seems undemonstrable chemically. As with chlamydiae they may perform some function other than peptidoglycan synthesis. The latter exception may not be an accidental one, as chlamydiae were later suggested to be related phylogenetically to planctomycetes as members of the PVC superphylum. However, it may be that peptidoglycan is synthesized during particular phases of chlamydial growth, a hypothesis not yet confirmed, and chlamydiae have many homologs of peptidoglycan synthesis genes (McCoy and Maurelli 2006). It is known that among other PVC superphylum bacteria, at least *Verrucomicrobium spinosum* possesses enzymes typical of synthesis of the Gram-negative bacterial type of peptidoglycan containing diaminopimelate in its tetrapeptide side chain (Nachar et al. 2012).

Recent proteomic studies suggested that there may be planctomycete-specific wall proteins with characteristic motifs such as YTV (Hieu et al. 2008), but further work has suggested that such proteins may be part of a surfaceome not necessarily solely part of the cell wall itself (Voigt et al. 2012). It is not clear however that the YTV proteins detected in the surfaceome via surface biotinylation and recovery of biotinylated proteins are not after all connected to the structural wall, as might occur if similar methods were applied to teichoic acids of Gram-positive bacteria.

The question of the existence and nature of the cytoplasmic membrane (or plasma membrane when discussing cell structure comparison with eukaryotes) in planctomycete cells is a significant one, since it is central to recognizing the unique nature of the paryphoplasm compartment. Evidence for the existence of a cytoplasmic membrane in planctomycetes comes from several sources, starting with electron microscopy of sectioned cryosubstituted cells of the *Pirellula* group (*Pirellula staleyi* and *Blastopirellula marina*), in which the cytoplasmic membrane can be seen clearly in some regions of partial plasmolysis where the membrane has retracted in very limited regions from the cell wall (Lindsay et al. 1997) (Fig. 2.2). Freeze fracture replicas of *Gemmata obscuriglobus* confirmed its existence just below the cell wall (Lindsay et al. 2001), and cytochemistry of ATP synthase distribution in the anammox planctomycete "*Candidatus* Kuenenia stuttgartiensis" demonstrated that such an enzyme consistent with generation of pmf to be expected across a cytoplasmic membrane was present in the wall-associated cytoplasmic membrane of that planctomycete (van Niftrik et al. 2010). Shrinkage of wall in hyperosmolar conditions is consistent with a membrane that may be attached tightly to wall preventing significant plasmolysis in native living cells (Lindsay et al. 1995), but the invagination of cytoplasmic membrane during the endocytotic protein uptake phenomenon (see below) suggests that the membrane must be free to invaginate at some point away from wall. A significant difference between planctomycete CM and that of other bacteria is that ribosomes are never found attached to planctomycete CMs so that co-translational protein secretion into periplasm and external milieu clearly does not occur in planctomycetes with the same topology as occurs in other bacteria. The absence of ribosomes in contact with CM is analogous to the exclusively internal location of membrane-bounded ribosomes in the eukaryote cell, where they are

attached to rough endoplasmic reticulum. This has been considered by some to be a fundamental distinction between prokaryotes and eukaryotes.

Recent BLAST-based analysis of planctomycete genomes has identified putatively annotated marker genes for lipopolysaccharide insertion, outer membrane biogenesis, and outer membrane proteins (Speth et al. 2012) and on this alone it has been suggested that what was previously considered the proteinaceous wall-associated cytoplasmic membrane of planctomycetes is actually an asymmetric bilayer analogous to the outer membrane of classical Gram-negative bacteria. At least one other group seems to follow a similar scheme seeing no difference between Gram-negative bacterial walls and those of planctomycetes other than invagination of the intracytoplasmic membrane, which is considered by them as the genuine cytoplasmic membrane (Reynaud and Devos 2011). There is no experimental or electron microscopic data supporting this interpretation of planctomycete wall and membranes, and localization of ATP synthase in the anammox planctomycete "*Candidatus* Kuenenia stuttgartiensis" in the wall-associated outermost membrane is in conflict with this view. There seems no reason to center on the cytoplasmic membrane as the location of proteins homologous with Gram-negative outer membrane proteins. These OMPs could be embedded in the proteinaceous wall itself, in a similar manner to the way in which porins are embedded in the peptidoglycan walls of the Gram-positive *Mycobacterium* (Faller et al. 2004; Trias et al. 1992). Until experimental evidence is forthcoming confirming the location of outer membrane proteins or lipids within the outermost wall-associated membrane, we believe that this planctomycete cell membrane should continue to be called "cytoplasmic membrane."

2.2.3 Paryphoplasm

The paryphoplasm (from Greek *paryphe*, meaning border woven along a robe) is a region between the cytoplasmic membrane and the intracytoplasmic membrane which appears in sectioned cryosubstituted cells to be completely free of ribosomes (Fig. 2.2) though not necessarily of RNA. It varies in electron density of its contents depending on the species; e.g., in *G. obscuriglobus* it is relatively electron dense, while in anammox "*Candidatus* Kuenenia stuttgartiensis" it can appear relatively electron transparent in sections of cryosubstituted cells (van Niftrik et al. 2009). It can appear to be mainly concentrated around the rim of the cell or it can occupy extensive areas within the cell while still connected to the paryphoplasm at the rim and still bounded at its inner edge by intracytoplasmic membrane. In members of the Pirellula group such as *Pirellula staleyi* and *Blastopirellula marina* it tends to form polar regions ("polar caps") such that one pole has more paryphoplasm than the other and such polar regions can be quite extensive relative to the area of cells seen in thin section, implying that they occupy a large volume of the cell of possibly up to 45 % of the whole cell (Lindsay et al. 1997). In *Rhodopirellula baltica*, the paryphoplasm contains vesicles similar in structure to small versions of the pirellulosome but without nucleoids (Schlesner et al. 2004). In Pirellula group members

including *Rhodopirellula baltica* rosettes formed from several cells suggest that the paryphoplasm may be associated with the cell pole involved with attachment to other cells or perhaps multiple budding events (Lindsay et al. 1997; Schlesner et al. 2004), but the budding pole of cells is not invariably the one with a polar cap (Lindsay et al. 1997). In *Planctomyces limnophilus*, the cell plan is similar to that in *Blastopirellula*, but the paryphoplasm shape changes with different cells, and changes along the z-axis of individual cells in serial sections, and it is thus not rotationally symmetrical (Jogler et al. 2011). Nevertheless, the identification of planctomycete paryphoplasm with a periplasm homologous with that of Gram-negative bacteria is assumed in some literature—see Reynaud and Devos (2011). However, its position underneath a confirmed cytoplasmic membrane is one argument against its being some form of periplasm and its small but established content of some form of RNA is a second (Lindsay et al. 1997). A third argument is that during protein uptake in *Gemmata obscuriglobus*, the cytoplasmic membrane invaginates into the paryphoplasmic space forming vesicles in that space containing incorporated protein (see below) (Lonhienne et al. 2010). The contents of such vesicles are topologically equivalent to the external medium or the periplasm in contact with it, but the paryphoplasm itself forms a space distinct from the vesicle contents and periplasm, one in which the vesicles lie and which is separated from their contents by a vesicle membrane. In this sense it is similar to the cytoplasm in which endosomes form in eukaryotes, not to a periplasm outside the cell contents topologically. A fourth argument is that in planctomycetes such as *Pirellula* and *Blastopirellula*, the paryphoplasm is distributed asymmetrically such that a polar accumulation occurs rather than an even distribution only around the cell rim—suggesting an asymmetry incompatible with a periplasm closely following the line of the cell wall where turgor pressure applies under hypotonic conditions pressing cytoplasmic membrane to the wall. There may well be a periplasm in planctomycetes, but it is more likely to be placed between the proteinaceous wall and the closely apposed cytoplasmic membrane, as proposed for some Gram-positive bacteria (Matias and Beveridge 2006), or trapped between the columns of a surface protein wall as proposed for some Archaea (Engelhardt 2007; Nickell et al. 2003). The paryphoplasm was first defined in planctomycetes of the *Pirellula* group, but was found to be a shared feature of all planctomycete species in pure culture examined (Lindsay et al. 2001) as well as of the anammox planctomycetes, a group yet to be isolated in pure culture (van Niftrik et al. 2008a).

Cytochemical methods applied to anammox planctomycetes are also consistent with the concept of the paryphoplasm in planctomycetes as a genuine cytoplasmic compartment. The paryphoplasm compartment of anammox planctomycete Kuenenia does not display peroxidase staining, and this is consistent with this compartment being a cytoplasmic rather than a periplasmic compartment (van Niftrik et al. 2008a). This indirectly supports the existence of a true cytoplasmic membrane bounding this paryphoplasm cytoplasm at its outer boundary.

Consistent with the concept of a cytoplasmic paryphoplasm compartment inside the cytoplasmic membrane, in *G. obscuriglobus*, the endocytotic uptake of protein (see below) apparently occurs via infolding of the cytoplasmic membrane to form

vesicles within the paryphoplasm—protein taken up from external milieu was bound to membranes forming such vesicles.

In the anammox planctomycete *Candidatus* Kuenenia, the occurrence of a cell division ring and its component non-FtsZ kustd1438 protein in the paryphoplasm (van Niftrik et al. 2009) is also consistent with the cytoplasmic nature of paryphoplasm, as the analogous Z ring in bacteria such as *E. coli* and *B. subtilis* forms in the cytoplasm during cell division. However, homologs of kustd1438 have not been detected in non-anammox planctomycetes (Jogler et al. 2012). In budding planctomycetes like *G. obscuriglobus*, the paryphoplasm may play a role in the budding division process at the reproductive pole (see Chap. 1), since the paryphoplasm is the only compartment clearly involved when budding first commences (Lee et al. 2009). Cell wall components such as the major YTV proteins (Hieu et al. 2008) must also accumulate in the paryphoplasm before transport through the cytoplasmic membrane and assembly into new wall, and thus via techniques such as immunogold should in future experiments be identifiable in the paryphoplasm as well as wall. It appears that several cell biology activities may be performed in the paryphoplasm, e.g., endocytotic protein trafficking and budding initiation, making it an active compartment of the planctomycete cytoplasm despite its apparent absence of ribosomes. Ruthenium red staining of sections in *Pirellula* and *Blastopirellula* indicates presence in the paryphoplasm of either polysaccharide or glycoprotein (Lindsay et al. 1997), and RNase-gold cytochemistry in both the Pirellula group species and in *G. obscuriglobus* indicates the presence of ribonuclease-degradable RNA significantly above background levels despite the absence of ribosomes but the identity of such RNA remains a mystery (Lindsay et al. 1997, 2001). Paryphoplasm appears to be the site of accumulation of protein incorporated from the external medium via an endocytosis-like mechanism, which appears to accumulate this protein within vesicles in the paryphoplasm. Paryphoplasm composition remains to be investigated in detail, especially regarding its proteome, but is important for understanding the functions of internal membranes and compartmentalization in planctomycetes, including the cell biological process of endocytosis previously unknown outside the eukaryotes.

2.2.4 Intracytoplasmic Membrane

The intracytoplasmic membrane (ICM) forms the inner border of the paryphoplasm. It is a single trilaminar membrane often much more clearly visible than the cytoplasmic membrane in electron micrographs of sectioned cryosubstituted cells (see Fig. 2.2). In *Planctomyces limnophilus* it is a bilayer ca. 6 nm wide, the same width as the cytoplasmic membrane in these cells (Jogler et al. 2011). However, the ICM never appears in direct contact with the cytoplasmic membrane or to display continuity with the cytoplasmic membrane so that it does not appear to directly derive from cytoplasmic membrane in the way which has been reported for other bacterial intracytoplasmic membranes (e.g., cyanobacterial thylakoids, magnetosome membranes). However, since the cytoplasmic membrane appears to infold during

endocytotic protein uptake and form vesicles within the paryphoplasm (Lonhienne et al. 2010), it may be that there is via such vesicles some membrane trafficking of components of the cytoplasmic membrane with those of the ICM, though there is no compositional evidence of this yet.

2.2.5 *Pirellulosome*

The pirellulosome is a major compartment of all planctomycetes, containing all the ribosomes and nucleoid DNA of the cell, and bounded by the ICM. In the case of *G. obscuriglobus*, the ICM seen in section actually invaginates into the pirellulosome to form the outer membrane of the envelope of the nuclear body region containing the nucleoid, but in planctomycetes such as those of the Pirellula group the ICM always forms a continuous boundary to the ribosome- and nucleoid-containing pirellulosome compartment, and the compartment was named for its discovery in the Pirellula group (Lindsay et al. 1997). It was identified in the species *Pirellula staleyi* and what is now known as *Blastopirellula marina* as a region containing a condensed fibrillar nucleoid and electron-dense ribosome-like particles ca. 9–18 nm in diameter, and bounded by a single ICM membrane up to 5 nm wide in *B. marina* but as thin as 3.2 nm wide in *Pi. staleyi.* (Lindsay et al. 1997). As expected from the ribosome-like particles, the pirellulosome in these species contains RNA as assayed via RNase-gold labeling of sectioned cells. A second region of the cell surrounding the pirellulosome was termed initially a polar cap region but was later known as the paryphoplasm after it was found to be a type of compartment shared by all planctomycetes examined.

In *Rhodopirellula baltica*, however, also a member of the Pirellula group, what seem to be several smaller versions of membrane-bounded pirellulosomes are enclosed within the paryphoplasm, probably containing ribosomes but apparently without nucleoids (Schlesner et al. 2004). This needs reexamination with tomography to exclude a complex 3D shape of a single pirellulosome resulting in this 2D appearance—the underlying cell plan may be identical to that of other Pirellula group members. *Planctomyces limnophilus* appears to have the same type of pirellulosome and pirellulosome contents as other members of the Pirellula group, but with complex shapes and organization giving rise to possible similarities to the smaller versions of pirellulosome seen in *Rhodopirellula baltica* (Jogler et al. 2011). In *Isosphaera pallida* the ICM can invaginate to form a large lobe of the paryophoplasm so that the pirellulosome forms a crescent-shaped region at the cell margins, with a similar phenomenon also observed in *Planctomyces maris* (Lindsay et al. 2001). In the Pirellula group, ribosomes of the pirellulosome can be seen in some regions to line the inner boundary of the ICM, suggesting that co-translational secretion of newly synthesized proteins into the paryphoplasm may occur.

In *G. obscuriglobus*, however, ribosomes appear to be arrayed linearly along both the inner and outer membranes of the nuclear envelope, at least in the case of sectioned cells prepared via high-pressure freezing followed by cryosubstitution.

This gives an appearance to the nuclear envelope of a eukaryotic endoplasmic reticulum. One assumes that the pirellulosome performs many of the essential metabolic functions of the cell such as glycolysis and because of the presence of DNA and ribosomes is almost certainly the location of all mRNA transcription and protein translation. However, future experiments locating for example glycolytic and other metabolic enzymes, DNA-dependent RNA polymerase, and the more abundant mRNA species and ribosomal proteins via immunogold techniques combined with transmission electron microscopy of sectioned cells are needed to confirm this.

2.2.6 Condensed Nucleoid

All planctomycete species so far examined by TEM of sectioned cells which have been prepared by cryofixation followed by cryosubstitution possess condensed fibrillar nucleoids residing within the pirellulosome or in the case of *G. obscuriglobus*, the membrane-bounded nuclear region within the pirellulosome. Such nucleoids display various types of folding of the fibrils within the nucleoid, suggesting a high degree of condensation even relative to nucleoids of bacteria such as *E. coli* where a "coralline" nucleoid extends throughout the cell volume. In the species *G. obscuriglobus,* at least where cell division for a planctomycete has been more extensively examined than for other planctomycetes, the nucleoid appears to remain condensed throughout the cell cycle, even during passage of the nucleoid through the bud neck into the new bud where the fibrils appear to unfold to some extent but remain associated (see below). Electron tomographic reconstruction of the *G. obscuriglobus* nucleoid from sectioned cryosubstituted cells demonstrates a liquid crystalline cholesteric organization (where molecules such as DNA filaments are ordered in each of a series of helically arranged layers where molecules in each layer are rotated relative to each other), resulting in the DNA arranged in a series of nested arcs visible in TEM of sectioned chromosomes similar to that found on the chromosomes of eukaryotic dinoflagellates (Yee et al. 2012). The condensation of nucleoids in planctomycetes has implications for how transcription and DNA replication may be organized in these organisms, and the location of RNA polymerases via immunogold electron microscopy may help to illuminate this arrangement. There is some indication that folded nucleoids in *G. obscuriglobus* may divide as a unit (Yee 2012), and the occurrence of multiple nucleoids in *G. obscuriglobus* suggests that segregation of the chromosomes of nucleoids may occur within cells before or during budding division.

It has been suggested that the condensation of the nucleoids of *G. obscuriglobus* may be one mechanism of its pronounced resistance to both UV and gamma radiation (Lieber et al. 2009a). It is not known whether other planctomycetes also display such resistance, but this would be predicted if condensation is a major radiation resistance mechanism.

2.3 Planctomycete Russian Dolls: Cells with Three Compartments

The simplest compartmentalized cell plan in planctomycetes is that there are only two compartments, the paryphyoplasm and pirellulosome, separated by the major internal ICM membrane (Fig. 2.1). However, at least two groups of planctomycetes contain within their pirellulosome a third compartment bounded by a membrane or membranes. So viewed in three dimensions, these cells consist of at least three nested containers, each defined by a membrane separating one compartment from another—the paryphoplasm, the pirellulosome, and a third compartment within the pirellulosome. In anammox planctomycetes, there is a metabolic organelle, the anammoxosome, bounded by a single membrane, the structure and function of which are outlined in Chap. 4 of this volume. It contains enzymes of significance to the unique anaerobic ammonium-oxidizing ability of anammox planctomycetes, and the anammoxosome membrane contains ATP synthase (van Niftrik et al. 2010) consistent with the development of proton motive force across the membrane, making it a unique energy-generating internal organelle for bacteria. We will emphasize here the second example, occurring throughout strains within the *Gemmata* clade of planctomycetes, including the model *Gemmata obscuriglobus*. In this organism, the nucleoid is surrounded by an envelope which in many regions consists of two membranes closely apposed, in a manner analogous to that observed for the nuclear envelope of eukaryotes (Fig. 2.3). Serial sectioning of cryosubstituted cells (Lindsay et al. 2001) and more recently cryotomography (in preparation) confirm that the nucleoid and associated cytoplasm surrounded by membranes form a "nuclear body" region analogous to a eukaryotic nucleus. TEM of freeze-fracture replica is also consistent with a bounded nuclear region (Fig. 2.4). The analogy to a nucleus occurs in at least two ways—the envelope commonly consists of two closely associated membranes, and all the cell's DNA appears to be enclosed by a membranous envelope. In addition there are often ribosomes lining the outer membrane (Fig. 2.3b), as in the outer membrane of eukaryote nuclei. However, there also appear to be ribosomes or at least ribosome-like particles inside the nuclear body and lining the inner side of the inner membrane of the double-membraned envelope (Fig. 2.3b), thus differing from the situation in eukaryotic nuclear envelopes where only the outer nuclear membrane has bound arrays of ribosomes. Significantly, the Gemmata three-compartment cell plan including nuclear envelope with two membranes is conserved within at least six strains clustering in the *Gemmata* clade via 16S rRNA-based phylogenetics (Wang et al. 2002). This applied to strains both relatively distant and relatively close to *G. obscuriglobus*, as long as they clustered within a Gemmata group significantly supported as a separate clade within a phylogenetic tree of planctomycetes, suggesting that the ancestor of the Gemmata group also possessed this cell plan. Cell plan types may be conserved within planctomycetes—the Pirellula group members all have the relatively simple two-compartment plan, as do members of Planctomyces and Isosphaera groups. *Zavarzinella*, a genus related to the Gemmata group (Kulichevskaya et al. 2009), also appears to possess

a somewhat similar plan (Lee 2010), but though there is a double-membrane nuclear envelope, whether there is a fully enclosed nuclear body compartment needs to be tested by serial sectioning or tomography of sectioned cells.

It should be noted that at least under some growth conditions, e.g., high nutrient levels, the internal membranes of *G. obscuriglobus* can be quite complex and the nuclear body or bodies are difficult to delineate, especially if only the view from individual sections is considered, but networks of double membranes with attached ribosomes are usually seen even in these cases. In very old cultures, only a single membrane is sometimes seen to bound a large region containing condensed nucleoid and ribosomes, with large vesicles containing ribosomes through the paryphoplasm at the cell rim. In some studies, networks of double membranes have been seen associated with condensed nucleoids but not necessarily forming a bounding envelope in 3D (Lieber et al. 2009b).

Where enclosed nuclear bodies are seen in cells processed from young cultures (displaying a high proportion of motile cells), the outer membrane of the nuclear envelope is often seen to be continuous in one or more places with the ICM, sometimes forming a large region of paryphoplasm. In regions where this occurs, which may sometimes be extensive, only a single membrane (continuous with the inner nuclear envelope membrane) surrounds the nucleoid and associated ribosomes of the nuclear body. This is exactly equivalent topologically to the regions of the nuclear envelope of eukaryotes where endoplasmic reticulum is continuous with the outer nuclear membrane, but in the latter cases the region of single membrane is very limited. From this perspective, the paryphoplasm and especially the pericisternal space are equivalent to the lumen of the RER of eukaryotes. The "pericisternal space" between the inner and outer membranes of the nuclear envelope is clearly continuous topologically with the paryphoplasm, though it may not be functionally identical.

The apparently sealed nature of the nuclear envelope in *G. obscuriglobus* has implications for molecular biology processes in these cells. Transcription and DNA replication must occur in the nuclear body and be restricted to it, though this requires confirmation by techniques such as autoradiography or bromodeoxyuridine-based methods. We do know from in situ hybridization experiments on sectioned *G. obscuriglobus* cells via EM using labeled oligonucleotides and immunogold methods (Butler 2007) that 16S rRNA appears within the nuclear body and in the pirellulosome outside the body (but not within the ribosome-free paryphoplasm, as we would of course predict), compatible with the idea that translation might occur in both regions where ribosome-like particles are identified via EM.

As in eukaryote cells, there must be some provision for communication of macromolecules such as protein and RNA between the nuclear body interior where the genomic DNA resides and the rest of the ribosome-containing pirellulosome cytoplasm, assuming that these separated ribosomes are active in translation. Ribosomes bound to both sides of the nuclear envelope suggest secretion of some proteins across the envelope as they are synthesized, but the ribosomes bound to the outer nuclear membrane as well as the free ribosomes in the rest of the pirellulosomes must use mRNA transported across the nuclear envelope. Proteins translated on free ribosomes of the pirellulosome, if destined for the nucleus, must also be transported

across the envelope, but it is possible that these ribosomes are specialized to non-nuclear proteins of course. There are indications that the "double"-nuclear envelope is in fact a series of "folded single-membrane" flattened vesicles where the outer and inner nuclear membranes meet in continuity at certain points around the nucleus (Fuerst 2005). This immediately suggests that these cells may possess a mechanism of nucleocytoplasmic transport depending on some form of nuclear pore structure interrupting the envelope at points where inner and outer nuclear membranes meet. There are indeed preliminary indications of nuclear pore structures, and extensive proteomic studies on fractionated envelope membranes containing such pores are under way in the authors' laboratory to investigate the possibility of their containing homologous proteins to those of eukaryotic nuclear pore complexes.

The possible evolutionary pressures which might give rise to origins of nuclear structures in a bacterium or an ancestor of a bacterium are considered in Chap. 11. Clearly the occurrence of these "nested box" cell plans in bacteria is of great significance for understanding possible reasons for how and why a eukaryote nucleus may have originated and been sustained through later evolution. This is regardless of whether any evolutionary homology or relationship by descent exists between planctomycetes and eukaryotes. Even an analogous structure may help us understand why such a structure could have evolved and been of adaptive value to the organism in which it originated. In any case, *G. obscuriglobus* is likely to teach us a lot of new molecular biology and cell biology. When considering planctomycetes, it is advisable to understand also the only example of integrated endomembrane systems and membrane-bounded organelles we are able to study, that of the eukaryote cell. The discovery of endocytosis ability in *G. obscuriglobus*, possessing also such a fascinating membrane-bounded nuclear structure, may prove to be only the first such phenomenon of integrated cell biology with astonishing resemblance to similarly integrated eukaryote cell biology.

2.4 Cell Division in Planctomycetes and the Model of *Gemmata obscuriglobus*

The dominant form of division in planctomycetes is budding, in which a "mirror image" of a much smaller daughter cell is formed from a large mother cell. Members of genera such as *Pirellula*, *Blastopirellula*, *Rhodopirellula*, *Planctomyces*, *Gemmata*, *Isosphaera*, and *Aquisphaera* use this form of reproduction, but in *Isosphaera* intercalary buds are formed along a filament of cells (Giovannoni et al. 1987b) and they do not separate from the mother cell as they do in other budding planctomycetes. Binary fission occurs in a minority of planctomycetes, including the anammox planctomycete "*Candidatus* Kuenenia stuttgartiensis" (van Niftrik et al. 2008b, 2009)—see Chap. 4—and the marine genus *Phycisphaera*, proposed as a member of a distinct class *Phycisphaerae* of the planctomycete phylum (Fukunaga et al. 2009). In the anammox planctomycete "*Candidatus* Kuenenia stuttgartiensis," not only does the cell constrict at a midpoint during division, but there is also apparent division of the

anammoxosome organelle within the cell. A cell division ring is observed within the paryphoplasm (rather than in cytoplasm as in bacteria with FtsZ rings) and composed of a protein with no clear homology to the FtsZ protein of the divisome of other bacteria (van Niftrik et al. 2009). So even where budding is not found in a planctomycete, cell division can have unique features not found in other bacteria.

The life cycle of an organism now known to be a *Pirellula staleyi* strain, known initially as Morphotype IV of the "Blastocaulis-Planctomyces group," was early established to involve a motile swarmer cell phase (Tekniepe et al. 1981), and *Rhodopirellula baltica* and *Planctomyces limnophilus* appear also to have a similar cycle (Gade et al. 2005; Jogler et al. 2011; Wecker et al. 2010). In the *Pi. staleyi* strain (Tekniepe et al. 1981), after a swarmer (or more accurately "swimmer") cell matures into a mother cell (phase A) budding (phase B) over *ca.* 3 h involves formation of a protuberance on the mother cell gradually developing into a daughter cell increasing to about half the mother cell diameter and synthesizing a flagellum while attached. A motile "swarmer" (i.e., swimmer) cell is eventually detached. There are significant phases in the cycle in addition to budding itself involving lags (during which events such as DNA replication might occur)—detached daughter buds mature (phase A) and gradually enlarge until *ca*. 30 h after budding, they synthesize fimbriae but lose their flagellum—as mother cells they then enter budding taking 3 h (phase B). Multiple budding from a mother cell is possible, up to four times from the same pole, with significant "resting phase" lags (phase C) of 7–9 h before new bud formation, suggesting cycles similar to DNA replication S phases of eukaryote and budding *Caulobacter* bacteria cell cycles. In this Pirellula group organism, elegant immunoferritin labeling of cell surfaces of mother cells allowed to bud, combined with whole-cell TEM, has shown that de novo synthesis of surface antigens occurs over the bud, and that some intercalation of newly synthesized surface occurs also on the mother cell surface as it matures (Tekniepe et al. 1982).

However, it is not clear that all planctomycetes have a motile swarmer phase, and acidic wetland strains of at least two genera, *Singulisphaera* and *Telmatocola*, have only nonmotile cells (Kulichevskaya et al. 2008, 2012). *Isosphaera pallida* displays gliding motility over surfaces but not swimming motility. Mother cells as well as swarmer cells may have flagella in *Gemmata*.

In *Rhodopirellula baltica*, pear-shaped mother ("adult") cells are nonmotile and by phase contrast microscopy a polar organization is seen (consistent with electron microscopy of thin sections showing the Pirellula-type polar cap region); crateriform structures have a polar distribution at the broader cell pole and fimbriae originate from them (Gade et al. 2005). Rosettes can arise from attachment of the smaller nonreproductive cell pole via a holdfast substance. Budding occurs from the broader, reproductive cell pole and produces a smaller "mirror image" of the mother cell; buds have a uniform crateriform structure distribution and a subpolar flagellum so that they are released as a "swarmer" cell. Particular morphotypes could be correlated with distinct growth phases, swarmers decreased in later phases, and rosettes of several cells were which dominated stationary phase. Proteomics suggested that such changes could be correlated with protein regulation.

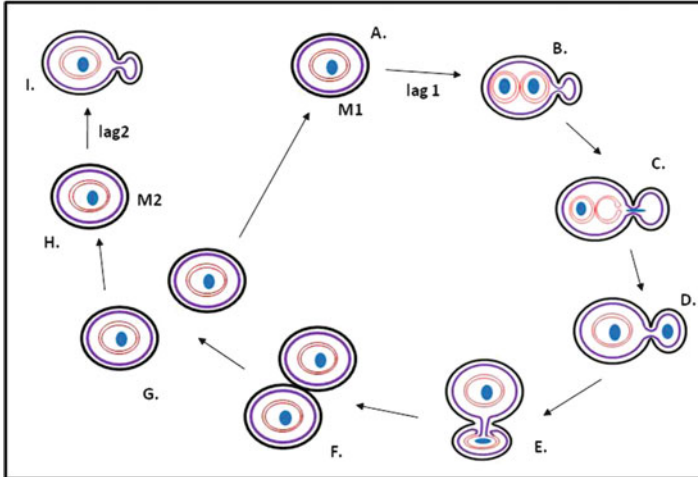

Fig. 2.6 Schematic diagram of the cell cycle and budding division of *Gemmata obscuriglobus*. In (A) and (B), a bud appears as a protuberance from one point on the mother cell M1, which possesses a nucleoid (*blue*) surrounded by a double-membrane nucleoid envelope (*red*). The mother cell ICM (*dark purple*) is continuous with the ICM of the bud (also *dark purple*). The bud initially does not possess a nucleoid. In stage C, the nuclear body double-membrane envelope opens up at the bud neck and the naked nucleoid passes through to the bud via the bud neck. (D) shows the result of that process where a single nucleoid is now present in the bud but without a membranous envelope at this stage. In (E), the bud nucleoid becomes enveloped by two membranes, an inner membrane (*red*) continuous with mother cell ICM and an outer membrane (also *red*) continuous with the bud ICM (*dark purple*). In (F), the bud nucleoid is completely surrounded by the two closely apposed membranes of the matured nuclear envelope (shown in *red*) where membrane fusion and pinching off have resulted in a double-membrane nucleoid envelope completely separated from ICM membranes. Incomplete separation is however commonly observed in micrographs. (F) Is the end-point of the mechanism where the bud reaches similar cell size to that of mother cell M1. (G) Shows the separation of the mother cell and the matured bud. The mother cell M1 initiates the next budding cycle after a 2–4-h lag (lag 1) while the matured bud M2 originating from M1 starts its first budding cycle (H and I) after a 3–5.5-h lag (lag 2). Courtesy of Kuo-Chang Lee (modified from (Lee et al. 2009)

In most of the above studies, the fate of the internal membranes critical to compartmentalization in planctomycetes has not been determined. It is clearly of interest to determine what happens concerning the internal structure during the process of division, and especially so in the case of *Gemmata obscuriglobus* with its membrane-bounded nucleoid and three compartments. Cells of *G. obscuriglobus* divide by budding over *ca.* 12 h until new bud separation and a mother cell can bud repeatedly as in other planctomycetes, taking several hours to start new bud formation (Fig. 2.6) (Lee et al. 2009). Both fluorescence microscopy with DNA stains and TEM of thin-sectioned cryofixed cells show that initially the new immature bud is devoid of nucleoid, but that when the nucleoid does appear in the bud it is initially naked and not surrounded by membranes. It appears that there is a stage in which a nucleoid is passed to the bud through the bud neck (Fig. 2.7a) so that the mother cell nuclear envelope must be open at some point of their extent. Later on membranes

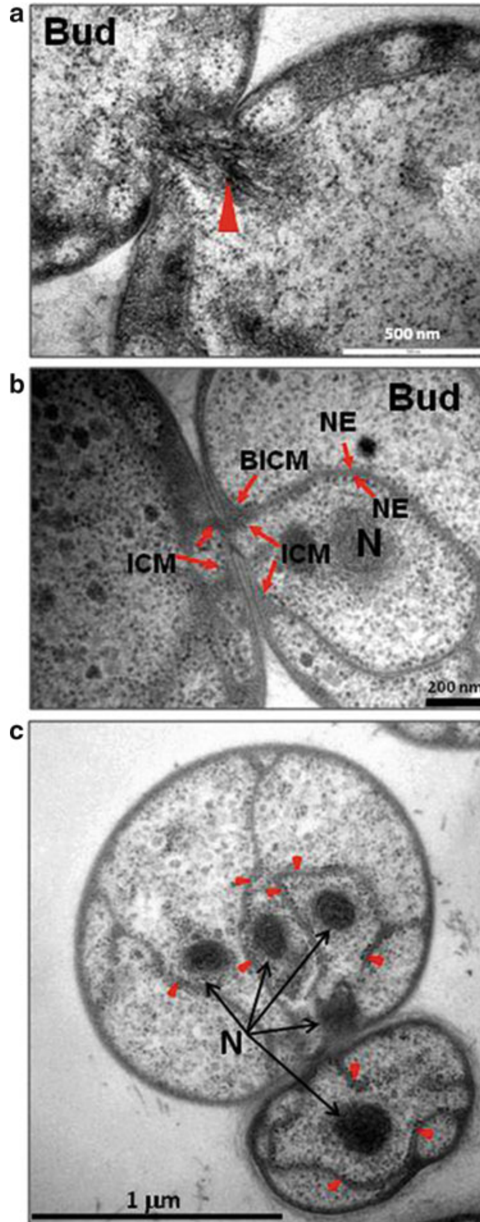

Fig. 2.7 Transmission electron micrographs of sectioned cells of *Gemmata obscuriglobus* processed by cryosubstitution, illustrating selected stages in cell division. (**a**) Fibrillar nucleoid material (*red arrow*) is shown passing through the neck of a newly formed bud on the *left*. Bar 500 nm. (**b**) A later stage where the nucleoid (N) has passed into the bud, and the process of formation of a new nuclear envelope around a naked nucleoid is nearing completion. The new nuclear envelope (NE) is now formed of two membranes, each with bound ribosomes—the outer membrane of the NE is continuous with the intracytoplasmic membrane of the bud (BICM), while the inner membrane of the nuclear envelope is continuous with the ICM of the mother cell (ICM). Bar marker 200 nm. (**c**) Budding cell of

appear to enclose the bud nucleoid until a complete double-membrane envelope is generated, and this seems to occur by an intriguing process perhaps unique within living organisms where the ICMs of both mother cell and bud contribute to the double membrane of the bud's nuclear envelope. At one late stage in formation of the mature bud, the inner membrane of the bud nuclear envelope shows continuity with the ICM of the mother cell, while the outer membrane of the bud nuclear envelope is continuous with the ICM of the bud (Fig. 2.7b). This suggests that the mode of distribution of nucleoid and nuclear body is not analogous to either closed mitosis as occurs in yeast or open mitosis of animal cells where nucleoid envelope disassembles. However, there may be some similarities with some aspects of open mitosis where new nuclear envelope formation is derived from existing endoplasmic reticulum membrane (Anderson and Hetzer 2007), which planctomycete nuclear envelope and its outer membrane resemble in some ways (e.g., regarding ribosome binding). There is no evidence however of any microtubules which could form mitotic spindles, so such analogies are limited.

Both fluorescence microscopy (Lee et al. 2009) and EM of thin-sectioned cells suggest that at least under some growth conditions the mother cell may possess multiple nucleoids and multiple nuclear bodies, perhaps up to four (Fig. 2.7c). Flow cytometry estimating DNA content of cells from an actively dividing culture is consistent with this (unpublished results, Fuerst lab; (Lee 2010)). Mother cells may replicate DNA and prepare nucleoids for transfer to a new bud on the next division, and this would be consistent with the lag observed between budding events.

Concerning molecular mechanisms underlying division in planctomycetes the key bacterial cytoskeletal protein and divisome component FtsZ appears to be absent from the available genomes examined. This implies another type of division system, as occurs in some Archaea where homologs of the eukaryotic ESCRT proteins function in division. One of the few components of the FtsZ-linked divisome in planctomycetes appears to be FtsK. In *G. obscuriglobus*, there is some evidence using antibodies against it in conjunction with immunogold that it is associated with the chromosomal DNA of the nucleoid, even as the nucleoid passes into the bud during division (Lee 2010). This is consistent with the known role of FtsK in chromosome segregation but is not informative concerning processes of constriction at the bud neck. Since this process seems not to involve peptidoglycan one expects it to be distinct from septation in other bacteria.

Allowing for limitations of primary structural bioinformatics homology searches, a comparative genomics study of planctomycetes suggests that many genes homologous with cell division genes of other bacteria may be present in all non-anammox

Fig. 2.7 (continued) *G. obscuriglobus*, the mother cell, half of which contains multiple nucleoids (N) and complex nuclear envelope double membranes (indicated by *red arrowheads*) surrounding the nucleoids. These nucleoids appear to be in separate nuclear body compartments suggesting possible division of nuclear bodies as a whole during the DNA replication S phase of the cell cycle preceding budding. The bud has one nucleoid but the nuclear envelope is not fully closed at this point. Ribosomes line nuclear envelope membranes in both mother cell and bud. Bar marker 1 μm

planctomycetes for which genomes are available including homologs of genes for ClpP, CpaE, ddl, FtsE, FtsK, ParA, MraW, and MraY (Jogler et al. 2012), while homologs of for example MraZ were only found in *Planctomyces* and *Blastopirellula*, of FtsW only in *Planctomyces*, and other genes for proteins such as homologs of the significant shape-determining actin-like cytoskeletal protein MreB also only seem present in some genera (e.g., *Planctomyces* and *Blastopirellula* but not *Gemmata*). Some species have homologs of the FtsZ-like FtsZl-1 proteins, which do not in any case seem likely to form Z-rings such as those composed of FtsZ. Since planctomycetes do not synthesize peptidoglycan, and FtsZ-based divisomes are largely based on interaction with peptidoglycan synthesis enzyme systems, we might not expect many division gene homologs related to peptidoglycan. The alternative ESCRT system of Archaea homologous to eukaryote ESCRT-III proteins does not seem to be present in planctomycetes, but some homology with VPS4 components of such a system is found, albeit modified in such a way as to throw doubt on homology of their function with the archaeal system.

2.5 Endocytosis in Planctomycetes as a Functional Correlate of Cell Compartments

If we had all the genes and the architectural cell plan for a synthetic planctomycete, with all its unique internal membrane and compartment features, we might expect such a cell to also have abilities unique to planctomycetes among the bacteria. One of the characteristics we would expect for a cell based on the plan of *Gemmata obscuriglobus* is the ability to take up proteins from the external medium. This ability is not found elsewhere in the bacteria but is expected of a eukaryote capable of the process called endocytosis. *G. obscuriglobus* can incorporate proteins including green fluorescent protein (GFP), immunoglobulin, and bovine serum albumin, and competition between uptake of such proteins is consistent with a receptor-mediated process and one capable of saturation of what must be a rather nonspecific protein receptor (Lonhienne et al. 2010). Such proteins as bovine serum albumin appear to be degraded once inside the cell, suggesting a nutritional function for the protein uptake. Evidence from fluorescence microscopy for GFP and immunogold electron microscopy of sectioned cryosubstituted cells (Fig. 2.8) shows that the protein is taken up only into the paryphoplasm compartment and does not appear elsewhere in the cell. It does not appear in the pericisternal space between the two nuclear envelope membranes even though this space is topologically continuous with the paryphoplasm. It appears to be taken up via small (*ca.* 50 nm diameter) vesicles within the paryphoplasm resulting from an initial invagination of cytoplasmic membrane to which the protein cargo binds, presumably via receptors on the external leaf of the cytoplasmic membrane bilayer (see Fig. 2.9 and Fuerst and Sagulenko (2010); Lonhienne et al. (2010)). Such vesicles appear to possess associated proteins homologous to MC proteins of eukaryotes such as the clathrins known to be necessary for receptor-mediated endocytosis in eukaryotes.

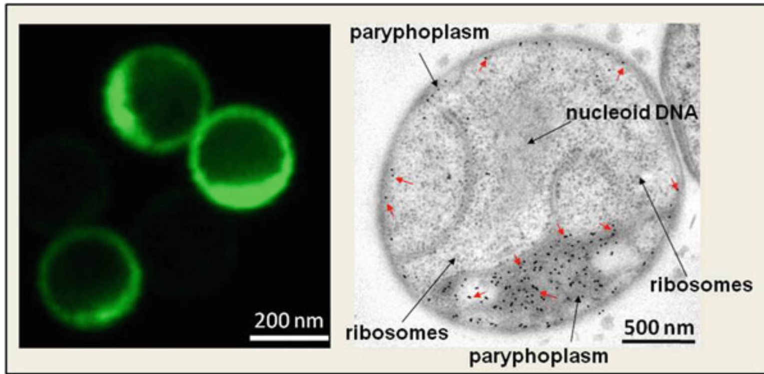

Fig 2.8 (**a**) Fluorescence micrograph illustrating endocytosis-like uptake of green fluorescent protein (GFP) by *Gemmata obscuriglobus*. Note that GFP (*green*) is localized to a distinct outer region of each cell consistent with a localization in the paryphoplasm. Bar: 2 μm. (**b**) Transmission electron micrograph of sectioned cell of cryosubstituted *G. obscuriglobus* illustrating internal GFP protein that has been incorporated by an endocytosis-like process. The 10 nm colloidal gold particles (*red arrowheads*) result from immunogold labeling using anti-GFP antibody, and are localized only in ribosome-free electron-dense paryphoplasm regions. Ribosomes are confined to the pirellulosome including the nuclear body containing the single nucleoid. Bar: 500 nm

The process of protein uptake in *G. obscuriglobus* bears a number of similarities to receptor-mediated, clathrin-dependent endocytosis in eukaryotes. Uptake of individual specific proteins appears to compete with the uptake of other proteins (e.g. uptake of bovine serum albumin or a mouse immunoglobulin inhibits uptake of green fluorescent protein, and uptake can reach saturation with increasing concentration of the external protein). The mechanism of uptake via vesicles budded from plasma membrane is also consistent with this type of endocytosis.

Finally, the presence in the genome of *G. obscuriglobus* of homologs of the eukaryote MC protein family, and association of one of the proteins with vesicles within sectioned whole cells of *G. obscuriglobus* (Santarella-Mellwig et al. 2010), is consistent with this mechanism. The significance of MC proteins for planctomycetes is indicated by their being a component of the core genome of eight different planctomycete species defined relative to *E. coli* and *Bacillus* genomes (Jogler et al. 2012).

The uptake of proteins in *G. obscuriglobus* occurs only via the paryphoplasm or vesicles in the paryphoplasm—other cell compartments are not labeled in the process. This provides strong confirmation of the functional reality of compartmentalization in this planctomycete and is consistent with the integrity of the paryphoplasm as a compartment separated from other cell compartments in this organism. Protein uptake also involves degradation of the protein, which seems to also be confined to the paryphoplasm, so that the function of the endocytosis process may depend on compartmentalization, confining processing of external proteins such that proteins in other compartments are protected. It seems likely that either specialized lysosome-like vesicles will be found or the paryphoplasm itself is something like a lysosome,

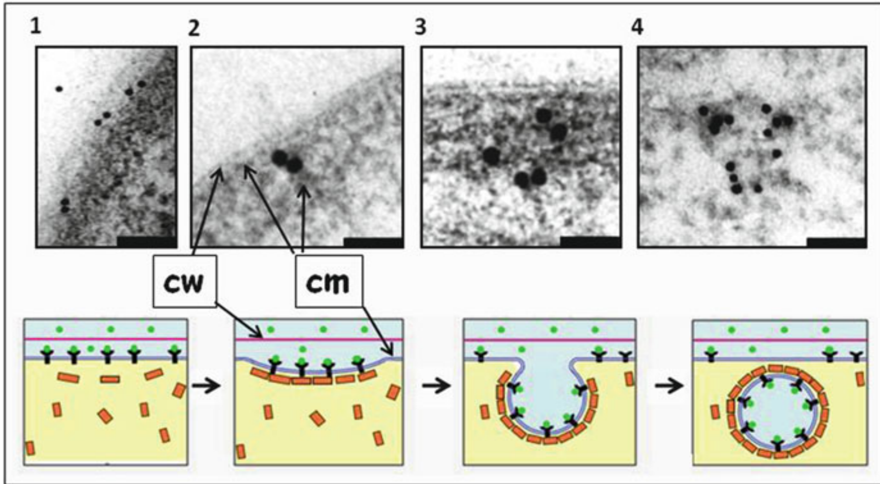

Fig. 2.9 TEM of sectioned high-pressure frozen cryosubstituted *Gemmata obscuriglobus* cell preincubated with GFP (*upper row* of *panels*), and a proposed model of the events involved in uptake of extracellular proteins (*lower row* of *panels*). Micrographs in the *top row* show enlarged views of the potential different stages of GFP uptake at peripheral cytoplasmic membrane and paryphoplasm in cells of *G. obscuriglobus*. Cells were incubated with GFP, immunolabeled with anti-GFP antibody and then cryofixed, processed via cryosubstitution, and sectioned for TEM. Gold particles indicating GFP can be seen associated with cytoplasmic membrane (1 and 2) and then with the membrane of vesicles inside the paryphoplasm region in the cell interior (3 and 4). The schematic series of diagrams in the *bottom row* show corresponding suggested stages for the GFP uptake process and its mechanism, consistent with the evidence from micrographs; stage 1 shows binding of GFP to plasma membrane, and stages 2 and 3 show initial steps of invagination of plasma membrane and association of GFP with vesicles in the process of being generated. At the final stage (stage 4) the vesicle formation is complete. In this model GFP ligand in the external milieu binds to receptors in the cytoplasmic membrane, MC-like (clathrin-like) proteins coat the outside of the vesicle, possibly in association with other ancillary adaptin proteins (not shown), and ligand becomes oriented to the inside membrane surface of the vesicle during its formation due to the effect of infolding. Infolding and formation of vesicles occur in the paryphoplasm. Arrows indicate cytoplasmic membrane (cm) indicated in blue and cell wall (cw) is indicated in magenta; green circles in the model indicate GFP; orange rectangles, MC-like proteins; black Y's, protein receptor. Paryphoplasm is indicated in *yellow*. Bars, 200 nm (micrograph 1), 50 nm (micrographs 2 and 3), and 100 nm (micrograph 4). Modified from Fig. 1b of Fuerst and Sagulenko (2010)

though the former is more likely. If so vesicles with acid pH containing specialized degradative enzymes operating at such pH might be found. Consistent with the nature of the cytoplasmic membrane is that vesicles can be seen to be budding into the paryphoplasm from that membrane (Fig. 2.9) so that it is indeed the outermost membrane of the planctomycete cell, external to the ICM bounding the inner side of the paryphoplasm, as mentioned above (Sect. 2.2.2).

Homologs of MC proteins of eukaryotes occur in *G. obscuriglobus* as well as other planctomycetes and at least some other members of the related PVC superphylum (see Chap. 3). In eukaryotes such MC proteins include clathrin forming the "cages" around vesicles formed during receptor-mediated endocytosis. The MC protein gp4978 which has been found associated with vesicles of *G. obscuriglobus* including those associated with endocytosed protein not only bears structural

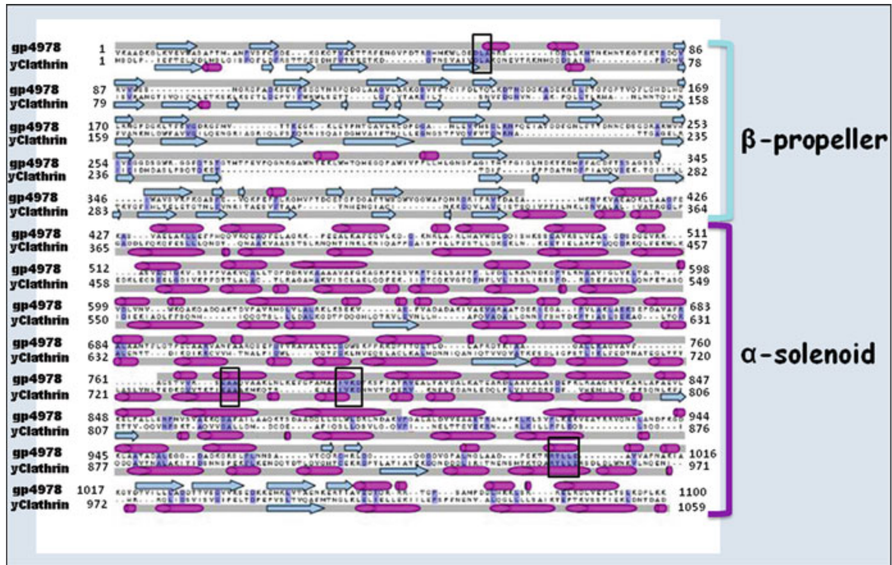

Fig. 2.10 Alignment and secondary structure comparison of gp4978 MC protein homolog from *Gemmata obscuriglobus* and yeast clathrin heavy chain (Uniprot ID: P22137- CLH_YEAST) from *Saccharomyces cerevisiae*. Protein profiles were aligned with the "hhalign" program. Secondary structures (alpha-helices and beta-strands) predicted with the PSI-PRED program are represented as *pink cylinders* (alpha-helices) and *cyan arrows* (beta-strands) above and below the aligned sequences, respectively. Where regions containing either beta-sheets or alpha-helices conform to domains corresponding to beta-propeller or alpha-solenoid domains, the predicted (for gp4978) and known (for clathrin) domains of these beta-propellers and alpha-solenoids are highlighted by a *grey shading* of the secondary structures. Amino acids in the alignment where identities occur in three or more consecutive amino acids are indicated by *boxes outlined in black*

similarity to eukaryotic MC proteins in its possession of alpha-solenoid and beta-propeller domains but even at the primary amino acid sequence level shows some remarkable identities with a protein such as clathrin heavy chain of yeast, scattered through the sequence (Fig. 2.10).

2.6 Molecular Aspects and Implications of a Compartmentalized Cell Plan

2.6.1 The Potential Role of Sterols in Membrane Rigidity Regulation and Compartment/Vesicle Formation

Two strains of at least one genus of planctomycete, *Gemmata*, including the model organism *G. obscuriglobus*, as well as soil strain Wa1-1, synthesize simple sterols such as lanosterol and parkeol (Pearson et al. 2003). Lanosterol is an intermediate in the synthesis of compounds such as ergosterol and cholesterol by eukaryotes, but

Gemmata strains stop at the lanosterol stage. Amounts (e.g. 20 mg/g of cell biomass) are some ten times of those found in cells of another bacterial sterol producer, *Methylococcus capsulatus*. In *Gemmata*, parkeol is actually produced in even higher amounts relative to lanosterol, a unique synthetic distribution for any organism. Interestingly, extractable lipid fractionation largely misses these sterols, which require direct hydrolysis to be released. Although some other groups of bacteria synthesize sterols, the pathway present in *G. obscuriglobus* may be the simplest known (Pearson et al. 2003). There appears to be both a putative squalene monooxygenase and an oxidosqualene cyclase, a minimum needed for lanesterol and parkeol synthesis, but the WPV motif in the oxidosqualene cyclase indicates a nondiscriminate enzyme similar to that observed in some laboratory mutations of plant cycloartenol synthase studied in yeast (Hart et al. 1999). There is some evidence that the enzymes may have homology with those of eukaryotes (Pearson et al. 2003; Summons et al. 2006) but they are not related closely to any contemporary group of eukaryotes, and this suggests that if lateral gene transfer from eukaryotes is to account for sterol synthesis genes in bacteria, then the transfer must have been quite ancient (Chen et al. 2007; Desmond and Gribaldo 2009). The oxidosqualene cyclase of *G. obscuriglobus* differs from all other such enzymes in particular amino acid position substitutions at F444L and S445G close to T381 and C449 and it has been suggested that they may be significant in biasing the particular sterol production yield towards the unusual parkeol "protosterol" (Summons et al. 2006). Since sterols may enable deformation of membranes more easily than other lipid components, the ability to synthesize sterols may be relevant to the ability of planctomycetes to form compartments via internal membranes and to form endomembrane vesicles during endocytosis (Summons et al. 2006). Sterol synthesis may have assisted compartment and vesicle formation by enabling greater membrane deformation ability (Bacia et al. 2005). Planctomyetes also synthesize hopanoids (Damste et al. 2004) more typical of bacteria and also thought to alter membrane properties, so planctomycetes may use both mechanisms for modifying membrane properties. It is conceivable that sterols are important for endocytosis and if so there may be a need for regulation of sterol synthesis by elements similar to the sterol regulatory element-binding proteins of eukaryotes (Castoreno et al. 2005).

2.6.2 Protein Secretion, Signal Peptides, and Signal Transduction Systems

The existence of internal compartments separated from other compartments of the cell by membranes, in some cases single membranes such as the ICM and anammoxosome membranes, suggests that mechanisms for protein transport across such membranes must exist, and perhaps also unusual features of signal transduction connected with the existence of the compartments and the difficulty of transmission of signals across compartments, for example for factors acting via transcription to yield signal output. Unusual signal peptides have been predicted for many planctomycete proteins of *Rhodopirellula baltica*, the species with the first complete genome

sequence among the planctomycetes (Studholme et al. 2004). There may be signal transduction systems especially suited to transmission of signals across internal membranes, as in the extracytoplasmic function (ECF) sigma factor system (Jogler et al. 2012), and these seem to have special properties in the planctomycetes.

A bioinformatic study of the genome of the anammox planctomycete "*Candidatus* Kuenenia stuttgartiensis" is highly relevant to both the potential systems for transport of proteins across membranes in a three-compartment planctomycete and the function of both cytoplasmic membrane and the membrane of the anammoxosome organelle (Medema et al. 2010). The Tat translocation system appears to be exclusively located in the organellar membrane, while the Sec system seems to occur on both cytoplasmic and anammoxosome membrane. Remarkably, the cytoplasmic membrane was predicted to be mainly a transport membrane rather than a membrane essential to metabolism containing cytochromes and the electron transport systems. Such metabolic functions were confined to the anammoxosome membrane, an internal organelle membrane, so that a situation analogous to a typical eukaryote with a mitochondrion seems to apply. This needs confirmation experimentally, but is consistent with some cytochemical data on cytochrome distribution in anammox planctomycetes (van Niftrik et al. 2008a).

Comparative genomics studies of non-anammox planctomycetes including *Rhodopirellula baltica* and seven species of *Pirellula*, *Blastopirellula*, *Planctomyces*, *Isosphaera*, and *Gemmata* have yielded some insights into the unusual features of the signal transduction systems of planctomycetes (Jogler et al. 2012). Planctomycetes possess all three known signal transduction systems operating in bacteria, one-component systems (1CSs), two-component systems (2CSs), and the ECF system.

However, planctomycetes show significant variations within these systems from other bacteria. Serine/threonine protein kinases (STPKs), common in eukaryotes, form a much more significant proportion of the 1CS proteins than in other bacteria, where DNA-binding proteins dominate. It may be that the STPKs acting via direct protein phosphorylation rather than DNA-binding reflect the compartmentalization and separation of nucleoid from much of the cytoplasm. The 2CS system proteins of planctomycetes also display unusual features, many consisting of multiple potential input domains that represent hybrid complex histidine kinases, consistent with regulation via response regulators in complex phosphorelays needed for integrating different stimuli as found for the decision to sporulate in *Bacillus* for example. The 2CS response regulators include a novel type that appears unique to planctomycetes.

The ECF system involves alternative σ factors for RNA polymerase working in conjunction with negative regulation via anti-sigma factors, which may be transmembrane proteins able to release sigma factor for interaction with corresponding promoters on receiving a signal external to the membrane (Helmann 2002). *G. obscuriglobus* ECFs have substantial C-terminal extensions, which can be much longer than the core sequence of this protein (Jogler et al. 2012). The extensions of many proteins in the family distinguished as ECF01-Gob contain multiple WD40 repeats conceivably important for assembling multi-protein complexes. Most ECF01-Gob proteins are membrane anchored, harboring 1–3 putative transmembrane helices between the ECF core and the C-terminal extension and it has been

proposed that these transmembrane ECFs may enable signal transduction across internal membranes such as across the nuclear envelope. If this is so we should find evidence for this type of signaling protein in future *Gemmata* genomes when sequenced. This study also identified ECF groups functionally linked to STPKs, suggesting a novel signaling mechanism. All these bioinformatics deductions from genomic analysis need experimental verification, but the results are what we might predict if planctomycete cells must use novel signaling mechanisms necessitated by their internal compartmentalization.

2.6.3 Ribosome Binding to Membranes and Co-translational Secretion

In planctomycetes including members of the Pirellula group and especially *Gemmata obscuriglobus*, ribosomes can be seen lining internal membranes, in the case of *G. obscuriglobus* those of the nuclear envelope itself. In bacteria like *E. coli*, the signal recognition particle receptor FtsY is needed for targeting of ribosomes to membranes. However, in constructs depleted in signal recognition particle (via Ffh depletion) or translocons (via SecE depletion), intracellular membrane structures can be formed in *E. coli* cells which display ribosomes bound to these membranes (Herskovits et al. 2002). It is conceivable that accumulation of FtsY–ribosome complexes in such may even induce formation of intracellular membranes to which they become bound. Ribosome targeting to membranes and formation of an intracellular membrane could be linked in this model. If this applies to planctomycetes, it may not be very difficult or involve many genetic changes to evolve from a classical bacterium the complex wild-type state for planctomycete cells with intracellular membranes and the ribosomes bound to them. The phylogenetics and regulation of proteins such as FtsY, SecE, and Ffh in planctomycetes may be especially interesting for study as a key to the mechanism of internal membrane formation and internal occurrence of membrane-bounded ribosomes in planctomycetes. The selective pressure for retaining such internal compartments and protein secretion is not obvious, but could be linked to the need for separation of functional compartments connected with endocytosis and protein degradation (see Chap. 11).

2.6.4 Transcription and Translation Separation and Consequences

Transcription and DNA replication and thus RNA polymerase and DNA topoisomerase distribution are predicted to be predominantly restricted to the nuclear body. However with respect to association with the nucleoid DNA, this is not necessarily vastly different distribution to that reported for a bacterium with a free-floating nucleoid such as *Bacillus subtilis*—even without a nuclear membrane transcription and translation this can be separately located in bacterial cells (Lewis et al. 2000). The presence of the nuclear envelope has implications for RNA transport if we

assume that the ribosomes in the pirellulosome outside the nuclear body are active translationally, since mRNA must be transported across the envelope to those extra-nuclear ribosomes. This is so even if some translation actually occurs on the ribosomes in the nuclear body, the nature of which in relation to activity is not clear.

2.6.5 Endocytosis and Related Processes

We would expect that if the receptor-mediated endocytotic protein uptake in *G. obscuriglobus* is mediated via the MC protein clathrin homologs, homologs of other elements of clathrin-mediated endocytosis in eukaryotes might also be found and function in the mechanism. These might include homologs to the adaptins, membrane-curving proteins such as epsins and amphiphysins and Rab GTPases, and also the actins and myosins (Galletta and Cooper 2009). Affinity pull-downs using MC proteins combined with proteomics are one way to retrieve and identify such proteins beyond boinformatic prediction.

In addition to receptor-mediated or MC (clathrin)-dependent endocytosis we would expect that fluid-phase endocytosis perhaps for transport of macromolecules other than proteins would occur and there is some preliminary evidence for this in a wider range of planctomycetes than the *Gemmata* strains exhibiting protein uptake (Sagulenko et al. in preparation). There is no evidence so far for any caveolae or caveolin-dependent process of cytosis.

Endocytosis in *G. obscuriglobus* implies some form of exocytosis as well, by which cytoplasmic membrane captured during protein uptake can be returned to the membrane. There is a need for turnover of membrane via an endocytic–exocytic cycle similar to that occurring in eukaryote cells. This is regardless of any homology of the process at the molecular level with eukaryotic processes. Secretory vesicles might occur in paryphoplasm for this process, and be involved in secretion of material such as cell wall proteins destined for the exterior of the cytoplasmic membrane and enlargement of cytoplasmic membrane as well as wall during budding division. Transcription must occur in the nuclear compartment and protein synthesis is unlikely to occur in the ribosome-free paryphoplasm, so proteins for the cell membrane and wall must be transported across the intracytoplasmic membrane—vesicles moving from ICM to cytoplasmic membrane and trafficking macromolecules via such transport may be a way to do this. Regulatory pathways for governing exocytosis relative to endocytosis can be predicted, perhaps even being controlled spatially in terms of membrane sites for secretion (Chamberlain et al. 2001).

2.6.6 Condensed Nucleoids: Is a Histone-Like Protein Needed for Nucleoid Folding in Planctomycetes?

It is striking that in sectioned cryosubstituted cells of named planctomycete species that have been so far examined by TEM, the nucleoid is highly condensed, such condensation occurs throughout the cell cycle, and the organization of DNA fibrils

displays a cholesteric liquid crystal organization similar to that seen for eukaryotic dinoflagellate chromosomes (Yee et al. 2012). Such condensation could conceivably involve homologs to eukaryote DNA-binding proteins such as histone-like proteins. So far bioinformatics searches for such proteins have not yielded clear candidate homologs of eukaryotic histones, though there is some evidence for homologs of the HU proteins occurring in other bacteria such as *E. coli*, unusual for the occurrence of two different versions in the same *G. obscuriglobus* species, one with an N-terminal and one with a C-terminal extension, unknown to occur together in other bacteria (Yee 2012; Yee et al. 2011). Condensation of nucleoid DNA has been proposed as a possible mechanism for resistance to both UV and gamma radiation displayed by *G. obscuriglobus* (Lieber et al. 2009a). It would be useful to test other planctomycetes for such radiation resistance, as correlation with nucleoid condensation follows from the hypothesis of that resistance mechanism. Proteomics of isolated nucleoid DNA may yield data concerning DNA-binding proteins responsible for condensation, but it is conceivable that such condensation is not due to protein but due to divalent cation interactions as suggested for dinoflagellate condensed chromosomes (Levi-Setti et al. 2008).

2.7 Conclusions

We have seen that there is both unity and diversity in the structural plans of planctomycete cells involving compartments defined by internal membranes. The universal occurrence of the major paryphoplasm and pirellulosome (riboplasm) compartments defined by the universal ICM endomembrane provides unity, while occurrence of a third compartment within the cell plan of *Gemmata* strains and anammox planctomycetes such as "*Candidatus* Kuenenia" is an example of the major type of variation within the shared cell plan. In both *Gemmata* and *Kuenenia*, the structural plans are correlated with function—endocytotic uptake of proteins into the paryphoplasm in the case of *Gemmata* and metabolic reactions for the biochemistry of ammonium oxidation in the case of *Kuenenia*. In the latter case, the anammoxosome organelle within the pirellulosome seems to be a case where the anammoxosome membrane is the site of proton motive force generation via an ATP synthase, analogous in some ways to the pmf generation across a eukaryotic mitochondrion membrane but unlike the mitochondrial biochemistry completely anaerobically. In the former case of the nucleated *Gemmata obscuriglobus*, protein uptake is via a mechanism bearing startling resemblance to receptor- and clathrin-mediated endocytosis of eukaryotic cells, down to the involvement of vesicles and associated clathrin-like proteins. Clearly the planctomycetes are significant for our understanding of the evolution of cell complexity, endomembranes, and the nucleus itself (see Chap. 11). Recent comparative genomic analysis suggests that even though we might expect sets of genes in addition to MC protein homologs shared by compartmentalized PVC superphylum members, we may have to experimentally determine their functions clearly (Kamneva et al. 2012). Future experimentation as well as comparative genomics

should yield some interesting answers and perhaps even more evolutionary questions about some of the major transitions in the history of the cell. Planctomycetes may also form a model for the simplest possible compartmentalized cell, perhaps forming the basis for a future synthetic biology construction of a complex cell with some characteristic functional features of eukaryote cell biology.

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Chapter 3
Structural Aspects of MC Proteins of PVC Superphylum Members

Damien P. Devos

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Abbreviations

PVC Planctomycetes-Verrucomicrobiae-Chlamydiae
MC Membrane coat
NPC Nuclear pore complex
nup Nucleoporin protein of a nuclear pore complex

3.1 The Protocoatomer Hypothesis

Probably the most striking feature of the eukaryotic cell is the endomembrane system that divides it into compartments of specialised functions. This includes the endoplasmic reticulum, the Golgi and the nuclear membrane surrounding the cell's

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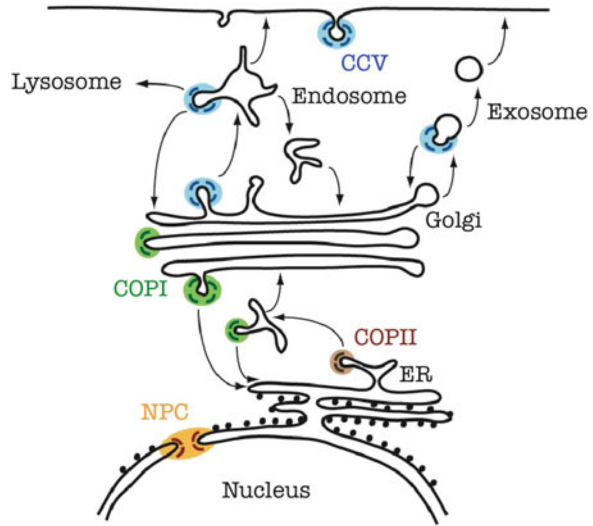

Fig. 3.1 Membrane coat proteins in the eukaryotic endomembrane system. A classical organisation of eukaryotic cell is presented in a schematic form, with the main complexes associated with curved membranes represented by coloured shapes. Clathrin-, COPI-, COPII-coated vesicles and the nuclear pore complexes are in *blue*, *green*, *brown* and *yellow*, respectively. Inside each complex, membrane coat proteins are represented by *darker curves*

genetic material. The origin of such a complex and dynamic system is still poorly understood. Early on, De Duve and Wattiaux proposed that a protoeukaryote acquired the capacity to invaginate its membrane and began to organise its intracellular space by specialisation of the newly created compartments (De Duve and Wattiaux 1966). The protocoatomer hypothesis supports this proposal by providing the needed mechanistic concepts (Devos et al. 2004). A key role inside the protein complexes shaping membranes in the eukaryotic cell is played by membrane coat (MC) proteins. MCs include coated vesicle components, like clathrin,[1] COPI α- and β′-subunits, and COPII Sec31, but also nucleoporins (nups), like Nup85 and Nup133. A common origin to all MCs was originally inferred from the detection of shared structural features in some of them suggesting a related origin to all protein complexes and thus to all compartments that they sustain in the eukaryotic cell (Devos et al. 2004). In clathrin-coated vesicles and in COPII, the MCs, respectively clathrin and the Sec31/Sec13 dimer, form the scaffold of the protein coat, also known as the cage, that surrounds the vesicle membrane. A similar organisation is likely to take place in the COPI cage with the α- and β′-subunits (Frederick 2010). The organisation of the MCs in the nuclear pore complex (NPC) is still unclear, with various hypotheses based on different data available (Hoelz et al. 2011). However, all models recognise a central role of the MC nups (nucleoporin proteins of the eukaryotic nuclear pore complex) in forming the scaffold of the NPC. Thus, at the core of each eukaryotic complex, MC proteins play key roles including the one of forming the backbone of the complex (Fig. 3.1). This organisation is present in all investigated eukaryotes, including divergent ones, and as such is likely to have been present in the last common ancestor of all eukaryotic

[1]Nomenclature referring to the yeast proteins throughout the manuscript and the figures.

cells, suggesting an early occurrence of the endomembrane system (Degrasse et al. 2009; Field and Dacks 2009; Neumann et al. 2010). The protocoatomer hypothesis thus states that "a simple coating module containing minimal copies of the two conserved folds evolved in protoeukaryotes as a mechanism to bend membranes into sharply curved sheets and invaginated tubules" (Devos et al. 2004). The major evolutionary innovation that this mechanism represented, allowing among other possibilities the elaboration of internal subcompartments and endosymbiosis, ensured the success of those organisms possessing it. Duplication and divergences then allowed for specialisation, with the partitioning of different functions into separate, interconnected compartments, each with their own specialised set of coating modules. Thus, the protocoatomer hypothesis predicts that the acquisition of MC proteins was a turning point in eukaryotic origin.

3.2 MCs Structural Commonalities and Differences

Most MCs are composed of variations around a typical protein architecture, consisting of an Nt-β-propeller domain (a fold formed by four to eight blades of four beta-strands shaped in a circular fashion) followed by a Ct-SPAH domain (a fold formed by stacked pairs of alpha-helices, hence its name[2]) (Devos et al. 2006, 2004). Some degree of similarity can be observed at the secondary structure level or schematically (Fig. 3.2). Crystallographic data have since then revealed some of the molecular details of the similarities and differences between eukaryotic MCs (Brohawn and Schwartz 2009; Hoelz et al. 2011). The surprises lay in the structural variations found in the two domains defining the MC architecture and their interaction (Fig. 3.3). Most of the MC structural features are so far only found in eukaryotic MC proteins.

Clathrin is formed by a single complete Nt-β-propeller domain and an elongated Ct-SPAH domain mainly straight with two bends (Fotin et al. 2004). COPI subunits α and β′ have two β-propellers followed by a shorter mainly straight SPAH domain. The α-subunit additionally contains a Ct-domain of mixed, α-helical and β-sheet composition after an unstructured fragment. COPII component Sec31 presents a variation where a complete β-propeller domain is followed by a single β-blade before the SPAH domain. Unexpectedly, in a feature so far only found in the MCs, the partner of Sec31, Sec13 forms an incomplete β-propeller that is complemented by the lone Sec31 β-blade (Fath et al. 2007). An additional variation of the MC structural features found in Sec31 and some nups is that the SPAH domain folds back on itself. In the case of Sec31, the turn corresponds to an unstructured fragment of the protein, whereas in the nups, the turn is structured and α-helical

[2]It has been argued that eukaryotic MC helical domains are distinct from a regular α-solenoid arrangement. This only illustrates the inconsistent usage of the term (Field et al. 2011). For this reason, we prefer to use the denomination of SPAH (stacked pairs of alpha-helices) domain, which only refers to the commonality of the fold types.

Fig. 3.2 Secondary and tertiary structures of eukaryotic MCs. Representative yeast MCs are illustrated. *Left*: predicted secondary structure. The amino acid scale is represented at the *top*. The *black horizontal line* represents the sequence of each MC protein. The predicted secondary structures (McGuffin et al. 2000), α-helices (*magenta*) and β-strands (*cyan*), are indicated by *coloured bars* above each line. The height of the bars is proportional to the confidence of the predictions. Sequences are aligned around the transition from mainly β-sheet to mainly α-helical. *Right*: Schematic tertiary structure: Predicted or observed structural features are represented by coloured shapes, *cyan circle* or *triangle* for β-propeller or β-blade and *magenta* shape for SPAH domain

(Lee and Goldberg 2010). Four scaffold nups, Nic96, Nup84, Nup85 and Nup145C share this particular organisation of the SPAH domain with COPII Sec31, manifesting the common evolutionary ancestry between the two membrane coats. This relationship is further supported by the fact that Nup145C and Sec31 complement the incomplete β-propeller Sec13 in an almost indistinguishable way. The incomplete β-propeller complementation found in the Nup145C/Sec13 and Sec31/Sec13 is mirrored in the Nup85/Seh1 pair. Seh1, homologue to Sec13, also present an incomplete β-propeller fold that is complemented by the Nt-β-blade of Nup85. The likely homology between Nup85 and Nup145C is not detectable at the sequence-only level. An extreme variation of the MC architecture is found in the Nup84 and Nic96 nups, which are structurally similar to Nup85 and Nup145C but lack the Nt-β-blade. Another case of domain fission and fusion is found in the polyprotein formed by Nup145N and Nup145C. In most organisms, Nup145C is synthesised as a fusion protein with another nup, Nup145N.[3] Nup145N then auto-catalyses its own cleavage from Nup145C. This autocleavage seems to be a conserved feature of Nup145N. Those cases of domain fission and fusion illustrate the extreme plasticity in protein

[3]The pair is also referred to as Nup96-Nup98, e.g. in human.

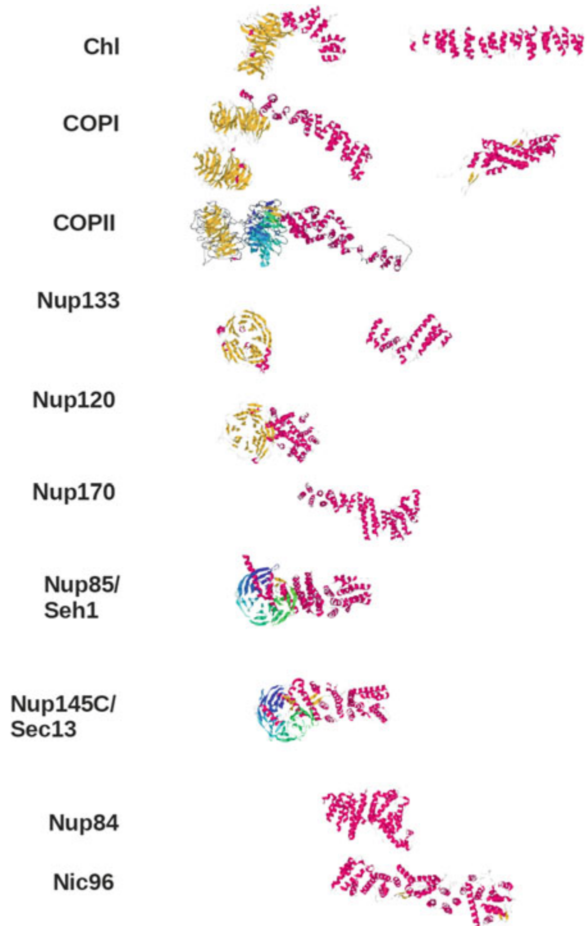

Fig. 3.3 Crystallographic structures of eukaryotic MCs. The crystallographic structures of described eukaryotic MCs are represented with the β-strands in *yellow*, and the α-helices in *pink*. The incomplete β-propellers Sec13 and Seh1 are *rainbow coloured*, from Nt-*blue* to Ct-*red*

domain organisation found in the MCs. In addition, the nucleoporin MCs present variations of the combinations of those structural features in different proteins (Hoelz et al. 2011). In the case of Nup170 and Nup133, a more extended and stretched SPAH domain is found after the β-propeller, whereas the SPAH domain of Nup120 turns back on itself to interact tightly with the β-propeller.

The important variations in the structure of the proteins also have an important impact on their multimerisation and thus the cage they are forming. This is not the scope of this chapter but has been discussed elsewhere (Field et al. 2011; Frederick 2010). Sequence similarity is remarkably absent between most MCs, even between the most structurally similar ones. The structural similarities confirm a relationship that is otherwise undetectable at the sequence level. This kind of evolutionary relationship with extreme sequence and structure deviation represents an important challenge for our current homology detection tools based on sequences only.

3.3 Bacterial MCs Detection and Analysis

Using sequence information only, proteins having the MC architecture are found exclusively in eukaryotic proteins (Devos et al. 2004). However, structure is more conserved than sequence in evolution. Thus, we turned to structure to search for proteins related to the eukaryotic MCs. We structurally screened all available genomes for proteins containing a β-propeller or a SPAH domain. Because the sequence and structural divergences are so pronounced even between eukaryotic MCs, and since we aimed at maximising the sensitivity of detection, we used one of the most sensitive tools, HHPred (Söding 2005), with a permissive cut-off and were very lax in our detection of fold type and considered β-propeller and SPAH domains at a very broad level. We then restricted this set to proteins having both domains in the MC organisation, a β-propeller preceding a SPAH domain and where most of the protein is covered by those two domains. We identified all known and various unknown eukaryotic MCs. We were unable to identify any such protein in archaea and in bacteria, with the exception of members of the PVC superphylum and Bacteroidetes (Table 3.1, Fig. 3.4)

Table 3.1 Number of membrane coat-like proteins detected in bacterial genomes

Phylum/species	MC-like proteins
Planctomycetes	
Gemmata obscuriglobus UQM 2246	8
Planctomyces maris DSM 8797	11
Planctomyces maris DSM 8797	11
Planctomyces brasiliensis DSM 5305	11
Blastopirellula marina DSM 3645	9
Pirellula staleyi DSM 6068	11
Rhodopirellula baltica WH47	5
Rhodopirellula baltica SH 1	9
Isosphaera pallida ATCC 43644	7
Lentisphaerae	
Lentisphaera araneosa HTCC2155	9
Verrucomicrobia	
Verrucomicrobium spinosum DSM 4136	16
Chthoniobacter flavus Ellin428	14
Pedosphaera parvula Ellin514	9
Verrucomicrobiae bacterium DG1235	2
Bacteroidetes	
Dyadobacter fermentans DSM 18053	9
Algoriphagus sp.	6
Spirosoma linguale DSM 74	6
Haliscomenobacter hydrossis DSM 1100	5
Leadbetterella byssophila DSM 17132	3
Maribacter sp.	3
Robiginitalea biformata HTCC2501	2
Chitinophaga pinensis DSM 2588	1
Pedobacter heparinus DSM 2366	1

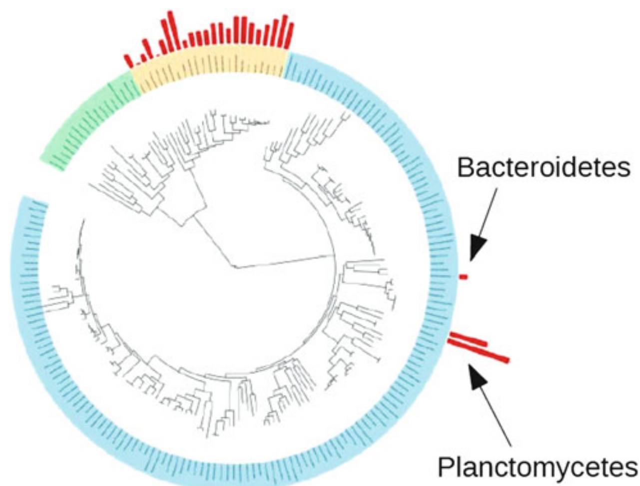

Fig. 3.4 Detection and features of MCs. Global phylogeny of some of the organisms screened for MC proteins, drawn with iTOL (Letunic and Bork 2007). Eukaryotes, archaea and bacteria are grouped with *orange*, *green* and *blue* backgrounds, respectively. The number of MC proteins found in each proteome is indicated on the external arc with *red bars*. Note that this tree includes only two members of the PVC superphylum (both are planctomycetes) and one Bacteroidetes

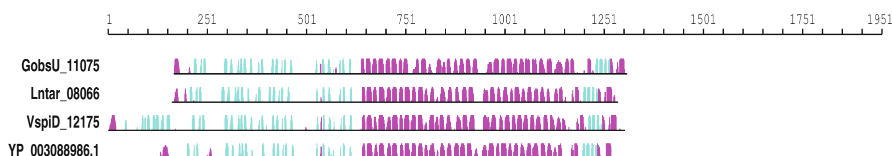

Fig. 3.5 Bacterial MC-like protein predicted secondary structure. Representative bacterial MC-like proteins are illustrated with the same convention as Fig. 3.2. NCBI ID codes are provided; GobsU for *Gemmata obscuriglobus*, LNTAR for *Lentisphaera araneosa*, VspiD for *Verrucomicrobium spinosum* DSM 4136. The last protein is from the Bacteroidetes member *Dyadobacter fermentans* DSM 18053

(Santarella-Mellwig et al. 2010). There is no doubt that the identified bacterial proteins have the MC architecture. At the sequence level, the sequence profiles (using hidden Markov models) identified the β-propeller and SPAH domains with statistically significant *e*-value, and predicted secondary structures are also similar to the ones observed for the eukaryotic MCs (Fig. 3.5), while at the structural level, the full-atom three-dimensional models derived are satisfyingly evaluated using mean force potentials. Thus, these bacterial proteins have the MC architecture. Most of the bacterial proteins also have an additional 140–150 amino acid domain of mixed α/β

composition. The contribution of this domain to the MC function is so far unknown. As in the eukaryotic ones, variability is observed inside the predicted structural features of the bacterial MCs (Fig. 3.5). It is difficult to predict the exact structural features found in the bacterial MC-like proteins. However, the predicted structures of bacterial MCs do not differ more from eukaryotic MCs than eukaryotic MCs do between themselves. For example, the eukaryotic MC that is most structurally similar to clathrin (PDB Code 1b89A) is COPI coatomer beta′ (3mkqC) with 124 structurally equivalent residues, and the second closest is Nup85 (3f3fD) with 113. The same clathrin structure has 118 residues that are structurally equivalent with the classical HEAT repeat protein (1b3uB), predicted to represent the bacterial MCs. Thus, structurally, the bacterial and eukaryotic MCs are at least as similar as eukaryotic MCs are to one another (Devos 2012). Unlike them however, most of the bacterial MCs show significant sequence similarity, suggesting a reduced evolutionary pressure to diverge after duplication or recent duplication events.

Given the uneven distribution of MC proteins in members of the PVC superphylum, it is likely that their common ancestor already possessed a few copies of those proteins, which have been retained and duplicated in some lineages, while lost in some others during divergence of the species (Santarella-Mellwig et al. 2010). The presence of such proteins in Bacteroidetes is also surprising but could be explained by the fact that some Bacteroidetes, like some Planctomycetes, are marine organisms; it is therefore likely that members of these two groups of bacteria or their ancestors have exchanged coding genes.

3.4 Endomembrane Localisation and Endocytosis

Because of its peculiar internal membrane organisation, we focused on the planctomycete *Gemmata obscuriglobus*. We thus cloned the ORF coding for one such *Gemmata* protein, GobsU_11075, and heterologously expressed and purified it. We then raised antibodies against this protein to reveal its localisation in the bacterial cell by cryo-electron microscopy. See Chap. 2 for an alternative and more detailed description of the *Gemmata* membrane organisation. However, for simplicity, we interpret here the available images in a classical bacterial membrane organisation, where the space between the inner and outer membranes called the paryphoplasm in Planctomycetes is equivalent to the bacterial periplasm. We found that the majority of the GobsU_11075 protein is located in the paryphoplasm of the *G. obscuriglobus* cells (Fig. 3.6). In addition, a significant proportion, between one third and one half, was found in close proximity to a bent membrane. In a related experiment (see Chap. 2), endocytosis was shown to take place in this organism, and the MC protein GobsU_11075 was found in tight association with the endocytosis vesicle membranes (Lonhienne et al. 2010). Thus, in addition to the similarity of architecture, the bacterial MCs (or at least GobsU_11075) appear to have a function similar to the eukaryotic one.

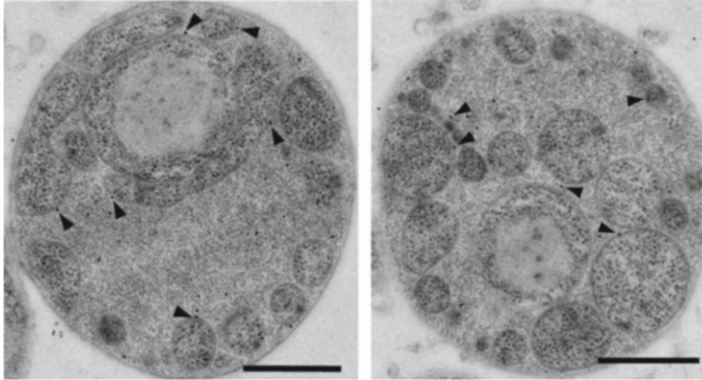

Fig. 3.6 Localisation in *Gemmata obscuriglobus* of bacterial MCs. Electron micrographs of GobsU_11075-immuno-labelled *G. obscuriglobus* cells. Gold particles associated with membranes are indicated by *arrowheads*. The paryphoplasm can be distinguished from the cytoplasm by the lack of ribosomes and the presence of gold particle-labelled antibodies. Scale bars: 500 nm

3.5 Evolutionary Considerations

Nups or coated vesicles? MCs are part of two complexes in eukaryotes: nuclear pores and coated vesicles. Nuclear pore complexes bridge a double membrane, formed by a tightly bent single membrane, while vesicle coats surround a single membrane vesicle. GobsU_11075 is unlikely to be a component of a nuclear pore-like structure in *G. obscuriglobus* as it is associated with a single membrane. In addition, our own ongoing work on the *G. obscuriglobus* endomembrane system suggests that no nuclear envelope-like compartments are present in these bacteria, nor are there any NPC-like structures found associated with the membranes.

How could the similarities between the bacterial and eukaryotic endomembrane systems and proteins be interpreted? There is no statistically significant sequence signal that can link the eukaryotic and bacterial proteins. However, this is also true for the relationship between the likely homologous eukaryotic MCs, illustrating the flexibility of sequences of such folds. Due to the lack of sequence similarity, a possible homology between bacterial and eukaryotic MCs is difficult to evaluate. The interpretation of the observed structural, architectural and functional data as similarities might be erroneous and the bacterial and eukaryotic proteins might not be related at any level at all. This possibility has been discussed elsewhere (Devos 2012; Reynaud and Devos 2011). Accepting the relevance of some similarities, the presence of MCs in bacteria can be explained by convergent evolution, lateral gene transfer to or from a eukaryote or direct homology. In the case where the eukaryotic and bacterial proteins are not related or the result of convergent evolution, it would still be interesting to characterise more deeply the endomembrane system of the

PVC bacteria to understand alternative pathways of emergence of such a complex feature. Strong evidences establishing the homologous relationship between bacterial and eukaryotic MCs are lacking. However, the fact that no homology is detectable at the sequence level between the eukaryotic MCs demonstrates that this is not sufficient to rule out vertical transmission. No evidence of a recent lateral gene transfer from eukaryotes has been observed (Santarella-Mellwig et al. 2010). The possibility of an ancient lateral gene transfer between a eukaryote and a PVC (or ancestors of PVC members) is still possible. If the presence of MCs in bacteria is the result of lateral gene transfer, analysing the origin of transfer (to or from a eukaryote) and the subsequent evolution would present fascinating data about the adaptations of such a system in a new cellular environment. We believe however that this is unlikely to result in the organisation of a complex endomembrane system. In other words, the MCs are necessary but not sufficient to achieve this organisation. More proteins are needed and as such the number of genes laterally transferred should have been important.

Finally, the similarity of structural features, architecture and function are important supports in favour of a possible vertical relationship between the two sets of proteins. This would suggest that PVCs have lain on the path of eukaryotic endomembrane system development (Reynaud and Devos 2011).

3.6 Conclusions and Foregoing Research

The protocoatomer hypothesis states that MC proteins were key to the origin of the eukaryotes because they form the scaffold of their membrane manipulating systems. Despite their common origin, eukaryotic MCs display impressive variations due to divergence in sequence, structure, architecture, interaction and cage formation. Using a structural approach, proteins with the MC architecture have been detected in bacteria, in the PVC superphylum and in Bacteroidetes. In one bacterial species, *G. obscuriglobus*, at least one MC-like protein is in tight interaction with the internal membranes and involved in endocytosis. This represents the first molecular link between a bacterial endomembrane system and the eukaryotic endomembrane system, suggestive of a possible evolutionary relationship between both systems.

Further characterisation of GobsU_11075 and of the other bacterial MCs is ongoing in our and other laboratories. We have now produced antibodies against the other *G. obscuriglobus* MCs and are testing their localisation in the cell. We will investigate interactions between the bacterial MCs and search for their partners. In the near future, we hope to achieve a characterisation of the *G. obscuriglobus* endomembrane system that is as deep as possible.

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Chapter 4
Cell Biology of Anaerobic Ammonium-Oxidizing Bacteria: Unique Prokaryotes with an Energy-Conserving Intracellular Compartment

Sarah Neumann, Muriel C.F. van Teeseling, and Laura van Niftrik

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Abbreviations

Anammox	Anaerobic ammonium oxidation
LUCA	Last universal common ancestor
PVC	*Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae*
SBR	Sequencing batch reactor
MBR	Membrane bioreactor
FISH	Fluorescence in situ hybridization
PCR	Polymerase chain reaction
DNRA	Dissimilatory nitrate reduction to ammonium
WWTP	Wastewater treatment plant
OMZ	Oxygen minimum zone
PBP	Penicillin-binding protein
TEM	Transmission electron microscopy
pmf	Proton motive force
Sec	Secretory
TAT	Twin arginine translocation
GC-MS	Gas chromatography mass spectrometry
HAO	Hydroxylamine oxidoreductase
AOB	Aerobic ammonium-oxidizing bacteria
HZS	Hydrazine synthase
HDH	Hydrazine dehydrogenase

4.1 Introduction

4.1.1 History of Anammox Research

During the 1940s to 1970s several studies indicated that the nitrogen cycle (Fig. 4.1) might contain more reactions than was known at that time and that a microbe was missing from nature that should be able to anaerobically oxidize ammonium, with nitrate or nitrite, to dinitrogen gas (Hamm and Thompson 1941; Richards 1965; Broda 1977). This was based on thermodynamical calculations and field observations which indicated that far less ammonium accumulated in anoxic water bodies than expected from Redfield stoichiometry. The existence of anaerobic ammonium

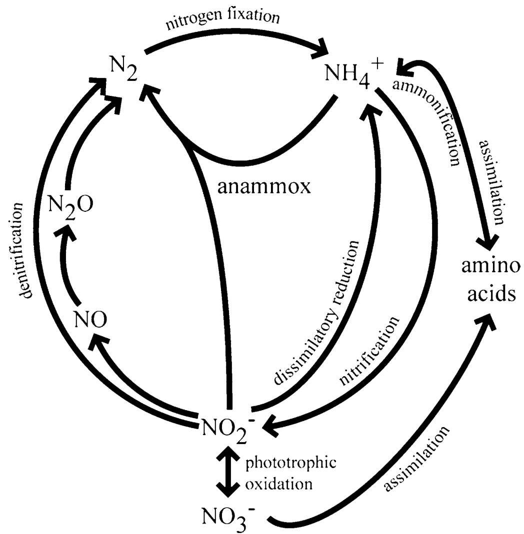


Fig. 4.1 Simplified scheme of the biological nitrogen cycle

oxidation was verified in the early 1990s in an anoxic fluidized bed bioreactor at the Gist-Brocades yeast factory in the Netherlands where ammonium was found to be converted to dinitrogen gas at the expense of nitrate. It still took a couple of years before the bacteria responsible for the anaerobic ammonium oxidation (anammox) were enriched and identified as a new planctomycete (van de Graaf et al. 1995; Strous et al. 1999a). Since then, anammox research has been focused on many different areas of research: genomics, molecular biology, biochemistry, physiology, cell biology, ecology, and application.

4.1.2 Evolutionary Implications

What no one could have predicted was that in addition to being the missing link in the nitrogen cycle, these anammox bacteria would also defy other microbiological concepts. Anammox bacteria do not conform to the typical characteristics of bacteria but instead share features with all three domains of life, Bacteria, Archaea, and Eukarya, making them extremely interesting from an evolutionary perspective. Indeed, the origin of the *Planctomycetes* has been nourishing food for evolutionary thought. Using ribosomal RNA phylogeny, it has been suggested that the *Planctomycetes* are an ancient lineage situated at the root of the bacterial tree (Brochier and Philippe 2002). On the other hand, others have argued that the number of nucleotide positions used in this phylogenetic analysis is too low to support this conclusion and that the bacterial ancestor is a hyperthermophile (Di Giulio 2003). Here, the *Planctomycetes* are not placed at the root but at the third branch of divergence in the domain of the Bacteria. From the sequencing of the complete genome

of some of the *Planctomycetes* it appears that the evolutionary relationship of this phylum is indeed not straightforward. Though the genome of the planctomycete *Gemmata obscuriglobus* revealed eukaryotic signature proteins (Staley et al. 2005), the metagenome of the anammox bacterium "*Candidatus* Kuenenia stuttgartiensis" indicated that anammox bacteria are more related to the *Chlamydiae*, obligate intracellular parasites, than to Eukarya and might have evolved from a Gram-negative bacterium (Strous et al. 2006). So the question remains: Are the *Planctomycetes* a relatively new phylum that evolved their compartmentalization separately from the Eukarya? Or is the last universal common ancestor (LUCA) eukaryotic-like and are the *Planctomycetes* the last examples to survive (Fuerst 2005) before evolution proceeded to the less complex but perhaps more efficient prokaryotic cell types? Recently, it was argued that the unusual features of the *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae* (PVC) superphylum (Wagner and Horn 2006) could indeed imply the existence of continuity and intermediate steps between the domains Bacteria, Archaea, and Eukarya and suggest that LUCA was complex (Devos and Reynaud 2010; Reynaud and Devos 2011). This hypothesis was disputed by others arguing that comparative genome analyses have never revealed a link between Eukarya and *Planctomycetes* and that the PVC features are either a result of convergent evolution or acquired through lateral gene transfer (McInerney et al. 2011). The *Planctomycetes* have thus divided scientists into two camps concerning their evolutionary origin. Are *Planctomycetes* an ancient evolutionary line with characteristics homologous to Eukarya and Archaea or are they relatively recent additions to the Bacteria that through convergent evolution and/or lateral gene transfer have acquired characteristics analogous to Eukarya and Archaea?

4.1.3 Classification

Of the known species capable of the anammox reaction, none are yet available in pure culture. It is, however, possible to enrich anammox bacteria in laboratory cultures until they make up approximately 95 % of all organisms in the enrichment culture (van der Star et al. 2008). As a result, all anammox genera currently have the status of "*Candidatus*," which designates prokaryotic organisms that have been described based on sequence information and phenotypic studies, but have not been isolated in a pure culture (Murray and Stackebrandt 1995).

Anammox bacteria are a distinct phylogenetic group within the phylum *Planctomycetes* and form a separate order named "*Candidatus* Brocadiales," which features only a single family: "*Candidatus* Brocadiaceae" (Jetten et al. 2010). This family consists exclusively of anammox bacteria and encompasses all known genera capable of the anaerobic oxidation of ammonium. To date, five different genera have been described (Fig. 4.2): "*Candidatus* Brocadia," "*Candidatus* Kuenenia," "*Candidatus* Scalindua," "*Candidatus* Anammoxoglobus," and "*Candidatus* Jettenia." The genera *Brocadia* and *Scalindua* consist of more than one species. These are "*Candidatus* Brocadia anammoxidans" (Strous et al. 1999a) and "*Candidatus* Brocadia fulgida" (Kartal et al. 2008) and "*Candidatus* Scalindua

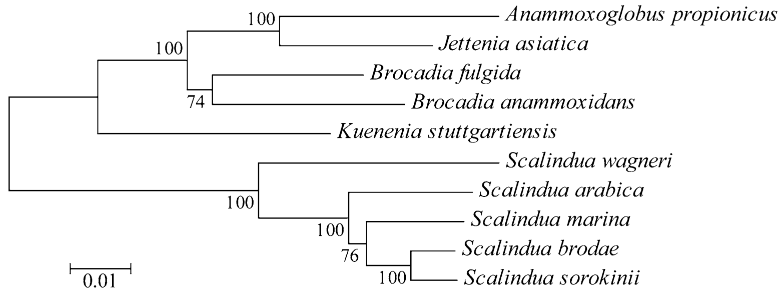


Fig. 4.2 Phylogenetic 16S rRNA gene tree showing the known anammox genera and species. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 0.36221871 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated, resulting in a total of 1,359 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). Courtesy of Huub Op den Camp

brodae" (Schmid et al. 2003), "*Candidatus* Scalindua sorokinii" (Kuypers et al. 2003), "*Candidatus* Scalindua wagneri" (Schmid et al. 2003), "*Candidatus* Scalindua marina" (Brandsma et al. 2011), "*Candidatus* Scalindua profunda" (van de Vossenberg et al. 2013), and "*Candidatus* Scalindua arabica" (Woebken et al. 2008), respectively. The remaining genera are presently represented by a single species: "*Candidatus* Kuenenia stuttgartiensis" (Schmid et al. 2001), "*Candidatus* Anammoxoglobus propionicus" (Kartal et al. 2007b) and "*Candidatus* Jettenia asiatica" (Quan et al. 2008). The divergence between the five different anammox genera is relatively large (Fig. 4.2) and the sequence identity on 16S rRNA gene level ranges from 87 to 99 % (Jetten et al. 2010). The largest divergence exists between *Scalindua*, which is usually found in marine environments, and the remaining genera, which thrive in man-made ecosystems such as wastewater treatment facilities, but have also been detected in soil and natural aquatic systems, e.g., estuaries, in independent clone libraries (Penton et al. 2006; Clark et al. 2008; Hirsch et al. 2011; Li et al. 2011; Yoshinaga et al. 2011). By the same technique, representatives of the genus *Scalindua* have been shown to be present outside of marine environments, i.e., in estuarine systems and freshwater lakes and rivers (Risgaard-Petersen et al. 2004; Schubert et al. 2006; Zhang et al. 2007; Rich et al. 2008).

4.1.4 Culturing Conditions

The doubling time of anammox bacteria is very long and can vary from one to several weeks depending on the growth conditions (Strous et al. 1998; Kartal et al. 2011a). Traditional microbiological techniques, e.g., cultivation on agar plates, are

Fig. 4.3 Image of an anammox sequencing batch reactor showing the aggregated anammox cells

therefore not applicable for cultivation of these bacteria. The successful enrichment of anammox bacteria was facilitated by the development of the sequencing batch reactor (SBR). The SBR is specifically designed for the enrichment of very slow-growing microorganisms. It features an efficient biomass retention system, which retains about 90 % of the growing biomass. The reactor is continuously fed with medium and extensively stirred. After a given period of time, the inflow of medium and the stirring are stopped and the biomass is left to settle before a part of the liquid in the reactor is pumped off. The enrichment level of anammox bacteria in an SBR reaches approximately 74 % (Strous et al. 1998). Besides the biomass retention, the keys to success of the system are its reliability and stability under substrate-limiting conditions. It selects strongly for fast-settling biomass and as a result, the enriched anammox biomass is present in flocs (Fig. 4.3). Although the SBR generates a lot of biomass, this is often not suitable for fundamental experiments. The reason is the heterogeneity of a floc, both in species composition and in activity of anammox bacteria (Kartal et al. 2011a). In order to remove the selection pressure of the settling time of the biomass, the settling step in the reactor operation has been discarded. Instead, the reactors have been equipped with a membrane unit (membrane bioreactor; MBR) that holds back the anammox cells, through which the effluent of the culture has to pass. This results in planktonic cultures of anammox cells, which have been reported to be enriched in anammox bacteria by more than 95 % (van der Star et al. 2008).

For the enrichment of anammox bacteria, the culture is inoculated with a suitable starting material, e.g., sludge or marine sediment, and is fed with gradually increased amounts of ammonium and nitrite as substrates for the anammox reaction and bicarbonate as carbon source. In an SBR, nitrate is also added in small amounts to avoid too low a redox potential. Due to the high anammox activity, which leads to the indigenous production of nitrate (see Sect. 4.7), this is not necessary in an MBR (Kartal et al. 2011a). The headspace of the reactor is kept anaerobic by addition of a gas mixture of Argon and CO_2 (95 %/5 %). The temperature of the culture is usually kept at 33 °C and the pH at a value of 7.3 (Kartal et al. 2011a), although anammox bacteria have been shown to tolerate temperatures between −2 and 43 °C (Jetten et al. 2009) and pH values between 6.7 and 8.3 (Strous et al. 1999b). In an SBR, it takes approximately 90–200 days for the culture to change to a characteristically bright red color, which stems from the heme proteins inside the anammox cells and indicates the enrichment of anammox bacteria. Additionally, the medium can be supplemented with substances such as organic acids to promote the growth of specific anammox species (see Sect. 4.7) (Kartal et al. 2007b, 2008). For the cultivation of marine anammox species, 2.5 % sea salt is added to the medium.

4.1.5 Detection Methods

A range of techniques is available to detect the presence of anammox bacteria in the environment. These can be divided into molecular tools, lipid analysis, fluorescence in situ hybridization (FISH), and activity measurements. Commonly, several of these techniques are used in concert to investigate the presence and activity of anammox bacteria.

The molecular tools make use of anammox-specific primers in a polymerase chain reaction (PCR). These are used to detect the 16S rRNA gene or functional genes of anammox bacteria by amplification of DNA isolated from environmental samples. Universal primers targeting the prokaryotic 16S rRNA gene and primers designed for the phylum *Planctomycetes* are not suitable to detect anammox bacteria, because their 16S rRNA gene sequence is too divergent (Kartal et al. 2011a). For more information on the abundance and the activity of anammox bacteria, a PCR can also be used to quantify them by real-time PCR on their 16S rRNA or a functional gene, e.g., *nirS*. In order to link the occurrence of anammox bacteria to their activity, the transcription levels of several functional genes can be monitored by quantitative reverse transcription PCR (Lam et al. 2009).

Another way of investigating the presence of anammox bacteria is by analyzing the lipids of the bacterial population in a given environment. Anammox bacteria possess unique lipids not found in any other species (see Sect. 4.6) and their detection therefore points to the occurrence of anammox bacteria. In ancient sediments, these lipids can also be used to investigate past anammox activity (Jaeschke et al. 2008).

FISH is a semiquantitative visualization technique for bacteria: specific 16S rRNA-targeting oligonucleotide probes carrying a fluorescent label are used to identify specified groups of bacteria by fluorescence microscopy. It provides an estimation for the relative abundances of different groups of organisms. For anammox bacteria, several FISH probes have been designed that are specific down to the species level (Kartal et al. 2011a).

In order to measure anammox activity and its contribution to the element cycling in a natural habitat, activity measurements using stable isotope labeling of nitrogen compounds are usually employed. If either nitrite or ammonium is labeled with the heavy isotope of nitrogen (^{15}N), anammox cells will produce $^{29}N_2$ (^{14}N-^{15}N) by combining one heavy isotope of nitrogen from the labeled substrate with a light isotope from the unlabeled substrate in a 1:1 ratio. To current knowledge, the resulting $^{29}N_2$ could not be produced by any other microbial process and is therefore characteristic for anammox activity (Kartal et al. 2011a). Although widely used, this technique is prone to underestimate anammox activity, because anammox bacteria are capable of producing ammonium from nitrate by performing the dissimilatory reduction of nitrate to ammonium (DNRA) (see Sect. 4.7). Since this ammonium is then used in the anammox reaction, $^{28}N_2$ or $^{30}N_2$ will be produced as an end product, depending on which of the two substrates has initially been labeled. This makes the dinitrogen gas produced by anammox bacteria indistinguishable from the one produced by denitrification, where two molecules of one nitrogen compound, i.e., nitrite, are combined to form $^{28}N_2$ or $^{30}N_2$ (Kartal et al. 2007a).

4.1.6 Occurrence and Ecological Significance

Most anammox bacteria have been found in the ocean and in anaerobic or oxygen-limited wastewater treatment plants (WWTPs). All genera of anammox bacteria have been detected in WWTPs, but only representatives of *Scalindua* are commonly found in marine environments, i.e., sediments and oxygen-minimum zones (OMZs) of the ocean (Jetten et al. 2009). A *Kuenenia*-like anammox bacterium was also localized in a marine environment in proximity to a deep-sea hydrothermal vent (Byrne et al. 2009). Reports of anammox bacteria in natural freshwater systems are few: an anammox species closely related to *S. brodae* was found in an African freshwater lake and a close relative of *B. anammoxidans* was detected in a Chinese river (Schubert et al. 2006; Zhang et al. 2007). Until recently, reports on the detection of anammox bacteria in soil systems were also few and based on retrieval of 16S rRNA gene sequences (Penton et al. 2006; Clark et al. 2008), but anammox bacteria related to *J. asiatica* and *B. fulgida* have now successfully been enriched from peat soil (Hu et al. 2011).

The anammox activity in OMZs appears to make a big contribution to the global nitrogen cycle. Studies in different OMZs around the world indicated that anammox bacteria are the key players responsible for the nitrogen loss from these

systems. Only a very small percentage of the oceans consist of OMZs. They are marked by very low oxygen conditions. The oxygen is depleted due to a combination of poor aeration and a high primary production in the surface waters. The degradation of the biomass consumes a major part of the oxygen in the waters. There is extensive anaerobic prokaryotic activity in OMZs, which is estimated to cause up to 50 % of the fixed nitrogen loss from the oceans. There is large evidence indicating that anammox bacteria are mainly responsible for the conversion of inorganic nitrogen compounds into dinitrogen gas in these parts of the ocean (Jetten et al. 2009; Lam and Kuypers 2011). As such, anammox bacteria would have a major impact not only on the oceanic nitrogen cycle, but also on the nitrogen cycle on a global scale. The nitrite for anammox activity is thought to be derived from both aerobic ammonium-oxidizing bacteria (AOB) and Archaea (Lam et al. 2007), but could also be produced by nitrate-reducing bacteria. The ammonium is usually assumed to be derived exclusively from demineralization processes in the suboxic zone. However, this may be an underestimation of the substrate availability for anammox bacteria, because they are capable of generating their own ammonium from nitrate via DNRA, as has been shown to be the case for anammox bacteria in the Omani shelf (Kartal et al. 2007a; Jensen et al. 2011). Therefore, anammox bacteria might be even more important in the oceanic nitrogen cycling than is currently recognized.

Besides these natural partners, anammox bacteria might also compete with other microorganisms for available substrates. In anaerobic systems where nitrite and both ammonium and methane are present, the coexistence of anammox bacteria and bacteria that couple anaerobic methane oxidation to nitrite reduction (Ettwig et al. 2010; Zhu et al. 2010; Luesken et al. 2011) ("*Candidatus* Methylomirabilis oxyfera") has been shown. However, even though anammox bacteria and *M. oxyfera* compete for the same electron acceptor (nitrite) they are able to coexist in nature.

4.1.7 Industrial Significance

Over the last decade, water demand and wastewater discharge have increased due to growing populations and industrialization. The European Union has been implementing more and more stringent directives in order to protect the potable water reservoirs from the discharge of untreated industrial (and domestic) wastewaters. Wastewater treatment facilities have especially been faced with more stringent emission standards for nitrogen. The conventional way that nitrogen is removed from wastewater is through nitrification and denitrification processes. These conventional systems use a high energy input to generate aerobic conditions for nitrification and can only deal with the increasing nitrogen loads (and more stringent emission standards) by adding methanol as the carbon and energy source for denitrification, leading to substantial costs and carbon dioxide emissions. For this reason, the demand for new and sustainable systems for nitrogen removal has

increased dramatically. The anammox reaction is emerging as an attractive alternative to conventional nitrogen removal from wastewater (Kartal et al. 2010). The anaerobic and autotrophic anammox bacteria do not require oxygen or organic matter as a carbon source. The first 75 m^3 anammox WWTP has been operating in Rotterdam (the Netherlands) since 2002 and is used to remove nitrogen from concentrated sludge reject water. Currently there are at least five full-scale anammox® WWTPs in the Netherlands and China treating sewage, food ingredient processing, yeast, tannery, and potato processing wastewater. The expected benefits compared to the conventional (nitrification/denitrification) technology are (source Paques BV; http://www.paques.nl) high nitrogen removal, up to 90 % reduction of CO_2 emission, up to 50 % less space requirement, up to 60 % reduction of power consumption, up to 90 % reduction of operational costs, no methanol consumption, and a minimal production of excess sludge. In this way, anammox contributes directly to our environment and economy.

4.2 The Anammox Cell

Anammox bacteria are interesting not only from an applied, ecological, and evolutionary point of view but also for their unusual metabolism and cell biology which will be the focus of this chapter. Like all other species of phylum *Planctomycetes* (Lindsay et al. 2001), the cells of anammox bacteria are compartmentalized by individual bilayer membranes. In the case of anammox bacteria, the cells are divided into three separate compartments (Fig. 4.4). The cytoplasmic membrane lies at the boundary of the outermost compartment called the paryphoplasm. Next, an intracytoplasmic membrane surrounds the riboplasm compartment and finally the anammoxosome membrane encloses the anammoxosome compartment. In the next paragraphs, the different anammox cell compartments and their proposed functions are discussed in more detail.

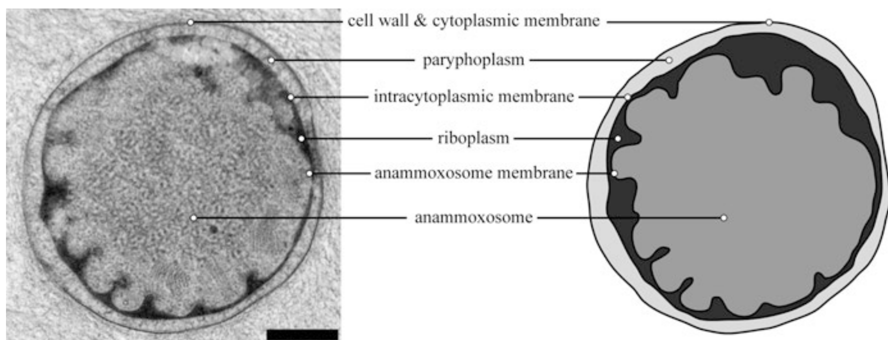


Fig. 4.4 Transmission electron micrograph and schematic drawing of the cell plan of anammox bacteria. Scale bar: 200 nm

4.3 The Cell Envelope

Each living cell is encircled by a cell envelope that shields the cell from the environment. The cell envelope consists of the cytoplasmic membrane, as a real physical barrier of the cell from its environment, and, in most Bacteria and Archaea, the cell wall. The definition of a cell wall is not very strict, but can best be described as "all cellular structures on the outside of the cytoplasmic membrane, whose main function is to provide rigidity to the cell" (Seltmann and Holst 2002). Historically, bacterial cells are divided into two main classes, Gram-positive and Gram-negative, based on the staining mechanism described by Gram in 1884 (Gram 1884) and much later, following development of electron microscopy, confirmed as correlated with cell wall structure and thickness in many species. However, with time it has become clear that the distinction between Gram-positive and Gram-negative cells is not that clear-cut in nature, with more and more cell plans being discovered that do not fit in this dichotomy (Maniloff 1983; Zuber et al. 2008).

Even while it is known that not all cells of bacteria are either Gram-positive or Gram-negative with respect to wall structure, the typical characteristics of these two can still be used to compare different cell walls in the domain Bacteria. Gram-positive cells have a cell wall consisting of a thick, 30–100 nm (Silhavy et al. 2010), cross-linked peptidoglycan layer, with in some cases secondary polymers linked to it. The cell wall of Gram-negative bacteria consists of a much thinner peptidoglycan network, up to maximally 12 nm (Vollmer and Höltje 2004), encircled by a highly asymmetric outer membrane. The outer membrane is relatively permeable or "leaky" compared to the cytoplasmic membrane. This can be attributed to the presence of porins in the outer membrane (Koebnik et al. 2000), which facilitates the relatively easy passive diffusion of small hydrophilic and/or charged molecules over this membrane. Archaea also have several cell wall structural classes, some of which correlate with stain reactions. Though polymers analogous to peptidoglycan are sometimes present, Archaea all lack muramic acid-containing peptidoglycan or typical outer membranes and in some cases the cell walls consist solely of glycoproteins (Albers and Meyer 2011).

Although the cell wall composition of anammox bacteria has not been determined experimentally, some conclusions have been based on electron microscopy. On the basis of these observations, the cell envelope of anammox bacteria has often been described as unusual because it is proposed to lack both peptidoglycan and an outer membrane, as is the case for all *Planctomycetes*. Therefore, the anammox cell wall would be neither Gram-positive nor-negative in the sense of structural cell wall type, and such Planctomycete walls bear comparison with some archaeal wall types.

4.3.1 A Peptidoglycan-Less Cell Wall in Anammox Bacteria?

In 1984, the cell walls of eight *Planctomycete* strains were biochemically analyzed and found to lack peptidoglycan (König et al. 1984). The *Planctomycete* strains that

were analyzed are *Planctomyces maris* (ATCC 29201T and IFAM 1317), *Pirellula staleyi* (ATCC 27377T), *Rhodopirellula baltica* (IFAM 1310), *Planctomyces brasiliensis* (IFAM 1448T), and three uncharacterized *Pirellula* strains (IFAM 1319, 1358, and 1441). In concordance with this analysis and the absence of any apparent peptidoglycan in the cell wall of anammox bacteria as visualized by transmission electron microscopy (TEM), it has been suggested that the cell wall of anammox bacteria is also devoid of peptidoglycan (Jetten et al. 2003).

When the metagenome of the *K. stuttgartiensis* became available, it was found that 19 of the 21 genes needed for the biosynthesis of peptidoglycan are present in the metagenome (Strous et al. 2006). A new investigation using the same data compared to the KEGG peptidoglycan biosynthesis pathway (http://www.genome.jp/kegg/pathway/map/map00550.html) indicates that all essential genes are present, except those encoding the penicillin-binding protein (PBP) 1a and 1b. In *Escherichia coli* a double mutant lacking both PBP1a and 1b is lethal, while the single mutants are not (Suzuki et al. 1978), indicating that either PBP1a or 1b is essential for cell survival. Both proteins, classified as PBP class A proteins, are described as bifunctional enzymes that catalyze both a penicillin-insensitive peptidoglycan transglycosylase reaction and a penicillin-sensitive peptidoglycan transpeptidase reaction (Yousif et al. 1985) required for the insertion of peptidoglycan precursors into polymeric peptidoglycan (Ghuysen 1997). The status quo in *K. stuttgartiensis* seems to resemble that in *Chlamydia trachomatis* in which class A PBPs are also absent (Ghuysen and Goffin 1999). In the case of *C. trachomatis* it has been hypothesized that the cells might form a glycan-less wall polypeptide (Ghuysen and Goffin 1999; McCoy and Maurelli 2006), which could be the case in *K. stuttgartiensis* as well. In the *K. stuttgartiensis* metatranscriptome, a small number of reads is detected for all peptidoglycan synthesis genes present in the metagenome, but none of these are detected in the metaproteome (except for the protein D-alanine–D-alanine ligase, Ddl that produces the peptidoglycan precursor D-alanine–D-alanine) (Kartal et al. 2011b). In conclusion, genetically *K. stuttgartiensis* could produce a glycan-less polypeptide to substitute for the canonical peptidoglycan polymer, but so far there are no indications that this actually is the case. Whether *K. stuttgartiensis* and all other anammox bacteria produce some form of pseudo-peptidoglycan thus needs further investigation.

4.3.2 No Outer Membrane in Anammox Bacteria?

In concordance with the cell plan of *Planctomycetes*, the outermost membrane of anammox bacteria has been defined as the cytoplasmic membrane (see Sect. 4.3.3). Nevertheless, the metagenome (Strous et al. 2006) of *K. stuttgartiensis* encodes proteins characteristically ascribed to the periplasm and outer membrane of Gram-negative bacteria. Several porin homologs can be found (van Niftrik et al. 2010), of which a few are also expressed (Kartal et al. 2011b). In addition, a complete TonB system, including five outer membrane receptors, is encoded (van Niftrik et al. 2010). Although the recent metatranscriptomics study did show some transcription

of these genes, no expression of the proteins involved in this complex, which transfers energy from the cytoplasmic to the outer membrane to facilitate transport, could be detected in the metaproteome (Kartal et al. 2011b). In addition, both the metagenome and the metaproteome show the presence of a number of typical Gram-negative multidrug exporters (Kartal et al. 2011b). Ultrastructural research (electron microscopy) on anammox bacteria has, however, never been able to detect an additional (outer) membrane outside the outermost, cytoplasmic, membrane of the anammox cell. Immunogold localization of the described proteins reminiscent of a typical Gram-negative outer membrane could indicate whether these are really functionally expressed and, if so, where they are located.

4.3.3 Identity of the Outermost Membrane of Anammox Bacteria

The identity of the outermost anammox membrane as either a cytoplasmic or an outer membrane typical of Gram-negative bacteria has been under debate (see Sect. 4.3.2). The definition of the cytoplasmic membrane is very broad: e.g., "a semipermeable barrier that separates the cytoplasm from the environment" (Madigan et al. 2012). The cytoplasm of prokaryotes is defined as "the fluid portion of a cell, bounded by the cytoplasmic membrane" (Madigan et al. 2012). From these two definitions it is difficult to identify the outermost membrane of anammox bacteria as either a cytoplasmic or an outer membrane. The outermost membrane, surrounding the paryphoplasm on its outer edge, has been defined as the cytoplasmic membrane of anammox bacteria. The most important argument for this is the detection of RNA inside the paryphoplasm, which has been demonstrated by using ribonuclease coupled to gold particles as a probe for RNA and observing them with TEM (Lindsay et al. 1997; Lindsay et al. 2001). Since typically one would expect RNA to be located inside the cell, the detection of RNA inside the paryphoplasm (see Sect. 4.4.1) makes the surrounding membrane a cytoplasmic membrane. However, this argument is not conclusive since it cannot be guaranteed that the RNAse-gold labeling is actually targeted at RNAs located inside the paryphoplasm. Because of the relatively small size of the anammox paryphoplasm compartment and the length of the antibody–target complex it could be that some of the gold labels closest to the intracytoplasmic membrane surrounding the riboplasm actually correspond to RNA in the riboplasm compartment. In addition, extracellular RNA sometimes occurs in bacteria (Demain et al. 1965). On the other hand, the immunogold localization of an F-type ATPase (Fig. 4.5a) on the outermost membrane of the anammox cell (van Niftrik et al. 2010) suggests the ability of gradients (e.g., proton motive force; pmf) to exist over this membrane and supports its identification as a cytoplasmatic membrane. Outer membranes typical of Gram-negative bacteria are relatively permeable to small molecules and ions and no pmf can exist across them as demonstrated by the fact that the pH in the periplasm is equal to the pH outside of the cell (Wilks and Slonczewski 2007). Therefore, the detection of ATPases in the outermost membrane indicates that this membrane is not an outer membrane typical of Gram-negative bacteria and is therefore best defined as the cytoplasmic membrane.

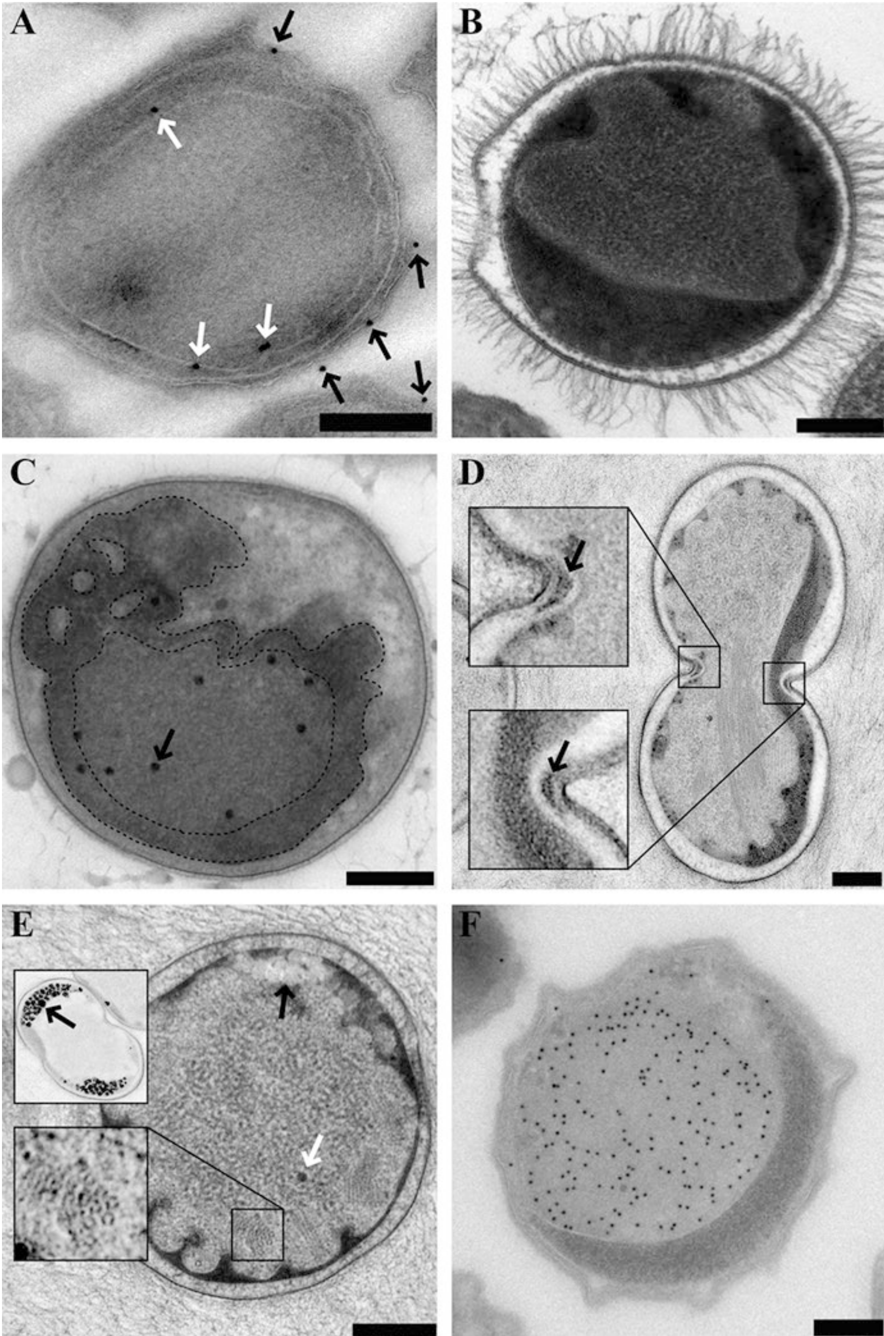


Fig. 4.5 Transmission electron micrographs of anammox bacteria. (**a**) Immunogold localization of the catalytic beta subunit of the F-ATPase-1 gene cluster localizes this ATPase to the outermost membrane (*black arrows*) and anammoxosome membrane (*white arrows*) in *K. stuttgartiensis* rehydrated cryosections. (**b**) High-pressure frozen, freeze-substituted, and Epon-embedded *Scalindua* spp.

4.3.4 Contents of the Cell Wall

Early biochemical studies concerning the composition of the cell wall of the same eight *Planctomycete* strains that were analyzed for peptidoglycan (see Sect. 4.3.1) have shown that the planctomycete cell wall consists for a substantial part of proteins (63–82 % of the cell envelope dry weight) (Liesack et al. 1986). Since biochemical analysis has not been performed on the cell wall of anammox bacteria yet, it is not known if the cell wall of anammox bacteria also has a proteinaceous character. In fact, the composition of the anammox cell wall remains completely unknown. To elucidate the composition of the anammox cell wall it should be isolated in order to perform biochemical and proteomics analysis.

4.3.5 Cell Appendages

Although cell appendages are normally not included in the definition of the cell envelope, they are located on the outside of the cell and (can) have important functions in bacteria (Jarrel 2009). The presence and function of cell appendages in anammox bacteria is not an extensively studied topic, but some first steps have been taken in this research. Pili-like appendages (Fig. 4.5b) with a length of approximately 130 nm have been observed in *Scalindua* sp. (van de Vossenberg et al. 2008; van Niftrik et al. 2008a). The exact identity and function of these appendages in this anammox species remain unknown. No cell appendages have ever been observed in the best studied anammox bacterium *K. stuttgartiensis*, but the metagenome seems to encode 22 of the 24 essential genes (and some nonessential genes) for the synthesis and movement of flagella. The metatranscriptomics and metaproteomics analysis of the *K. stuttgartiensis* enrichment culture showed some transcription of these genes, but only one protein was detected (Kartal et al. 2011b). The metagenome also showed some genes associated with the formation of pili (Strous et al. 2006) of which only one was found in the metaproteome (Kartal et al. 2011b). Future research focusing on *K. stuttgartiensis* grown under different conditions, using microscopy and proteomics analysis, could shed light on the question of whether flagella or pili can be expressed by this anammox bacterium under certain environmental or growth conditions.

Fig. 4.5 (continued) cell showing pili-like cell appendages. (**c**) Cytochrome peroxidase staining localizes cytochrome *c* proteins to the anammoxosome in chemically fixed and Epon-embedded thin sections of *K. stuttgartiensis*. Intense staining occurs within close proximity to the anammoxosome membrane, as outlined by the dashed lines. *Arrow*: Iron particle. (**d**) High-pressure frozen, freeze-substituted, and Epon-embedded *K. stuttgartiensis* cell going through cell division. Insets: The cell division ring in the paryphoplasm. (**e**) High-pressure frozen, freeze-substituted, and Epon-embedded *K. stuttgartiensis* cell showing tubule-like structures (*lower inset*), iron particle (*white arrow*), and glycogen storage (*black arrows* and *upper inset*). *Upper inset*: Glycogen-stained cell. (**f**) Immunogold localization of an anammox hydroxylamine oxidoreductase-like protein shows its location in the anammoxosome compartment in *K. stuttgartiensis* rehydrated cryosections. Scale bar: 200 nm

4.4 The Paryphoplasm

The paryphoplasm, a compartment found in all *Planctomycetes*, is defined as "a ribosome-free region between the cytoplasmic membrane and an internal membrane called the intracytoplasmic membrane" (Fuerst and Sagulenko 2011). The paryphoplasm is named after the Greek word *paryphe*, meaning "border woven along a robe" (Lindsay et al. 2001). It forms the outermost compartment of the planctomycete cell, located on the inside of the cytoplasmic membrane and cell wall. In anammox bacteria, the paryphoplasm is the compartment with the smallest breadth, on average 31 ± 8 nm (Fig. 4.4). Nonetheless the paryphoplasm makes up on average 21 % of the total cell volume in *K. stuttgartiensis*.

4.4.1 Contents of the Anammox Paryphoplasm

To understand the function of the paryphoplasm, it is necessary to first identify the contents of the paryphoplasm. In transmission electron micrographs, the paryphoplasm appears as the most electron-light compartment (Fig. 4.4), which suggests a relatively low density of material in this compartment. No ribosomes have been observed in the paryphoplasm of anammox bacteria and DNA seems to be absent too. RNAse-gold labeling has been observed in the paryphoplasm of the anammox bacterium *B. anammoxidans* indicating the presence of RNA (Lindsay et al. 2001) (see Sect. 4.3.3). Although this labeling is higher than the background, the localization in the paryphoplasm might be an artifact due to the length of the antibody–target complex and the narrowness of the anammox paryphoplasm (van Niftrik 2008). Cytochrome peroxidase staining (Fig. 4.5c), that was used to localize cytochrome *c* proteins in *K. stuttgartiensis*, was absent from, or below the detection limit in, the paryphoplasm (van Niftrik et al. 2008a). Despite, or because of, the apparent lack of structural detail in the paryphoplasm compartment, one structure stands out in dividing anammox cells: the cell division ring (Fig. 4.5d). The cell division ring can be seen as an electron-dense, bracket-shaped structure as has been investigated in *K. stuttgartiensis* and *B. fulgida* (van Niftrik et al. 2009). Unlike other *Planctomycetes*, which divide through budding, cell division in anammox bacteria is performed via the process of binary fission (van Niftrik et al. 2009). The cell division ring in anammox bacteria is not based on FtsZ (van Niftrik et al. 2009), which is the typical and widespread cell division ring-forming mechanism in prokaryotes and eukaryotic organelles (Margolin 2005). Instead, the cell division ring in anammox bacteria might consist of the protein encoded by the *K. stuttgartiensis* gene kustd1438. Immunogold labeling showed that this protein was located at the cell division ring in *K. stuttgartiensis* (van Niftrik et al. 2009) and might thus be the ring itself or a protein associated with it. The further composition of the cell division ring or divisome complex in anammox bacteria is unknown, but it seems plausible that more proteins are involved, as is the case in the FtsZ-based cell division ring. In the well-studied case of FtsZ-based division rings, it is known that GTP is bound and

hydrolyzed by FtsZ and needed for self-assembly of multiple FtsZ proteins into the cell division ring (Margolin 2005). Kustd1438 also contains a GTP/ATP-binding site and associated synergy loops (also called T7 loops, involved in the activation of GTPase activity) (van Niftrik et al. 2009), which suggests that GTP or ATP is hydrolyzed by this protein as well. It thus seems plausible that GTP or ATP is present near the cell division ring in the paryphoplasm. In addition to the proteins of the cell division ring, other proteins must be present in the paryphoplasm as well. All proteins present in the cytoplasmic membrane (including the F-type ATPase (van Niftrik et al. 2010)) and other components of the cell wall have to be transported to these structures from the riboplasm. Therefore, they have to pass the intracytoplasmic membrane and paryphoplasm compartment using a protein transport mechanism that has not yet been elucidated (see Sect. 4.5.2). In conclusion, besides the cell division ring, the contents of the paryphoplasm compartment remain unknown. A proteomics study of fractionated anammox cells could give an answer to the question of which proteins are present in the paryphoplasm.

4.4.2 Function of the Paryphoplasm

With the description of the compartmentalized *Planctomycetes*, the question immediately arose of whether the paryphoplasm was a cytoplasmic or a periplasmic compartment (Lindsay et al. 1997). The cytoplasm of prokaryotes has been defined as “the fluid portion of a cell, bounded by the cytoplasmic membrane” (Madigan et al. 2012). Therefore, the identity of the paryphoplasm as either periplasmic or cytoplasmic depends on which membrane is defined as the cytoplasmic membrane. As explained above (see Sects. 4.3.3 and 4.4.1), the outermost anammox membrane has been identified as the cytoplasmic membrane based on TEM observations, the detection of RNA inside the paryphoplasm, the apparent absence of cytochrome peroxidase staining in the paryphoplasm, and the detection of an F-type ATPase on the outermost anammox membrane. Based on these arguments, the paryphoplasm in anammox bacteria should be described as a cytoplasmic compartment.

At this moment, the function of the paryphoplasm in anammox bacteria remains unknown. The question whether the anammox paryphoplasm even has a cellular function or whether it has become rudimentary cannot be answered with the current knowledge. Future research into the contents of the paryphoplasm could give clues about the cellular function of this compartment.

4.5 The Riboplasm

Interior to the intracytoplasmic membrane lies the riboplasm of the anammox bacteria (Fig. 4.4). Its granular appearance stems from the ribosomes located in this compartment. The great majority of the RNA inside the anammox cell is present

here as was shown by RNAse-gold labeling (see Sect. 4.4.1). Additionally, the presence of DNA was confirmed with antibodies generated against both single- and double-stranded DNA molecules, which were also coupled to gold particles in immunogold labeling (Lindsay et al. 2001). The riboplasm is therefore thought to correspond to the classical bacterial cytoplasm in which the DNA metabolism and protein synthesis of a cell take place.

4.5.1 Carbon Fixation and Storage

The building blocks for protein synthesis are derived from carbon dioxide via autotrophic carbon fixation. The carbon fixation proceeds via the acetyl-CoA (Wood–Ljungdahl) pathway as is evident from the metagenome of *K. stuttgartiensis* (Strous et al. 2006). The use of this pathway for carbon fixation manifests itself in the isotopic composition of the carbon inside the cell components of the anammox cell: the pathway strongly discriminates against the heavy stable isotope of carbon (^{13}C) leading to a characteristic depletion of this isotope in the biomass of the cell. In *B. anammoxidans* and *S. sorokinii*, the depletion in ^{13}C lies between −48 and −58‰ compared to the standard Vienna Pee-Dee Belemnite (Schouten et al. 2004). The Wood–Ljungdahl pathway is the strongest discriminating pathway against ^{13}C of the known bacterial carbon fixation pathways. During fixation two molecules of carbon dioxide, preferably containing ^{12}C, which is the lighter stable isotope of carbon, are reduced and bound to coenzyme A forming acetyl-CoA. This compound forms the starting point for the formation of all cell constituents via the tricarboxylic acid cycle and glycolysis/glyconeogenesis as intermediate routes (Jetten et al. 2009). The gene coding for ATP citrate lyase is the only gene of these anabolic pathways that has not yet been identified in the metagenome of *K. stuttgartiensis*, but the activity of the two key enzymes carbon monoxide dehydrogenase and formate dehydrogenase of the Wood–Ljungdahl pathway has been demonstrated in broken and homogenized cell material (Strous et al. 2006). The anabolic pathways used in anammox bacteria have therefore been deduced by in silico or indirect methods, but are generally considered as reliable. The Wood–Ljungdahl pathway for the reduction of carbon dioxide depends on electrons with a high reducing power (low redox-potential). These are derived from the oxidation of the highly energy-containing compound hydrazine, which is an intermediate in the catabolism of the anammox bacteria (see Sect. 4.7).

Excess assimilated carbon is stored as glycogen granules (Fig. 4.5e) inside the riboplasm (van Niftrik et al. 2008a). Glycogen granules consist of branched polyglucose and constitute storage of energy and carbon. They are formed when carbon is not limiting, but another nutrient, i.e., ammonium, phosphate, sulfur, or amino acids, is not present in sufficient quantities. The synthesis of glycogen requires energy and is usually activated by intermediates of glycolysis, which probably serve as indicators for high carbon availability. The synthesis starts with the reaction of phosphorylated glucose with ATP to produce ADP-glucose, which is normally

added to an existing glycogen molecule via an α1,4-glycosyl linkage. Under stress conditions that might compromise the existence of a cell, glycogen is then degraded again to supply the cell with carbon and energy needed for survival (Preiss 1984).

4.5.2 Protein Transport

Ribosomes are found only in the riboplasm of the anammox cell. Therefore, newly synthesized proteins have to be transported to the other cell compartments, i.e., the anammoxosome and paryphoplasm. Anammox bacteria possess both the ubiquitous general secretory (Sec) pathway and the twin arginine translocation (TAT) system (Strous et al. 2006). This is evident from the metagenome data of *K. stuttgartiensis*. The prokaryotic secretion systems type I–V are absent. The TAT pathway transports folded or cofactor-containing proteins and is essential for the transport of many enzymes. Most of these are associated with redox reactions, e.g., cofactor-containing oxidoreductases or the Rieske subunit of the bc1 complex (Natale et al. 2008). Accordingly, the TAT system was predicted in silico to be located exclusively on the anammoxosome membrane, the cell compartment associated with the energy metabolism of the cell (see Sect. 4.6) (Medema et al. 2010). The translocation requires no input of energy except for a pmf across the membrane. The anammoxosome membrane is thought to separate a proton gradient between the riboplasm and the inside of the anammoxosome and can therefore deliver the energy necessary for the transport. In contrast to the TAT system, the Sec pathway transports only unfolded proteins or facilitates the insertion of membrane-bound proteins into a membrane. For some proteins, especially those that are to be inserted into a membrane, the translocation occurs concerted with the protein synthesis. The rest of the proteins are prevented from folding and translocated after complete synthesis has taken place. This pathway uses ATP or GTP as an energy source for the translocation (Natale et al. 2008). The Sec pathway is thought to facilitate transport to all cell compartments in anammox bacteria. Both translocation pathways usually recognize their targets via signal peptides. The signal peptides in anammox bacteria are dissimilar from the ones characterized in other species: the amino- and carboxyl-terminal part of the proteins are not thought to play a role in designating the protein to a location inside the cell (Medema et al. 2010). The exact mechanism of protein sorting in anammox bacteria is therefore different from well-studied transport systems in other prokaryotes and not yet understood. The major reason for the difficulty of predicting the locations of proteins are the large number of paralogues in proteins in the available metagenome, which leads to an apparent redundancy in many pathways in the cell, and difficulties in determining the correct start codon for genes in the metagenome. Furthermore, the signals for protein sorting are not always well conserved and in anammox bacteria these appear to differ extensively from known translocation pathways (Jetten et al. 2009). In conclusion, how anammox bacteria are capable of specifically transporting proteins from the riboplasm to

either the paryphoplasm or anammoxosome remains unclear. No specific signal peptides could be detected for different compartments and it is hypothesized that protein sorting might be achieved through both the Sec (both paryphoplasm and anammoxosome) and TAT (anammoxosome) systems with additional chaperones to achieve specificity and facilitate separate translocation routes (Medema et al. 2010).

4.6 The Anammoxosome

Inside the riboplasm lies the anammoxosome (Fig. 4.4). It is an independent cell compartment and as such it is quite unique among prokaryotic life. It takes up on average 61 % of the cell volume in *K. stuttgartiensis* and has a highly curved membrane (van Niftrik et al. 2008b). It harbors the energy metabolism of the anammox cell and probably conserves energy by use of a pmf across the anammoxosome membrane.

4.6.1 Ladderane Lipids and Hopanoids

The anammoxosome membrane consists of a single lipid bilayer, which is enriched in lipids that are unique for anammox bacteria. The lipids are called ladderane lipids, owing their name to the ladderlike arrangement of cyclobutane rings in the hydrocarbon tails of the lipids (Fig. 4.6). Studies with gas chromatography mass spectrometry (GC-MS), high-field nuclear magnetic resonance spectrometry, high-performance liquid chromatography electrospray ionization tandem MS, and matrix-assisted laser desorption/ionization time-of-flight MS showed that the majority of the polar head groups of the lipids are made up of phosphocholine, phosphoethanolamine, and phosphoglycerol linked to a glycerol moiety. They are bound to the hydrocarbon chain by ester or ether bonds with no known preference for ester or ether binding in

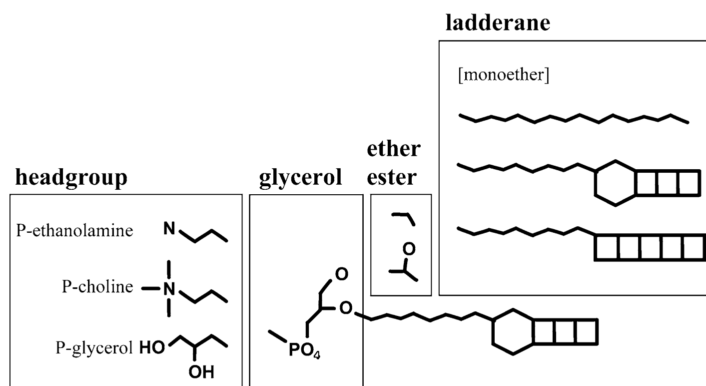


Fig. 4.6 General structure of anammox ladderane lipids. Modified with permission from Jetten et al. (2009)

distinctive ladderane lipids (Boumann et al. 2006; Rattray et al. 2008). Ester bonds are typical of Bacteria and Eukarya. Ether bonds are usually found in the membranes of Archaea, but have also been detected in a few bacterial species (Langworthy et al. 1983; Huber et al. 1996; Pasciak et al. 2003). Examples for mixed glycerol ester and ether bond containing membranes are scarce, but include both thermophilic and mesophilic bacteria (Huber et al. 1992; Rutters et al. 2001). The mixed nature of the bonds is therefore not unique for anammox bacteria, in contrast to the ladderlike structure of the hydrocarbon chains, which contain three or five linearly concatenated cyclobutane rings (Fig. 4.6) (Sinninghe Damsté et al. 2002, 2004b; Boumann et al. 2006). To date, such lipids have not been found in other bacterial genera and appear to be present exclusively in anammox bacteria. How the ladderane lipids are synthesized is unknown, but two pathways are proposed (Rattray et al. 2009). The ladderane lipids might be produced using polyunsaturated fatty acids as precursors. The so-called polycyclization mechanism or SAM radical cascading of polyunsaturated fatty acids could produce ladderane lipids. Another possibility would be that the extended lipid biosynthesis gene clusters detected in the anammox bacterium *K. stuttgartiensis* encode a presently unknown pathway for ladderane biosynthesis that feeds the ladderane moieties into fatty acid biosynthesis.

The possession of unique lipids suggests that these have evolved to meet particular requirements inside of the anammox cell. Although all membranes of anammox bacteria appear to contain ladderane lipids, these are especially abundant in the anammoxosome membrane. Molecular modeling suggests that the lipid structure of ladderanes confers a low degree of rotational freedom to the membrane and limits its permeability. This is also reflected in the inability of fluorescent dyes to pass through the anammoxosome membrane (Sinninghe Damsté et al. 2002). These dyes are apolar compounds and pass readily through conventional biomembranes, but are held back by the densely packed ladderane lipids in the anammoxosome membrane. Mixtures containing purified ladderane phospholipids that constituted both monolayers and bilayers further substantiated that in situ these lipid systems have a high lipid packing density and relatively rigid nature, but also convey a fluidlike behavior (Boumann et al. 2009a). The limitation of passive diffusion is strictly necessary for anammox bacteria due to their very slow growth speed. The rate of passive diffusion of protons over a conventional membrane would probably exceed the rate at which the pmf is generated in an anammox cell. Therefore, such a membrane could not sustain ATP synthesis and, consequently, survival of anammox bacteria. The diffusion of intermediates of the anammox reaction, e.g., nitric oxide and hydrazine, would further exacerbate the loss of energy and thus has to be limited as much as possible. This holds especially for hydrazine, which is also toxic and mutagenic, and its excessive diffusion through the cell and contact with the genetic material could be detrimental to the bacterium (van Niftrik et al. 2004).

Anammox bacteria contain another noteworthy type of lipids, which are probably present in all membranes of the cell: hopanoids. Hopanoids are pentacyclic isoprenoid lipids that serve as membrane rigidifiers in prokaryotic organisms and are analogous to sterols in Eukarya. Hopanoids are widespread among prokaryotes and were long thought to be synthesized only in aerobic organisms and to be absent in strict anaerobes (Rohmer et al. 1984). Therefore these lipids have previously been used as

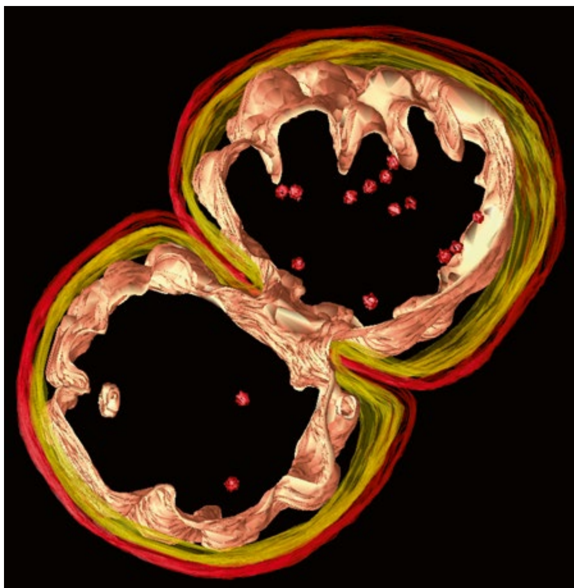


Fig. 4.7 Snapshot of *K. stuttgartiensis* electron tomography model showing the three anammox cell compartments, the curved anammoxosome membrane, and iron particles. From out- to inside: cytoplasmic membrane, intracytoplasmic membrane, anammoxosome membrane, and iron particles

biomarkers for aerobic bacteria and oxic environments. However, all anammox genera that have been analyzed by GC and GC-MS have been shown to contain hopanoids (Sinninghe Damsté et al. 2004a; Rattray et al. 2008). They were the first anaerobic prokaryotes for which the presence of these lipids was demonstrated. Genomic data of *K. stuttgartiensis* corroborated the finding by indicating the presence of a gene coding for squalene hopene cyclase, an important enzyme in hopanoid biosynthesis, in this bacterium (Strous et al. 2006). The hopanoids that have been detected in all anammox genera investigated so far are bacteriohopaneterol and a C_{27} hopanoid ketone. Some anammox species also contain diplotene and diplopterol. But in contrast to the ladderane lipids, these specific hopanoids are not confined to anammox bacteria and can also be found in other prokaryotes (Sinninghe Damsté et al. 2004a; Rattray et al. 2008). In the case of anammox bacteria, the hopanoids are suggested to play a role in maintaining the optimal equilibrium between membrane fluidity and rigidity (caused by the ladderane lipids) in anammox cells (Boumann et al. 2009b).

4.6.2 Putative Cytoskeleton and Storage Elements

The anammoxosome lies as an independent entity inside of the riboplasm. Its highly curved membrane has no links with other membranes of the cell. This has been shown with electron tomography, where a 3D image of a cell is generated by digitally combining TEM pictures of a semi-thick section (400 nm) of a cell taken at different angles (van Niftrik et al. 2008a, b) (Fig. 4.7). The folding of the membrane is thought to be connected to the central role the anammoxosome plays in the energy

metabolism of the cell: the curvature could enhance the metabolic rates of the bacteria by enlarging the surface area of the anammoxosome membrane, which probably harbors key metabolic enzymes (van Niftrik et al. 2004).

During cell division, the anammoxosome is equally divided among the two daughter cells (see Sect. 4.4.1). The mechanism for the division of the anammoxosome is presently unknown. It has been hypothesized to be connected to tubular structures inside the anammoxosome (Fuerst 2005). These are readily discernable in electron micrographs of ultrathin sections (Fig. 4.5e), but their composition, as well as function, has not yet been elucidated. The DNA of anammox bacteria is always associated with the outside of the anammoxosome. The attachment might be connected to the putative cytoskeleton inside of the anammoxosome and fulfill a function in the concerted division of the cell compartment and the hereditary material. If the tubule-like structures constitute cytoskeletal elements, they might function in maintaining the curvature of the anammoxosome membrane by an active process (Fuerst 2005; van Niftrik et al. 2008b). However, the putative tubules inside of the compartment might also consist of non-cytoskeletal proteins. The anammoxosome is relatively electron dense in electron micrographs owing to the high concentration of proteins on its inside. The tubular structures might therefore be highly abundant proteins in the anammoxosome, i.e., key enzymes of the anammox reaction, that have aggregated to form a structure that superficially resembles a cytoskeleton.

The mechanism by which the anammoxosome membrane is folded has not yet been investigated. Besides the already mentioned possibility of an active folding by a cytoskeleton, the membrane might also be actively or passively folded by other mechanisms: integral membrane proteins or special lipids can influence the curvature of a membrane and lead to its bending. Alternatively, the riboplasm surrounding the anammoxosome might have a higher osmotic value than the anammoxosome, leading to water flowing out of the cell compartment and thereby promoting the folding of the membrane (van Niftrik et al. 2008b).

Next to the tubule-like structures, the anammoxosome contains very electron-dense particles 16–25 nm in diameter (Fig. 4.5c, e). These have been shown by energy-dispersive X-ray analysis to be enriched in iron and possibly also in phosphorous and are thought to function as iron-storage particles (van Niftrik et al. 2008b). They could serve as an iron-reservoir for the multitude of heme-containing proteins that participate in the anammox reaction and are located inside of the anammoxosome, or perhaps constitute a pool of alternative electron acceptor for anammox bacteria, which are capable of iron respiration (Strous et al. 2006) (see Sect. 4.7).

4.6.3 Energy Conservation

The anammoxosome harbors the energy metabolism of the anammox cell and is therefore analogous in function to mitochondria in eukaryotic organisms. It is the first and only energy-conserving cell compartment in prokaryotes that has been discovered to date. Evidence for the role of the anammoxosome in the catabolism of

the cell initially came from immunogold labeling experiments in TEM on anammox cells. Antibodies against a proposed key enzyme of the anammox reaction, which is homologous to the hydroxylamine oxidoreductase (HAO) from aerobic AOB, were raised using purified proteins from *B. anammoxidans*. These were used on ultrathin sections of anammox cells and detected by a secondary antibody coupled to gold particles. The labeling was extensive and was observed only inside the anammoxosome (Fig. 4.5f), meaning that the HAO-like protein is only present inside of the compartment (Lindsay et al. 2001). For ATP to be generated from a pmf over a membrane ATPases are necessary. The anammoxosome has in fact been shown to contain ATPases inside its membrane (Fig. 4.5a). This was shown by immunogold labeling with an antibody against a heterologously expressed β-subunit of an F-ATPase, which is one of the four ATPase paralogues in the metagenome of *K. stuttgartiensis* (van Niftrik et al. 2010). Furthermore, electrons derived from oxidation reactions have to be shuttled towards the reductive pathways. In anammox bacteria cytochrome *c*-type proteins act as electron carriers to transport electrons from one redox reaction to the next. Using cytochrome peroxidase staining, those electron carriers were shown to be present exclusively in the anammoxosome (Fig. 4.5c) (van Niftrik et al. 2008a). This result not only reinforced that the anammoxosome is the location of the energy metabolism, but also that there is no link between the anammoxosome and any other cell compartment, since no other part of the anammox cell was stained for cytochrome peroxidase.

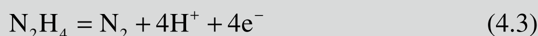
4.7 The Energy Metabolism of the Cell

Anammox bacteria derive the energy for growth from the anaerobic oxidation of ammonium inside a separate compartment, the anammoxosome. They use nitrite as electron acceptor and produce dinitrogen gas as an end product (Box 4.1; equation 4.4) ($\Delta G^{0\prime} = -357$ kJ mol^{-1}). The conversion of the substrates can be split into three separate reactions (Box 4.1): the one electron reduction of nitrite to nitric oxide

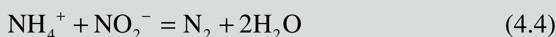
Box 4.1 Anaerobic ammonium oxidation reactions

$$NO_2^- + 2H^+ + e^- = NO + H_2O \quad (4.1)$$

$$NO + NH_4^+ + 2H^+ + 3e^- = N_2H_4 + H_2O \quad (4.2)$$



$$N_2H_4 = N_2 + 4H^+ + 4e^- \quad (4.3)$$



$$NH_4^+ + NO_2^- = N_2 + 2H_2O \quad (4.4)$$

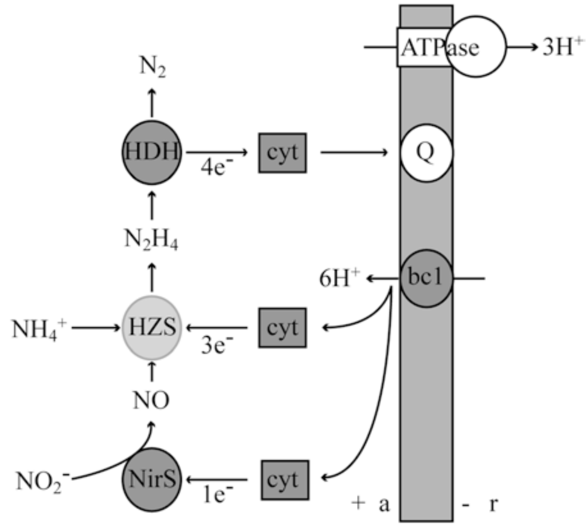


Fig. 4.8 Model for anammox energy metabolism. The process of anaerobic ammonium oxidation is coupled to the anammoxosome membrane resulting in a proton motive force and subsequent ATP synthesis in anammox bacteria. Heme-containing enzymes are shown in *grey*. *NirS* nitrite reductase, *HZS* hydrazine synthase, *HDH* hydrazine dehydrogenase, *cyt* cytochrome, *bc1* cytochrome bc1 complex, *Q* co-enzyme Q, *a* anammoxosome, *r* riboplasm

equation (4.1), the condensation of nitric oxide and ammonium to hydrazine requiring the input of three electrons equation (4.2), and the oxidation of hydrazine to dinitrogen gas, which yields four electrons equation (4.3) (Kartal et al. 2011b). The conversions proceed at a very slow rate (15–80 μmol N_2 per gram of dry weight per min), but with a very high affinity for the substrates nitrite and ammonium ($K_S < 5 \mu M$) (Jetten et al. 2009).

4.7.1 *Enzymatic Components of the Anammox Reaction*

The anammox reactions are carried out by several heme-containing proteins (Fig. 4.8). Equation 4.1 (Box 4.1) had long been hypothesized to generate hydroxylamine as an intermediate in the anammox reaction, but the sequence analysis of the metagenome of *K. stuttgartiensis* showed that the bacterium possesses a cd_1 nitrite::nitric oxide reductase (NirS; kuste4136). It is now recognized that nitric oxide is the intermediate in the anammox reaction (Kartal et al. 2011b): anammox activity is inhibited through addition of the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) and nitric oxide accumulates after the inhibition of the ammonium-activating step (Box 4.1; equation 4.2). Furthermore, anammox cells fluoresce when incubated with DAF2-DA, which reacts with NO to form a fluorescent compound (Kartal et al. 2011b). The reduction of nitric oxide and its simultaneous condensation with ammonium are done by hydrazine synthase (HZS; gene cluster kuste2859-61). This enzyme, which bears no homology to any other characterized enzyme, produces the intermediate hydrazine (N_2H_4) and is therefore capable of forming an N–N bond, a feature that is otherwise only known for the NO reductase of denitrifiers.

Nitric oxide is not only an intermediate but also a strong inhibitor of the anammox reaction. The same is true for hydroxylamine. These compounds inhibit the enzyme responsible for the last step in the anammox process (Kartal et al. 2011b): the oxidation of hydrazine by hydrazine dehydrogenase (HDH; kustc0694) to dinitrogen gas (Box 4.1; equation 4.3). This enzyme is homologous to the HAO enzyme from AOBs. However, there are ten different HAO-like paralogues encoded in the metagenome of *K. stuttgartiensis*. One of them (kustc1061) is known to function as a detoxification system in the cell, converting hydroxylamine to nitric oxide (Kartal et al. 2011b). By removing hydroxylamine, which is a compound inhibiting HDH, it simultaneously produces the useful substrate nitric oxide, which can be used by HZS. The function of the residual paralogues remains to be investigated.

Hydrazine is a very powerful reductant ($E_0'=-0.75$ V) and its oxidation releases four electrons. These are proposed to be transferred to a membrane-bound cytochrome bc1 complex (complex III). Via cytochrome *c*-type proteins as intermediate electron carriers, the electrons are shuttled to the reduction of nitrite and the synthesis of hydrazine. The electron transport could facilitate the translocation of protons by the bc1 complex across the anammoxosome membrane, which would acidify the inside of the anammoxosome. This would result in a proton gradient building up between the inside of the anammoxosome and the riboplasm. The energy stored in this gradient could be used by ATPases located in the anammoxosome membrane to combine ADP and phosphate to ATP in the riboplasm.

The substrates for the energy metabolism have to be transported through the anammox cell. This probably proceeds via transporters for ammonium (Amt), nitrite (FocA), and nitrate (NarK). Of these transport proteins anammox bacteria possess several paralogues. This is to be expected considering the ultrastructure of the cell that makes transport over three membranes necessary in order to reach the inside of the anammoxosome, where the energy metabolism is localized. The paralogues of the transport proteins most likely differ in their localization inside the different membranes of the anammox cell. But since to date the protein sorting machinery has not been elucidated (see Sect. 4.5.2), the exact mechanism of substrate transport through the anammox cell remains unknown.

4.7.2 Electron Redistribution Towards Carbon Fixation

Anammox activity is always accompanied by the production of small amounts of nitrate (Strous et al. 1999b). These are formed through the oxidation of nitrite by a nitrate::nitrite oxidoreductase (NarGH; kustd1700&03). The reason for this is connected to the carbon fixation pathway of anammox bacteria, which has been discussed earlier (see Sect. 4.5). The Wood–Ljungdahl pathway requires low-redox-potential (high reducing power) electrons derived from the oxidation of hydrazine. Some of the electrons derived from HDH are proposed to be shuttled via quinone or NADH towards the carbon dioxide reduction for carbon fixation. These electrons

are therefore not available for the reduction reactions (Box 4.1; equations 4.1 and 4.2) of the anammox process. The oxidation of nitrite closes the resulting gap in electrons in the energy metabolism. This oxidation, however, generates one high-redox-potential electron ($E_0{}'=+0.43$ V) per molecule of nitrite. The reductions of nitrite and nitric oxide require electrons of a higher reducing power. Therefore, in order to be able to compensate for the electrons invested in carbon fixation, these high-redox-potential electrons are probably pumped to a lower redox-potential through the investment of energy in reverse electron transport.

4.7.3 Anammox Bacteria as Generalists

Anammox bacteria were long regarded as specialists only capable of the conversion of ammonium with nitrite. There is increasing evidence that they are in fact generalists and can convert some organic as well as other inorganic compounds. Genomic data shows that transporters for organic acids and amino acids are abundant in the metagenome. Furthermore, a multitude of more than 200 genes were identified to be dedicated to respiration in the metagenome of *K. stuttgartiensis*. This is much more than commonly found in a bacterial species. The multiple paralogues of respiratory complexes are likely to be arranged in a branched respiratory chain, e.g., the 61 different cytochrome *c*-type proteins that have been identified are presumably helping to integrate the multitude of respiratory pathways. Thereby different energy sources and different electron acceptors can be exploited (Strous et al. 2006). *Scalindua* sp. and *K. stuttgartiensis* can use iron and manganese oxides as electron acceptors using formate as an electron donor. In fact, next to formate also acetate, propionate, and methylamines are oxidized by representatives of many anammox genera. Finally, at least *K. stuttgartiensis* respires nitrate using iron as electron donor (Strous et al. 2006; Kartal et al. 2007b, 2008; van de Vossenberg et al. 2008). But the anammox reaction, too, is not strictly dependent on the presence of ammonium in the environment. The bacteria are capable of oxidizing organic acids using nitrate as electron acceptor, which is via nitrite in a six-electron reduction reduced to ammonium, a process known as DNRA. The resulting ammonium can then be used in the anammox reaction together with nitrite to form dinitrogen gas. This gives anammox bacteria an advantage in ammonium-limited ecosystems. The combination of DNRA with anammox means that the activity of anammox bacteria in the environment can easily be overlooked and mistaken for denitrification (Kartal et al. 2007a), because both anammox bacteria and denitrifiers can live on organic acids and nitrate (see Sect. 4.1.6). The enzyme responsible for DNRA is a pentaheme protein (NrfA) identified in other bacterial species that can also perform DNRA. A homolog of NrfA is absent from the metagenome of *K. stuttgartiensis*. Anammox bacteria therefore must use a different, novel enzyme or pathway for the reduction of nitrite to ammonium. The generalization of the metabolism in anammox bacteria not only serves as a competitive advantage relative to other species such as heterotrophic denitrifiers but possibly also plays an important role in niche

differentiation among the different anammox species. These differ in their affinity for organic acids as alternative electron donor: *A. propionicus* has an advantage over other anammox species in the presence of propionate, whereas acetate promotes the growth of *B. fulgida* (Kartal et al. 2007b, 2008). *K. stuttgartiensis* thrives when only inorganic sources of nitrogen are present (Kartal et al. 2011a). For other species of anammox bacteria it is yet unknown which compound or condition causes them to become dominant in a particular system and remains the subject of future research.

4.8 Conclusions and Outlook

In the past 15 years anammox research has progressed at an enormous pace. The anammox process was discovered and the bacteria responsible for it identified. The initial focus was on the application, ecological significance, and genomics of the anammox process and bacteria. Since anammox bacteria kept on surprising us with their unusual properties, anammox research further advanced into the directions of molecular biology, biochemistry, physiology, and cell biology. Although big steps have recently been taken with regard to anammox physiology and the proposed model for anaerobic ammonium oxidation, many questions still remain concerning their unusual metabolism and cell biology. Some questions for future research have been summarized in Box 4.2. The first steps in future research will concern the function of the anammoxosome. The exact metabolic model needs to be further unraveled, and the generation of a pmf over the anammoxosome membrane and subsequent ATP synthesis need to be validated. In addition, future research will also concern the nature and function of the paryphoplasm and the cell wall. In conclusion, over the past decade anammox bacteria have proven to be of a high microbiological interest. Also, the application in wastewater treatment and the ecological significance of anammox bacteria make these unique prokaryotes an important object of future study.

Box 4.2 Questions for Future Research

The Cell Envelope

1. What is the composition of the cell wall of anammox bacteria?
2. Do anammox bacteria produce a form of pseudo-peptidoglycan?
3. What is the location and function of the expressed proteins that are normally associated with the outer membrane of Gram-negative bacteria?

(continued)

Box 4.2 (continued)

The Paryphoplasm

1. What are the contents of the paryphoplasm in anammox bacteria?
2. What is the function of the paryphoplasm in anammox bacteria?

The Riboplasm

1. How are newly synthesized proteins assigned to their correct destination in the compartmentalized cell?
2. By what mechanism is the DNA associated with the anammoxosome?

The Anammoxosome

1. How is the folding of the anammoxosome membrane organized?
2. How does the anammoxosome divide?
3. Are all the key metabolic enzymes of the anammox process located inside of the anammoxosome?

The Energy Metabolism of the Cell

1. How is the electron transport chain in the anammoxosome membrane organized?
2. Can a pmf across the anammoxosome membrane be demonstrated?
3. What is the function of the multitude of HAO-like enzymes encoded in anammox bacteria?
4. How is the transport of substrates for the energy metabolism organized in the compartmentalized anammox cell?
5. How is the use of alternative substrates, e.g., organic acids, integrated into the anammox metabolism?

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Chapter 5
Acidophilic Planctomycetes: Expanding the Horizons of New Planctomycete Diversity

Svetlana N. Dedysh and Irina S. Kulichevskaya

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Members of the bacterial phylum *Planctomycetes* are ubiquitous in a wide range of aquatic and terrestrial environments with diverse conditions (Fuerst 1995). Despite the reported widespread distribution, the known ecophysiological types of planctomycetes are quite limited. Most currently described planctomycetes are both mesophilic and neutrophilic. *Isosphaera pallida* is the only moderately thermophilic planctomycete so far described (Giovannoni et al. 1987). Psychrophilic and alkaliphilic representatives of the *Planctomycetes* have not yet been isolated. Acidophilic members of this phylum also remained unknown for a long time. The first report on the isolation of a planctomycete-like strain from an acidic environment, i.e., peat bog water (pH 4.2) of the Kaltenhofer Moor near Kiel, Germany, was published by Heinz Schlesner in 1994 (Schlesner 1994). This isolate, however, has not been described in detail. More recent research on acidic peatlands identified these ecosystems as a

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unique source of uncharacterized planctomycete diversity and resulted in isolation of several peat-inhabiting, acidophilic planctomycetes in pure cultures. This chapter gives an overview of planctomycete diversity in acidic wetlands, reports phenotypic traits of the newly described acidophilic planctomycetes, and discusses their functional role in natural environments.

5.1 Occurrence of *Planctomycetes* in Acidic Environments

Planctomycetes can be found in environments with pH values ranging from below 3 (Bohorquez et al. 2012) to above 11 (Schlesner 1994). The acidic range of this spectrum is represented mostly by terrestrial environments, such as acidic soils, wetlands, ore deposits, and mining areas. Cultivation-independent molecular recovery of 16S rRNA genes from various acidic (pH 4.2–5.5) soils has repeatedly demonstrated the presence of planctomycetes (Liesack and Stackebrandt 1992; Borneman and Triplett 1997; Jangid et al. 2008; Tsai et al. 2009). These bacteria have been detected among acidophilic microorganisms colonizing waste ore deposits at an acid (pH 3.0) mine drainage site in China (Hao et al. 2007). Analysis of the microbial community composition in an acidic (pH 2.7) hot (29 °C) spring of the Colombian Andes revealed a high relative abundance of 16S rRNA gene sequences from the *Planctomycetes* (Bohorquez et al. 2012). Members of this phylum were identified as one of the dominant bacterial groups in the suspended acidic (pH 4.0–5.1) water within bromeliad tanks in the tropical rainforest canopy (Goffredi et al. 2011). The most extensive acidic habitat colonized by planctomycetes, however, is *Sphagnum*-dominated northern wetlands (Dedysh et al. 2006; Kulichevskaya et al. 2006; Ivanova and Dedysh 2012). The ecology and biology of planctomycetes inhabiting these wetlands are discussed below.

5.2 Acidic Northern Wetlands as a Habitat for Planctomycetes

Wetlands are ecosystems in which the water table is permanently or periodically close to the soil surface. More than half of the global wetland area is located between 50°N and 70°N and is therefore referred to as northern wetland. *Sphagnum*-dominated peatlands represent one of the most extensive types of northern wetlands. *Sphagnum* moss is characteristic of peat bogs and poor fens. These ecosystems typically have pH values between 3.5 and 5.5 and are nutrient poor by nature. The total concentration of mineral nutrients is usually in the range of 5–50 mg L^{-1}. Therefore, transformations of mineral N, S, and Fe are of minor importance in these ecosystems, while degradation of plant litter is the basis of the microbial food chain (Dedysh 2011).

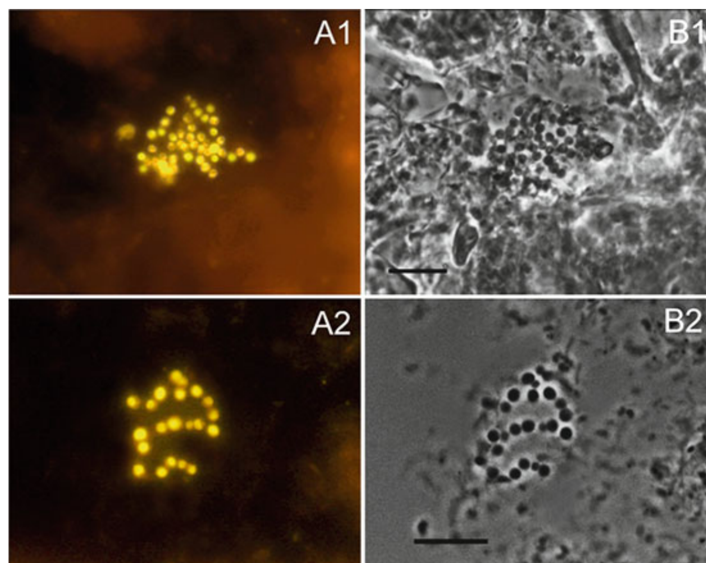

Fig. 5.1 Specific detection of planctomycete cells in acidic *Sphagnum* peat by FISH. Epifluorescent micrographs of in situ hybridizations with Cy3-labelled probes PLA46 and PLA886 (**a**) and the phase-contrast images (**b**) are shown. Bar, 10 **μ**m

As shown in several cultivation-independent diversity studies, nucleotide sequences of planctomycetes commonly comprise 2–5 % of all 16S rRNA gene sequences in the clone libraries made from *Sphagnum* mosses or *Sphagnum*-derived peat (Juottonen et al. 2005; Dedysh et al. 2006; Morales et al. 2006; Ausec et al. 2009). These data, however, have low quantitative value since planctomycetes are strongly underrepresented in clone libraries obtained with the widely used *Bacteria*-specific PCR primer 9-27f (Lane 1991). As shown by Vergin and colleagues (Vergin et al. 1998), this primer contains a mismatch with the corresponding sequence region in full-length 16S rRNA genes in planctomycete genome fragments deposited in fosmid clone libraries. The use of alternative primers may significantly increase the proportion of planctomycete-related sequences. For example, a clone library made from *Sphagnum fallax* with the use of 799f/1492r primers contained 22.5 % of sequences affiliated with the *Planctomycetes* (Bragina et al. 2012).

A combination of two oligonucleotide FISH probes PLA46 and PLA886 with the target specificity for the phylum *Planctomycetes* and the order *Planctomycetales*, respectively (Neef et al. 1998), was applied to determine the in situ abundance of these bacteria in peat sampled from nine *Sphagnum*-dominated wetlands of different geographic locations within European North Russia and West Siberia (Ivanova and Dedysh 2012). The probes hybridized to numerous spherical- or ellipsoid-shaped cells that were arranged in chains or in shapeless cell aggregates and were mostly attached to the particles of nondecomposed organic material (Fig. 5.1). Highest abundances of cells targeted with the probes PLA46 and PLA886 were

observed in the uppermost, oxic layers of the wetland profiles. The cell numbers of planctomycetes in these layers were in the range 1.1–6.7 × 10^7 cells per gram of wet peat, comprising 2–14 % of all bacteria. The abundance of planctomycetes in wetlands was positively correlated with pH value of peat water. Highest cell numbers of these bacteria were detected in mildly acidic (pH 5.3–6.0) fens, whereas low planctomycete abundance was more typical for more acidic (pH 3.7–4.5) ombrotrophic bogs, which are nutrient-deficient wetlands that receive water inputs primarily from precipitation. A sharp decline of planctomycete abundance with depth was observed in most peatland sites, suggesting the numerical predominance of aerobic moderately acidophilic planctomycetes.

5.3 Isolation Approaches

A well-established technique for the successful isolation of planctomycetes recommends the use of dilute media with antibiotics affecting the biosynthesis of peptidoglycan in growing cells (Staley 1973; Schlesner 1994; Staley et al. 1992). Dilute media are preferred since many planctomycetes do not grow on nutrient-rich media. In addition, the latter promote development of fast-growing bacteria, which easily overgrow planctomycetes. Therefore, one of the recommended enrichment procedures for samples from aquatic habitats is their long incubation without any addition of nutrients. The use of *N*-acetylglucosamine as a sole source of carbon and nitrogen was also proposed as one of the approaches that gives a selective advantage to planctomycetes (Schlesner 1994).

All of the above-mentioned approaches are clearly applicable for the isolation of planctomycetes from acidic *Sphagnum*-dominated wetlands. A more accurate simulation of the peat bog environment in the laboratory, however, requires the use of acidic (pH 4.0–5.8) media with low concentrations of mineral salts (Dedysh 2011). One example of an appropriate medium is medium M31 (Kulichevskaya et al. 2007a), which is a modification of medium 31 described by Staley et al. (1992). This medium has pH 5.8 and contains *N*-acetylglucosamine as the growth substrate. Several peat-inhabiting planctomycetes, which were further assigned to the novel genera *Schlesneria*, *Singulisphaera*, and *Zavarzinella*, were isolated using medium M31. These bacteria displayed a clear preference for growth in mildly acidic conditions but, in general, their phenotypic traits were quite similar to those in all neutrophilic planctomycetes described earlier. Phenotypically more unusual planctomycetes, *Candidatus* Nostocoida acidiphila and *Telmatocola sphagniphila*, were isolated on the mineral medium M1 containing polysaccharide Phytagel as a solidifying agent (Kulichevskaya et al. 2012b, c). The latter apparently served as the growth substrate for the newly isolated planctomycetes. Interestingly, both *Candidatus* Nostocoida acidiphila and *Telmatocola sphagniphila* were unable to develop on common “planctomycete-specific” media containing relatively high (0.05–0.2 %) concentrations of N-acetylglucosamine or sugars and displayed several other unusual features (see Sect. 8.4). These examples nicely demonstrate the ultimate need of the novel cultivation approaches for discovery of the novel planctomycete phenotypes.

The use of Phytagel (Gellan Gum, Gelrite) instead of agar has one additional advantage: this polysaccharide is free of contaminants which may inhibit growth of some bacteria. Several acidophilic planctomycetes, such as *Telmatocola sphagniphila*, *Candidatus* Nostocoida acidiphila, and some as-yet-undescribed isolates in our collection, were unable to develop on agar media.

Extended incubation time is also one of the important prerequisites for the successful cultivation of peat-inhabiting planctomycetes. Colonies of these fastidious bacteria appear on solid media only after 4–6 weeks or several months of incubation (Kulichevskaya et al. 2009, 2012c).

Finally, one of the most efficient screening tools to monitor the enrichment/isolation procedure and to recognize the presence of planctomycetes is the use of fluorescence in situ hybridization (FISH) with a combination of two *Planctomycete*-specific oligonucleotide probes PLA46 and PLA886 (Neef et al. 1998). FISH-based screening allows direct visualization of the target bacteria within the entire population of cells present in the sample (Fig. 5.1). This screening tool was successfully applied in our work for examination of microbial biofilms consisting of cells of peat-inhabiting bacteria that were obtained by using a Petri dish technique described by Schlesner (1994). Further work on isolation of planctomycetes from these microbial biofilms was also monitored by means of whole-cell hybridization with these probes, which greatly simplified all related identification/purification procedures (Dedysh et al. 2006; Kulichevskaya et al. 2006).

5.4 Cultivated Representatives of Acidophilic Planctomycetes

The use of the above-listed isolation approaches resulted in isolation of several peat-inhabiting, acidophilic planctomycetes in pure cultures. These isolates were further described as representing the novel genera *Schlesneria*, *Singulisphaera*, *Zavarzinella*, and *Telmatocola* (Kulichevskaya et al. 2007a, 2008, 2009, 2012a, c) (Table 5.1). As shown in Fig. 5.2, these newly described planctomycetes do not form a phylogenetically coherent cluster but belong to different subgroups within the family *Planctomycetaceae*. *Schlesneria paludicola* belongs to the phylogenetic lineage defined by the genus *Planctomyces*, *Singulisphaera acidiphila* and *S. rosea* are members of the *Isosphaera* group, while *Zavarzinella formosa* and *Telmatocola sphagniphila* affiliate with the lineage defined by the genus *Gemmata*. These planctomycetes possess different cell morphologies (Fig. 5.3) and differ with regard to some phenotypic traits but are quite uniform with respect to their pH preferences. Members of these genera are moderately acidophilic bacteria growing at pH values between 3.5–4.0 and 7.0–7.5, with an optimum at pH 5–6. In comparison to all previously described planctomycetes, representatives of the genera *Schlesneria*, *Singulisphaera*, *Zavarzinella*, and *Telmatocola* are clearly more acid tolerant (Fig. 5.4a). Some other characteristics of the newly described planctomycetes are given below.

Table 5.1 List of currently characterized acidophilic planctomycetes

Species name	Type strain	GenBank Accession No. for 16S rRNA gene	Relation to oxygen	pH growth range (optimum)	Carbon sources utilized
Schlesneria paludicola	MPL7^T (=ATCC BAA-1393^T = VKM B-2452^T)	AM162407	Facultative aerobe	4.2–7.5 (5.0–6.2)	Some sugars and heteropolysaccharides, *N*-acetylglucosamine, salicin
Singulisphaera acidiphila	MOB10^T (=ATCC BAA-1392^T = VKM B-2454^T)	AM850678	Obligate aerobe	4.2–7.5 (5.0–6.2)	Sugars and heteropolysaccharides, *N*-acetylglucosamine, salicin
Singulisphaera rosea	S26^T (=DSM 23044^T = VKM B-2599^T)	FN391026	Obligate aerobe	3.2–7.1 (4.8–5.0)	Most sugars, several organic acids and polyalcohols, some heteropolysaccharides *N*-acetylglucosamine
Zavarzinella formosa	A10^T (=DSM 19928^T = VKM B-2478^T)	AM162406	Obligate aerobe	3.8–7.2 (5.5–6.0)	Sugars and heteropolysaccharides, *N*-acetylglucosamine, salicin, pyruvate
Telmatocola sphagniphila	SP2^T (=DSM 23888^T = VKM B-2710^T)	JN880417	Obligate aerobe	4.0–7.0 (5.0–5.5)	Sugars and heteropolysaccharides, carboxymethyl cellulose, crystalline cellulose
Candidatus Nostocoida acidiphila	OB1	JQ067914	Obligate aerobe	3.2–5.5 (3.6–4.0)	Some heteropolysaccharides

5.4.1 Genus Schlesneria

Schlesneria paludicola is the only currently described species of this genus. It was described based upon a characterization of three strains, MPL7^{T}, MOB77, and SB2, which were isolated from the *Sphagnum* peat bog Bakchar (pH 4.0), West Siberia, and the peat bog Obukhovskoe (pH 4.2), Yaroslavl region, European North Russia (Kulichevskaya et al. 2007a). Members of this species are represented by budding, ellipsoid-shaped cells that occur singly, in pairs, or are arranged in rosettes (Fig. 5.3a). The cell surface is covered by crateriform pits and numerous fibrillar appendages; stalklike structures are short and rarely observed. These planctomycetes are facultatively aerobic chemoheterotrophs. They grow best in aerobic conditions on media with carbohydrates or *N*-acetylglucosamine. However, they are also capable of fermenting carbohydrates, which might be of special importance for bacteria that inhabit wetlands. *Schlesneria paludicola* possesses weak hydrolytic capabilities and can degrade fucoidan, laminarin, aesculin, pectin, chondroitin sulfate, pullulan, gelatin, and xylan. Growth occurs at pH values between 4.2 and 7.5 (optimum at 5.0–6.2) and at temperatures between 4 °C and 32 °C (optimum at 15–26°C). The major fatty acids are C16:0 and C16:1ω7c. The closest taxonomically described phylogenetic relative of *Schlesneria paludicola* is *Planctomyces limnophilus* (87 % sequence similarity). Several 16S rRNA gene cloned sequences that were retrieved from acidic peat and acidic soil display 98–99 % similarity to the corresponding gene sequence from *Schlesneria paludicola* (Fig. 5.2), suggesting that these planctomycetes are typical for acidic environments.

5.4.2 Genus Singulisphaera

At present, this genus includes two species, *S. acidiphila* and *S. rosea* (Kulichevskaya et al. 2008, 2012a). Both species are represented by spherical, nonmotile cells that occur singly, in pairs, or shapeless aggregates (Fig. 5.3b). These cells attach to surfaces by means of amorphous holdfast material; stalklike structures are absent. Members of this genus are chemoheterotrophic aerobes. They are not capable of fermenting carbohydrates but they grow very well in microaerobic conditions. The preferred growth substrates of these planctomycetes are sugars and *N*-acetylglucosamine. *S. rosea* utilizes also some organic acids including citrate, fumarate, lactate, malate, pyruvate, and succinate. Members of both species hydrolyze various heteropolysaccharides such as laminarin, aesculin, or pullulan. In addition, *S. acidiphila* degrades pectin, lichenan, and xylan, while *S. rosea* grows on starch. None of them utilize cellulose or chitin. The major fatty acids are C16:0, C18:1ω9*c*, and C18:2ω6*c*,12*c*; the latter is genus characteristic. *S. rosea* is more acidophilic (growth at pH 3.2–7.1, optimum at 4.8–5.0) (Fig. 5.4b) than *S. acidiphila* (growth at pH 4.2–7.5, optimum at 5.0–6.2). Members of the genus *Singulisphaera* are only distantly related to the thermophilic filamentous neutrophile from hot springs *Isosphaera pallida* (Giovannoni et al. 1987) and to the

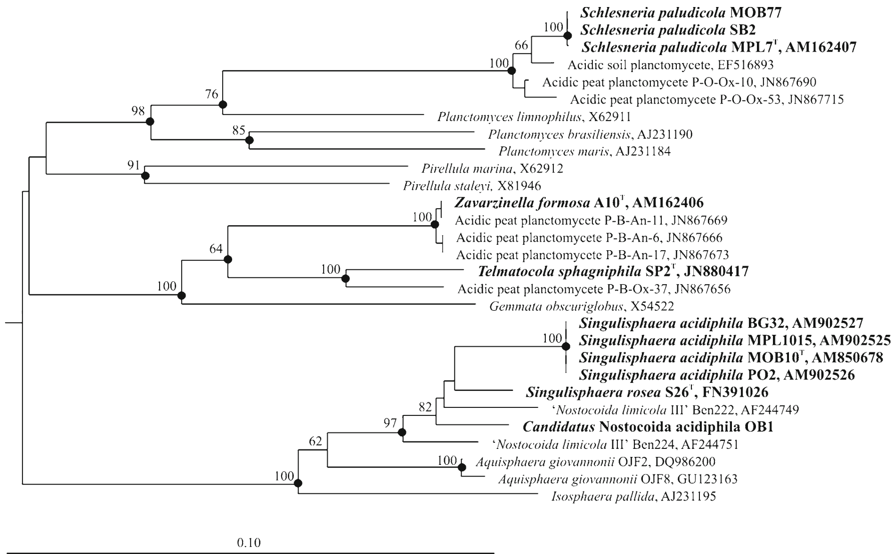

Fig. 5.2 16S rRNA gene-based neighbor-joining tree showing the phylogenetic position of acidophilic planctomycetes (in *bold*) in relation to other taxonomically characterized members of the family *Planctomycetaceae*. Bootstrap values (1,000 data resamplings) of >60 % are shown. *Black circles* indicate that the corresponding nodes were also recovered in the maximum-likelihood and maximum-parsimony trees. The root (not shown) was composed of five 16S rRNA gene sequences from anammox planctomycetes (AF375994, AF375995, AY254883, AY257181, AY254882). The scale bar represents 0.1 substitutions per nucleotide position

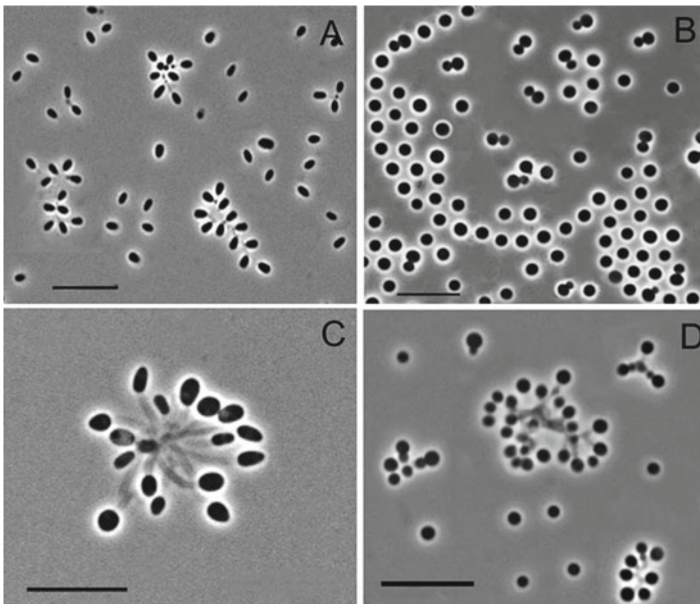

Fig. 5.3 Cell morphology of acidophilic planctomycetes: *Schlesneria paludicola* (**a**), *Singulisphaera acidiphila* (**b**), *Zavarzinella formosa* (**c**), *Telmatocola sphagniphila* (**d**). Bar, 10 μm

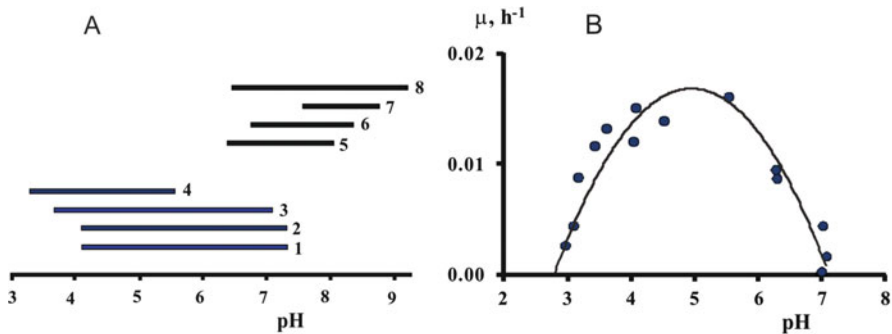

Fig. 5.4 (**a**) pH growth ranges of neutrophilic (*black*) and acidophilic (*blue*) planctomycetes: 1—*Schlesneria paludicola*, 2—*Singulisphaera acidiphila*, 3—*Zavarzinella formosa*, 4—*Candidatus* Nostocoida acidiphila, 5—*Planctomyces limnophilus*, 6—*Isosphaera pallida*, 7—*Gemmata obscuriglobus*, 8—*Aquisphaera giovannonii*. (**b**) Influence of medium pH on the growth of *Singulisphaera rosea* S26^T

mesophilic nonfilamentous neutrophile from the sediments of a freshwater aquarium *Aquisphaera giovannonii* (Bondoso et al. 2011) (89–90% and 92–93 % 16S rRNA gene sequence similarity) (Fig. 5.2). More close relationship (94.5–95.5 % sequence similarity) is observed with a group of filamentous planctomycete strains from activated sludge, "*Nostocoida limicola*" III (Liu et al. 2001), which also belongs to a wide phylogenetic clade defined by the genus *Isosphaera*.

5.4.3 Genus Zavarzinella

Zavarzinella formosa was described based upon a characterization of a single unique isolate, strain A10^T, obtained from the Siberian peat bog Bakchar (Kulichevskaya et al. 2009). Ellipsoid-shaped cells of this isolate are uniformly covered with crateriform pits and possess long (up to 10–15 μm) and unusually thick stalks, which assemble cells in large rosette-like clusters (Fig. 5.3c). Adult cells are immobile, while daughter cells are motile by means of one-two flagella. This planctomycete is an obligately aerobic chemoheterotroph. In contrast to *Schlesneria* and *Singulisphaera*, it is not capable of growth in microaerobic conditions. The growth substrates are sugars, N-acetylglucosamine, and pyruvate, but organic acids other than pyruvate are not utilized. The protein gelatin and the heteropolysaccharides laminarin, pectin, chondroitin sulfate, aesculin, starch, lichenan, and xylan are hydrolyzed, but chitin or cellulose are not. The major fatty acids are C18:0, C18:1ω5c, and C16:1ω5c. Members of this species grow at pH values between 3.8 and 7.2 (optimum at 5.5–6.0) and at temperatures between 10 and 30 °C (optimum at 20–25 °C). The closest described relatives are the neutrophile *Gemmata obscuriglobus* (90 % 16S rRNA gene sequence similarity) and the acidophile *Telmatocola sphagniphila* (86 % sequence similarity) (Fig. 5.2). A number of 16S rRNA gene clones displaying high similarity (99 %) to *Z. formosa* were retrieved from acidic *Sphagnum*-dominated wetlands (Ivanova and Dedysh 2012) and from an acidic hot spring of the Colombian Andes (Bohorquez et al. 2012).

5.4.4 Genus Telmatocola

Telmatocola sphagniphila is a recently proposed species, which accommodates morphologically and phenotypically unusual planctomycetes. Two characterized members of this species, strains SP2^T and OB3, were isolated from two North European *Sphagnum* peat bogs, Staroselsky moss and Obukhovskoye (Kulichevskaya et al. 2012c). When grown on solid media, spherical cells of these bacteria are arranged in unusual, dendriform-like structures (Fig. 5.3d). *Telmatocola sphagniphila* is an obligately aerobic chemoheterotroph, which is unable to grow in micro-oxic or anoxic conditions. The preferred growth substrates are various heteropolysaccharides and sugars, the latter being utilized only if provided in low concentrations (below 0.025 %). In contrast to other described planctomycetes, *Telmatocola sphagniphila* possesses cellulolytic potential and is capable of slow growth on carboxymethyl cellulose, microcrystalline cellulose, and fibrous cellulose prepared from Whatman filter paper. Growth occurs at pH 4.0–7.0 (optimum pH 5.0–5.5) and at 6–30 °C (optimum 20–26 °C). The major fatty acids are C16:1ω5c, C18:1ω5c, C16:0, and C18:0. *Telmatocola sphagniphila* is only distantly related to *Zavarzinella formosa* and *Gemmata obscuriglobus* (86% and 87 % 16S rRNA gene sequence similarity, respectively) (Fig. 5.2). Representatives of this species appear to be numerically abundant in acidic peatlands (see Sect. 8.5).

5.4.5 Candidatus *Nostocoida acidiphila*

One of the commonly observed bacterial morphotypes detected in acidic peat by means of hybridization with planctomycete-specific FISH probes is chains composed of spherical cells (Fig. 5.5a, b). For a long time, our attempts to cultivate these filamentous planctomycetes remained unsuccessful. Recently, however, one of these organisms was isolated in a co-culture with several chemoheterotrophic bacteria, which appear to feed on a polysaccharide sheath produced by the planctomycete (Fig. 5.5c, d) (Kulichevskaya et al. 2012b). The consortium consisting of the filamentous planctomycete and several satellite bacteria developed on a surface of a mineral medium solidified with Phytagel (1 %, w/v); the latter apparently was used as the source of carbon and energy. The phylogenetic position of this filamentous bacterium was determined by means of total DNA extraction from the consortium followed by PCR-mediated amplification of the 16S rRNA gene fragment using the combination of the *Planctomycete*-specific forward primer Pla46 (Neef et al. 1998) and the universal bacterial reverse primer Univ1390R. The retrieved 16S rRNA gene fragment displayed 95 % sequence similarity to the corresponding gene fragment in *Singulisphaera acidiphila* and 94.8–96.3 % similarity to 16S rRNA gene sequences of filamentous, taxonomically uncharacterized planctomycetes from activated sludge; those which have been termed by wastewater microbiologists "*Nostocoida limicola*" III (Liu et al. 2001) (Fig. 5.2). In contrast to moderately acidophilic

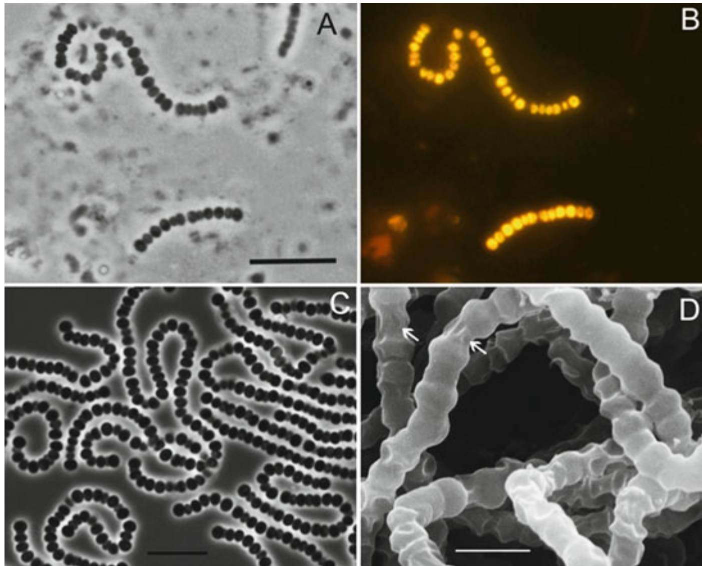

Fig. 5.5 (**a**, **b**) Specific detection of filamentous planctomycetes in acidic peat by FISH: epifluorescent micrograph of in situ hybridization with Cy3-labelled probes PLA46 and PLA886 (**a**) and the phase-contrast image (**b**). (**c**) Phase-contrast image of the acidophilic planctomycete *Candidatus* Nostocoida acidiphila OB1. (**d**) Electron micrograph of cell filaments of *Candidatus* Nostocoida acidiphila OB1 covered with a sheath (shown by *arrows*). Bars, 10 μm (**a**, **c**) and 3 μm (**d**)

members of the genera *Schlesneria*, *Singulisphaera*, *Zavarzinella*, and *Telmatocola*, this filamentous peat-inhabiting planctomycete showed a clear preference for growth in acidic conditions, with an optimum at pH 3.6–4.0. The only growth substrates utilized by this planctomycete were polysaccharides of microbial origin, such as Phytagel, Gellan Gum, or xanthan, which are produced by bacteria of the genera *Sphingomonas* and *Xanthomonas*. Since all attempts to obtain a pure culture of this slowly growing filamentous bacterium were unsuccessful, a tentative name *Candidatus* Nostocoida acidiphila was proposed for this planctomycete (Kulichevskaya et al. 2012b).

5.5 Phylogenetic Diversity and Functional Role of *Planctomycetes* in Wetlands

Phylogenetic diversity of the *Planctomycetes* in acidic *Sphagnum*-dominated wetlands was assessed in two recently published studies (Ivanova and Dedysh 2012; Kulichevskaya et al. 2012c). In the first study, PCR-mediated amplification of the 16S rRNA gene fragments (~1,350 bp) of peat-inhabiting planctomycetes was performed using the combination of the planctomycete-specific forward primer Pla46 (Neef et al. 1998) and the universal bacterial reverse primer Univ1390R. The resulting sequence

pool was highly diverse and included nearly all currently known major lineages of this phylum with the only exception of anammox planctomycetes (Jetten et al. 2005, 2010). The clone libraries constructed for two oxic peat samples from wetlands in West Siberia (peat bog Bakchar) and European North Russia (peat bog Obukhovskoye) had highly similar composition. More than half of all clones (53–61 %) in both clone libraries affiliated with the phylogenetic lineage defined by the genera *Isosphaera* and *Singulisphaera*. The second abundant group of sequences (25–35 % of all clones) obtained from oxic peat belonged to the phylogenetic lineage defined by the genera *Gemmata* and *Zavarzinella*. Finally, two minor groups of clones affiliated with *Planctomyces*- and *Pirellula*-like planctomycetes. By contrast, most 16S rRNA gene sequences (45 % of all clones) retrieved from the anoxic peat sample clustered within the group defined by the genus *Pirellula*. These sequences displayed only a distant relationship (85.7–89.7 % sequence similarity) to those from taxonomically described representatives of this planctomycete group, but were highly similar (95.2–98.7 %) to the environmental clone sequences Molly75 (AY775524), Molly19 (AY775494), and B86 (AM162476) obtained from *Sphagnum*-dominated wetlands in the USA and Russia (Morales et al. 2006; Dedysh et al. 2006).

The study of Kulichevskaya and coauthors (Kulichevskaya et al. 2012c) applied pyrosequencing-based *Bacteria* diversity analysis to get a deeper insight into diversity of peat-inhabiting planctomycetes. PCR in this study was carried out using the *Bacteria*-specific primers 907F and 1392R. A total of 1081 partial (average length ~490 bp) planctomycete 16S rRNA gene sequences were obtained from the *Sphagnum* peat sample collected from the peat bog Obukhovskoye. Taxonomy-based analysis, which was performed at a confidence threshold of 80 %, revealed that only 39 % of these sequences affiliate with phylogenetic lineages defined by taxonomically described organisms, including members of the genera *Isosphaera*, *Pirellula*, *Planctomyces*, *Schlesneria*, *Singulisphaera*, and *Telmatocola* (Fig. 5.6). The most frequently detected organisms were *Isosphaera*- and *Telmatocola*-like bacteria (16% and 10 % of all planctomycete-related sequences, respectively). The majority (61 %) of all planctomycete-related 16S rRNA gene sequences retrieved

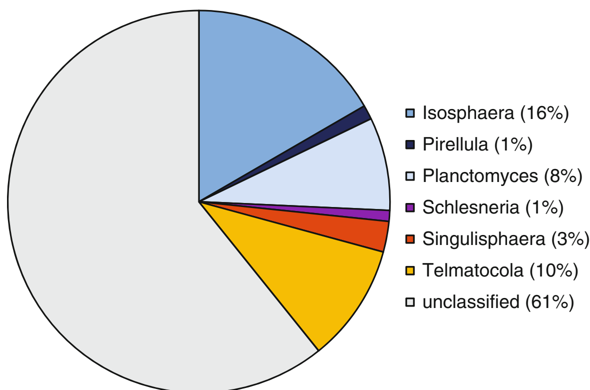

Fig. 5.6 The community composition of the *Planctomycetes* in an acidic (pH 4.0) peat sampled from the *Sphagnum*-dominated, ombrotrophic peat bog Obukhovskoye, European North Russia (58° 14′N, 38° 12′E), as assessed by pyrosequencing-based analysis (cited from Kulichevskaya et al. 2012c)

from acidic peat could not be assigned to taxonomically characterized already isolated or described organisms, thus highlighting the need for further cultivation efforts in uncovering the *Planctomycete* diversity in acidic northern wetlands.

The occurrence of anammox planctomycetes in acidic wetlands remains to be verified. As discussed above, these bacteria were absent in samples taken from the two *Sphagnum* peat bogs where available forms of nitrogen were at undetectable levels. However, anammox planctomycetes were detected in a mildly acidic swampy peat soil fed by nitrate-enriched local groundwater (Hu et al. 2011). The enrichment culture obtained from this peat sample displayed the highest specific activity at pH 7.1, suggesting the acid-tolerant, if not acidophilic, nature of these bacteria.

The functional role of planctomycetes in wetlands remains poorly understood. Based on our current knowledge, these bacteria are slow-acting decomposers of plant-derived organic matter. In our experiments, members of the *Planctomycetes* were identified as a numerically abundant component of a bacterial community participating in *Sphagnum* moss decomposition, which developed at the final stage of decomposition process (Kulichevskaya et al. 2007b). This is not surprising since all currently characterized peat-inhabiting planctomycetes are capable of degrading various heteropolysaccharides of plant and microbial origin. A weak ability to degrade cellulose, the major component of *Sphagnum*-derived litter, was recently demonstrated for *Telmatocola sphagniphila* (Kulichevskaya et al. 2012c). The existence of other, as-yet-uncultivated cellulolytic planctomycetes, therefore, cannot be excluded. Due to slow growth rates, the role of primary degraders is unlikely to be attributed to this group of bacteria. Yet, the biogeochemical role of planctomycetes in peatlands remains to be clarified since characterized representatives make up only a minor part of the whole planctomycete diversity in acidic wetlands.

5.6 Final Remarks

Despite the recent success in cultivation of the first acidophilic planctomycetes, most *Planctomycete*-related 16S rRNA gene sequences retrieved by molecular approaches from various acidic habitats display low similarity to those of characterized organisms. This also reasonably applies to planctomycetes in acidic wetlands, which were addressed in our studies. These bacteria inhabit both oxic and anoxic peat layers, while only aerobic representatives of peat-inhabiting planctomycetes have been characterized so far. Further work is needed, therefore, to characterize the unknown planctomycetes found in this habitat and to explore their physiology, genomic capabilities, and functional role in wetlands.

The existence of extremely acidophilic planctomycetes with growth optima at pH below 3.0 remains an open question. According to the currently available reports on molecular microbial diversity in extremely acidic habitats, members of the *Planctomycetes* are either absent or present in a relatively low abundance in these environments. More research is needed to extend our knowledge of the metabolic types and specific adaptations of planctomycetes that colonize extremely acidic habitats.

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Chapter 6
Toward the Development of Genetic Tools for *Planctomycetes*

Mareike Jogler and Christian Jogler

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6.1 Introduction

Members of phylum *Planctomycetes* are ubiquitous and environmentally important bacteria, the cell biology of which displays numerous unusual traits. Lacking peptidoglycan, they divide by polar budding, most likely without FtsZ (Fuerst and Sagulenko 2011). The cytosol of all planctomycetes contains at least one intracytoplasmic membrane (ICM) partitioning it into two compartments: the paryphoplasm and the pirellulosome (sometimes called riboplasm) (Lindsay et al. 1997, 2001). In all planctomycetes, the chromosome exists as a condensed nucleoid that lies within

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the pirellulosome and is, in some species, surrounded by an intriguing additional membrane system (Fuerst 2005; Lieber et al. 2009; Lindsay et al. 2001). Some planctomycetes are multicellular (Giovannoni et al. 1987), while others, such as *Planctomyces limnophilus*, form biofilms or aggregates and have complex life cycles involving both a sessile and a swimmer stage (Hirsch and Müller 1985; Jogler et al. 2011). Sessile cells attach to various surfaces using a "holdfast" that can be simply a secreted substance or, in some species, constitutes a distinct, stalked structure. Planctomycetes can adhere to form complex aggregates or biofilms using their holdfasts or additional fimbriae and thus can participate in the microbial communities dwelling within the macroscopic detrital aggregates known as marine or lake snow (DeLong et al. 1993).

Recent reports have suggested that planctomycetes might perform endocytosis-like uptake of proteins using putative membrane coat-like proteins that structurally resemble eukaryotic clathrins (Lonhienne et al. 2010; Santarella-Mellwig et al. 2010). The possibility that endocytosis, a trait widely regarded as a hallmark of eukaryotic cells, might exist in planctomycetes fueled the exciting hypothesis that these unusual bacteria might have played a significant role in eukaryogenesis, might even represent intermediate steps evolutionarily linking the prokaryotic and the eukaryotic cell plans (Devos and Reynaud 2010; Forterre and Gribaldo 2010; Fuerst and Sagulenko 2011). Such interpretations continue to be hotly debated because the procurement of convincing evidence has been hampered by the lack of genetic tools (Forterre and Gribaldo 2010; McInerney et al. 2011). Without the ability to genetically manipulate planctomycetes, most of the provocative features of these unique bacteria, including their possible endocytosis-like uptake of proteins, will remain poorly characterized (Fuerst 2005; Fuerst et al. 1997; Wagner and Horn 2006). Recently, we demonstrated the first successful gene transfer and the first generation of mutants in our model bacterium of choice, *Planctomycetes limnophilus* (Jogler et al. 2011), and this implies the promise of solutions to genetic analysis of planctomycete cell biology.

In this chapter, we will first discuss the pros and cons of the various model organisms previously used to study planctomycetes and will then summarize recent progress in the development of needed genetic tools. In conclusion, we present our priorities for future tool development with a focus on *P. limnophilus*.

6.2 Planctomycete Model Organisms

6.2.1 The Perfect Model Planctomycete for Genetic Tool Development

What attributes are most important when choosing one organism to serve as the model for a group of bacteria? An ideal model organism needs to be cultivatable in pure culture and should possess a short doubling time, the capability to grow in liquid medium, and the ability to form colonies on solid plates to allow clonal selection. Its chromosome and potential plasmids sequences should be known. Table 6.1

Table 6.1 Characteristics of sequenced planctomycetal genomes

	Rhodopirellula baltica SH1	*Planctomyces limnophilus*	*Pirellula staleyi*	*Isosphaera pallida*	*Planctomyces brasiliensis*	*Gemmata obscuriglobus*	*Planctomyces maris*	*Blastopirellula marina*
Chromosome	7.1 MB	5.4 MB	6.2 MB	5.5 MB	6.0 MB	9.1 MB	7.8 MB	6.7 MB
Contigs	1	2	1	2	1	922		64
Plasmid size	–	37 kb	–	56 kb	–	N.D.	N.D.	N.D.
G+C (%)	55.4	53.7 (57.0)	57.5	62.4 (67.0)	56	67.2	50.5	57.0
Bacteriophage	–	PI-89	PI-57	–	–	–	–	–
References	Glöckner et al. (2003)	Labutti et al. (2010)	Clum et al. (2009)	Göker et al. (2011)	JGI, NCBI	JGI, NCBI	JGI, NCBI	JGI, NCBI

presents an overview of the potential model planctomycetes sequenced thus far. We omitted the ammonium-oxidizing (anammox) planctomycetes for several reasons: (1) they are not available as pure cultures, (2) they grow extremely slowly with doubling times of 11–30 days (Strous et al. 1998), and (3) they require complex anaerobic culture conditions. In addition, their differences from other planctomycetes are such that they might even eventually be distinguished as a separate phylum since they form a distinct separate clade within planctomycetes at present. However, if a pure culture does become available, genetic tool development might become feasible for them, as their growth rate could be expected to be reduced under perfect cultivation conditions to, perhaps, "only" 1.8 days (Isaka et al. 2006). Anammox bacteria are excluded from further discussion in this chapter, but we hope that they will be revisited for genetic tool development in the future.

Planctomycetes in general are considered to be slow growing. However, with our recent advances in cultivation methods (Jogler et al. manuscript in preparation) we have reduced the doubling time for *Planctomyces limnophilus*. *Planctomyces maris* and *Gemmata obscuriglobus* exhibit doubling times of 13 h (Bauld and Staley 1976; Franzmann and Skerman 1984), while the doubling time of *Rhodopirellula baltica* ranges between 10 and 14 h (Rabus et al. 2002) and that for the filamentous *Isosphaera pallida* is 18 h at 42 °C (Ward et al. 2006). Still, for the planctomycetes, reasonable growth rates are measured in hours, not minutes. The second basic requirement for genetic manipulation—the ability to carry out clonal selection—is met for many planctomycetes that form colonies originating from single cells on agar plates (Ward et al. 2006). We recently demonstrated that *P. limnophilus* is one of the fastest growing of these, producing detectable colonies in 6–14 days (Jogler et al. 2011).

The third requirement for a model organism, that of having an available genome sequence, is currently met by all of the planctomycete species discussed above, though some of these, e.g., for *Gemmata obscuriglobus*, are still only draft sequences (Table 6.1). This will cease to be a major factor in the near future due to the advent of third-generation sequencing technologies. Full genome sequencing now lies within the budget of small research laboratories and has become a matter of weeks, thus obviating the need for a preexisting sequenced genome for future model planctomycetes (Check Hayden 2009). Notably, among the sequenced planctomycetes, both *Blastopirellula marina* and *P. limnophilus* are known to harbor a plasmid (Labutti et al. 2010; Ward-Rainey 1996). Such endogenous plasmids provide promising starting points for vector construction, thus making their host organisms more attractive for tool development. In addition, two bacteriophages of planctomycetes have been described: Pi-89 found in *P. limnophilus* and Pi-57 infecting *Pirellula* sp. IFAM 1358 (Ward et al. 2006). Like plasmids, phages represent valuable starting points for the development of gene transfer vectors.

Today's model planctomycetes are *G. obscuriglobus*, *R. baltica*, and *P. limnophilus*. When all aspects are carefully considered from the geneticist's point of view, *P. limnophilus* presents a particularly attractive choice for the development of a genetic system. It is reasonably fast growing and forms colonies on solid surfaces, its genome has been sequenced, it possesses a plasmid, and its cells are permissive

for phage infections (Jogler et al. 2011; Labutti et al. 2010; Ward et al. 2006). However, there was one major drawback. Until recently, only *G. obscuriglobus* and *R. baltica* had been intensively studied and almost nothing was known about the biology of *P. limnophilus*. In the following sections, we summarize the studies of cell biology, genomics, transcriptomics, and proteomics of all three that are relevant for future genetic experiments.

6.2.2 Gemmata obscuriglobus *as a Model Planctomycete*

Since details of the fascinating biology of *G. obscuriglobus* are presented elsewhere in this book (Chap. 2), we will focus on the potential development of genetic tools for this species. Devising such tools might turn out to be more difficult than for other planctomycetes. That the nucleoid of *G. obscuriglobus* is surrounded by two additional double membranes adds another layer of complexity to the planctomycetal cell plan (Fuerst and Webb 1991). While a number of observations have implied the existence of a closed "nuclear" compartment (Fuerst 2005; Fuerst and Webb 1991; Lee et al. 2009), including reconstruction of the basis of serial sectioning of cryofixed cells, this remains inconclusive as even cryo-electron tomography so far applied in at least one study has failed to determine the entire three-dimensional organization of this additional membrane system at high resolution (Lieber et al. 2009). Assuming a continuous membrane-bounded nucleoid consistent with at least some present evidence, then if electroporation or conjugation is used to transfer DNA into *G. obscuriglobus*, the vector might have to pass through twice the number of membranes compared to *P. limnophilus*. It had been speculated that even the intracytoplasmic membrane present in all planctomycetes, including *P. limnophilus*, might prevent DNA delivery to the host chromosome. However, we recently demonstrated that DNA can be transferred to *P. limnophilus* by electroporation (Jogler et al. 2011). The situation in *G. obscuriglobus* being far more complex, harsher treatment might be required in order to transport DNA across additional membranes. Deciphering the topography of the membranes surrounding its nucleoid at high resolution might help to predict the feasibility of gene transfer into this organism.

6.2.3 Rhodopirellula baltica *as a Model Planctomycete*

While the cell biology of *G. obscuriglobus* was being intensively studied, especially its endocytosis-like uptake of proteins, the biology of *R. baltica* was addressed via a more holistic approach involving genomic, transcriptomic, and proteomic analysis (Gade et al. 2005a, b, 2003; Glöckner et al. 2003; Rabus et al. 2002; Wecker et al. 2010). The diversity of the genus *Rhodopirellula* was explored at the species level using MLSA (Winkelmann et al. 2010), while refined enrichment and cultivation

techniques made a collection of different strains available (Winkelmann and Harder 2009). Thus far, *R. baltica* is the only planctomycete culturable in a chemostat and for which a potential biotechnological application has been demonstrated (Frank et al. 2011; Wallner et al. 2005). It has also emerged as an important model organism for the study of aerobic carbohydrate degradation in marine systems. In these environments, phototrophic microorganisms dwelling within the upper layers of the water column account for most of the biomass production. Since polysaccharides are the major component of their biomass, degradation of these carbohydrates is of major significance for global carbon cycles (Gade et al. 2005a).

Research on *R. baltica* received a major stimulus with the publication of the first planctomycetal genome sequence, that of *Pirellula* sp. strain 1, which was later identified as *R. baltica* SH-1 based on subsequent DNA–DNA hybridization, physiology, and chemotaxonomic tests (Schlesner et al. 2004). In 2003, the more than 7.1 MB genome of *R. baltica* ranked as the largest bacterial genome sequenced thus far (Table 6.1). Interestingly, analysis detected remnants of genes for peptidoglycan synthesis, as well as genes for lipid A biosynthesis and homologs of the flagellar L- and P-ring proteins (Glöckner et al. 2003). These findings still await clarification, since planctomycetes are known to lack both a peptidoglycan cell wall and a typical Gram-negative outer membrane containing lipid A (see Chap. 2 for details). Most strikingly, *R. baltica* was found to encode 110 sulfatases—more than a hundred times more of these enzymes than in any other bacteria. That 31 % of the potentially functional sulfatases contained a predicted signal peptide suggested their extracytoplasmic localization. In this location, the sulfatases might be used to access more efficiently the carbon skeleton of extracellular sulfated glycopolymers as an energy source. In addition, all genes required for heterolactic acid fermentation and key genes for the interconversion of C1 compounds were described (Glöckner et al. 2003). Based on cultivation experiments, this predicted metabolic capacity was proposed to serve basal energy requirements (Schlesner et al. 2004). As in methanogenic archaea, the enzymes for C1 metabolism in *R. baltica* utilize tetrahydromethanopterin as a C1 carrier. Since *G. obscuriglobus* also encodes these proteins, one view is that horizontal gene transfer from the *Methanosarcinales* currently appears to be their most likely source (Bauer et al. 2004), but the alternative that the C1 enzymes reflect vertical transmission from an ancestral C1 transfer enzyme organism has also been proposed (see Chap. 8 by Chistoserdova et al.).

R. baltica's genome sequence also afforded the identification of 15 novel protein domains and motifs, including an N-terminal export signal peptide and planctomycete-specific N-terminal domains containing heme-binding site motifs of cytochrome C (Studholme et al. 2004). Another study generated three theoretical proteome maps based on this genome sequence, one each for proteins with assigned function, hypothetical proteins, and conserved hypothetical proteins. Their overlay shows a different isoelectric distribution pattern than previously reported for any bacterial or archaeal proteome, one comprising a trimodal distribution with an additional peak around pH 7 that had formerly been seen only in eukaryotic genomes (Gade et al. 2005b). Intriguingly, isoelectric point (pI) distribution sometimes reflects the subcellular localization of the proteins and thus this finding might

correlate with the more complex subcellular compartmentalization of *R. baltica* (Gade et al. 2005b).

In addition to these in silico predictions, the *R. baltica* proteome was analyzed by four other studies (Gade et al. 2003, 2005a, b; Hieu et al. 2008). Investigation of the soluble protein fraction using two-dimensional gel electrophoresis (2-DE) with immobilized pH gradients (pH 4–7) yielded a reference gel (Gade et al. 2005b). The analysis of 1,000 protein spots from this gel by MALDI-MS and peptide mass fingerprinting generated a nonredundant data set of 626 distinct protein spots encoded by 558 different genes (Gade et al. 2005b). The most abundant proteins were housekeeping genes involved in glycolysis, the tricarboxylic acid cycle, amino acid biosynthesis, and protein quality control and translation (Gade et al. 2005b). Notably, the absence of predicted signal peptides indicated that these proteins were localized along with the ribosomes within the pirellulosome. In contrast to these housekeeping proteins, 146 proteins from the reference gel contained signal peptides and were annotated mostly as dehydrogenases, hydrolases for extracellular macromolecules, or signal transduction proteins. These potentially exported proteins might be located in the paryphoplasm, which may have contributed to the *R. baltica* soluble protein fraction analyzed. At the time of this study (2005), functions could be assigned to only 366 proteins (32 %); 54 % of those with unknown function were unique to *R. baltica* (Gade et al. 2005b). Interestingly, the majority of the hypothetical proteins had theoretical pIs above 7, with 37 % being greater than 10 (Gade et al. 2005b). Since integral membrane proteins tend to have a pI value around 9, it was speculated that *R. baltica* recruits these proteins for functions such as cellular development (e.g., the transition from a swimmer to a sessile cell) or for the translocation of proteins or solutes across the complex membrane structures (Gade et al. 2005b). The hypothesis that most of the 110 sulfatases from *R. baltica* serve for extracellular degradation of sulfated glycopolymers was supported by this study as nine of the ten identified sulfatases had a signal peptide and might have been sampled while in the process of being secreted (Gade et al. 2005b).

A second study employed, along with 2-DE, gel-free approaches and a combination of 1-D SDS-PAGE and nHPLC separation to generate a comprehensive *R. baltica* dataset under standard growth conditions (Hieu et al. 2008). The pH range for the 2-DE was also broadened (pH 3–10). These procedures detected 709 novel proteins and extended the *R. baltica* protein catalog to 1,267 entries. Of the 7,325 putative protein-coding ORFs, 17.3 % were expressed at the protein level under standard growth conditions. Of those 1,267 proteins, functional assignments could be made for 668 proteins, and these included housekeeping metabolic pathways of carbohydrate, amino acid, nucleic acid, and fatty acid metabolism. In addition, 190 hypothetical proteins were predicted to be unique to planctomycetes (Hieu et al. 2008).

Most remarkably, this work also included the first proteomic study of the rigid proteinaceous cell wall of *R. baltica*. Here 148 proteins were detected, some of which (RB850, RB7455, and RB2247) were rich in cysteine and proline consistent with earlier data for planctomycetal cell wall amino acid composition (Hieu et al. 2008; Liesack et al. 1986). Such proteins belong to a novel YTV family that consists of five members and that, at time of this study, had been found only in *R. baltica*

(Hieu et al. 2008; Studholme et al. 2004). Since YTV proteins are abundant in the *R. baltica* cell wall and disulfide bonds may form between their cysteine residues, they may serve as important structural components (Hieu et al. 2008). In addition, this study investigated the extracellular proteome. Surprisingly, only 13 proteins containing a predicted signal peptide were identified, suggesting that *R. baltica* most likely does not secrete degradative enzymes in substantial amounts but might instead attach such enzymes to the cell surface (Hieu et al. 2008).

The 2-DE reference gel from the first proteomic study provided a baseline for subsequent analysis of changes in *R. baltica* gene expression in response to external stimuli. During one of such follow-up study, *R. baltica* cells were cultured with one of eight different carbohydrates as their sole carbon source. The eight proteomes obtained using 2D-DIGE technology were compared against cultures grown with the reference carbohydrate, glucose. Spots for differentially regulated proteins observed on such gels were cut out and the proteins identified using peptide mass fingerprinting (Gade et al. 2005a). Most of the upregulated proteins were dehydrogenases, oxidoreductases, or proteins of unknown function. Few genes encoding these regulated proteins were found in a meaningful genomic context, the majority being apparently randomly distributed across the genome (Gade et al. 2005a). This finding could be expected because previous in silico analysis of the *R. baltica* genome revealed that functionally related genes are often not arranged in clusters or operons (Glöckner et al. 2003). However, this proteomic study demonstrated that, in *R. baltica*, functionally related proteins can be co-regulated despite the scattered genomic distribution of their encoding genes (Gade et al. 2005a). These findings suggest that *R. baltica* might employ an alternative regulation logic. Indeed, analysis of its transcriptional regulator pool revealed some unusual properties (Lombardot et al. 2005). Although in bacteria the proportion of genes encoding transcriptional regulators generally increases with genome size, only 2.4 % of *R. baltica*'s genes were predicted to encode transcription regulators (Lombardot et al. 2005). Many two-component systems (66) and sigma factors (49) were identified, with 76 % of the latter belonging to the extracytoplasmic function family of sigma 70 sigma factors (ECFs) (Lombardot et al. 2005). This prompted the proposal of a different regulatory mechanism for this organism and indicated a distinct bias toward direct environmental sensing (Lombardot et al. 2005). In addition, upregulation of several genes encoding proteins of unknown function suggested that *R. baltica* uses thus far unknown pathways for peripheral carbohydrate catabolism (Gade et al. 2005a).

The same lab also used whole-genome microarrays to analyze the transcriptional response of *R. baltica* to changing environmental conditions such as temperature and salinity (Wecker et al. 2009). In summary, this demonstrated that *R. baltica* is highly responsive to its environment, adjusting the expression of more than 3,000 of its 7,325 genes in response to changes in temperature and/or salinity (Wecker et al. 2009). Heat shock, cold shock, and salt stress produced measurable differential expression of 2,372, 922, and 1,127 genes, respectively, mostly genes encoding hypothetical proteins. That 152 of these genes were up- or downregulated for all stressors at any given time point suggests that they function in a general stress response (Wecker et al. 2009). While overall this response bears some resemblance

to the behavior of other bacteria studied under similar conditions, some differences were striking. For instance, *E. coli* orchestrates its heat shock proteins by employing alternative sigma factors encoded by *rpoH*, *rpoE*, and *rpoN*. Although all of those genes are present in *R. baltica*, they did not display differential regulation in the microarray experiments, thus suggesting a significantly different response cascade in *R. baltica* (Wecker et al. 2009). Furthermore, three of the 37 ECF-encoding genes were upregulated under all tested conditions, while the regulation of others was stressor specific. This result confirms that ECFs are involved in sensing and responding to stress in *R. baltica*.

Both proteomic and transcriptomic approaches have been used to survey regulation in *R. baltica* throughout its life cycle and during different growth phases (Gade et al. 2005a; Wecker et al. 2010). For the proteomic studies, *R. baltica* was grown with *N*-acetyl glucosamine as the sole organic carbon and nitrogen source. Samples were taken during exponential growth, at the start of stationary phase, and three times during stationary phase. As proof of principle, regulation of the dehydrogenase RB3330 was monitored during stationary phase and a fourfold downregulation was observed. RB3330 had been previously shown to be required for *N*-acetyl glucosamine utilization in *R. baltica*. Its downregulation correlated with the depletion of *N*-acetyl glucosamine in stationary phase (Gade et al. 2005a). This study found that during the shift from exponential to stationary phase, 48 proteins were upregulated while 27 were downregulated. Similarly, in late stationary phase (432 h), 101 and 76 proteins were found to be upregulated and downregulated, respectively, this being the phase with the most pronounced changes relative to the reference state (46 h). These included proteins related to carbon, amino acid, nucleotide, and general metabolism, as well as to inorganic compounds, regulation, translation, transport, and stress response.

In addition, 14 regulated proteins of unknown function that may be unique to planctomycetes were posited to be involved in the cell cycle and/or morphotype differentiation. The latter possibility is based on the growth habits of *R. baltica*. At low concentrations, one finds mostly solitary cells that are either budding cells containing holdfast substance at one pole or flagellated swimmer cells. In stationary phase, swimmer cells are absent and the remaining cells form aggregates (Gade et al. 2005a). However, predicting proteins to be morphotype related is problematic, as one would expect to find, for example, flagellar proteins to be downregulated in stationary phase. However, such findings were not reported. Rather, the protein profiles more likely reflect adaptation to the limited nutrients or increased toxic products characteristic of stationary-phase cultures. For example, enzymes involved in the biosynthesis of the amino acid proline decreased as cells entered stationary phase. Because proline is one of the main constituents of the proteinaceous cell wall of *R. baltica*, reduced proline synthesis would be expected to correlate with reduced cell growth (Liesack et al. 1986). Likewise, upregulated during stationary phase were global regulators such as the extracytoplasmic sigma factor σ^H that controls gene expression during adaptation to nutrient depletion in *Bacillus* (Gade et al. 2005a).

When comparing growth on agar plates against liquid cultures, two proteins were found to be upregulated RB5240 (4.7-fold) and downregulated RB6941 (11.4-fold)

while growing on solid surface (Gade et al. 2005a). While the function of both proteins is unknown, they were predicted to correlate with the sessile or free-living swimmer stage, respectively (Gade et al. 2005a). Among the upregulated known proteins were catalase and peroxidase, both indicative of oxidative stress in colonies on solid surfaces (Gade et al. 2005a). A complementary transcriptomic study employing microarrays compared samples from different time points against each other (Wecker et al. 2010). The greatest number of genes showing differential expression (863) was observed between early (82 h) and late (240 h) stationary phase. Again, most of those genes (58 %) encode proteins of unknown function. Among the genes putatively relevant for biotechnological applications, 12 sulfatases were found to be up- or downregulated (Wecker et al. 2010). Based on the distinctive expression pattern of sulfatase genes, a role in the structural remodeling accompanying morphological differentiation of *R. baltica* cells was proposed (Wecker et al. 2010).

While proteomic and transcriptomic analyses such as these suggested many potential directions for future research, the lack of genetic tools for *R. baltica* has hampered, in particular, any in-depth analysis of its many proteins of unknown function.

6.2.4 **Planctomyces limnophilus** *as a Model Planctomycete*

P. limnophilus was first isolated from a German freshwater lake and described in 1985 (Hirsch and Müller 1985). While planctomycetes such as *Planctomyces maris* and the *R. baltica* subjected to so many proteomics experiments are marine, the freshwater habitat of *P. limnophilus* makes it easier to handle in genetic experiments, especially for electroporation where the osmotic stress should be minimized, but almost salt-free conditions are required to prevent arcing (see Sect. 6.3.2). *P. limnophilus* is a budding bacterium with a mostly typical planctomycete architecture, and one somewhat simpler than the more complex architecture of the freshwater model planctomycete *Gemmata obscuriglobus* (Fig. 6.1) (Jogler et al. 2011). However, the pirellulosome of *P. limnophilus* appeared as several separated compartments in the TEM analysis of some thin sections (Jogler et al. 2011). When serial sections are viewed, it becomes more likely that these separate vesicle-like structures form a continuum. By analogy, picture a horizontal, slightly concave, human hand with the fingers spread. Depending on the position of a hypothetical horizontal section, one continuous compartment or several separated will become visible—the latter if the section passes through the heel of the hand and the fingers. Whether the structure of this pirellulosome is static or dynamic remains an enigma. In contrast to *R. baltica*, *P. limnophilus* forms a stalked holdfast structure, the stalk being formed by a bundle of fibers (Fig. 6.1) (Jogler et al. 2011; Hirsch and Müller 1985). By means of this structure, the cells attach to surfaces or to each other, thus forming characteristic rosettes (Hirsch and Müller 1985). We note also that the sessile stalked cells can form additional fimbriae to support biofilm formation and attachment to surfaces (Fig. 6.2I) (Jogler et al. 2011).

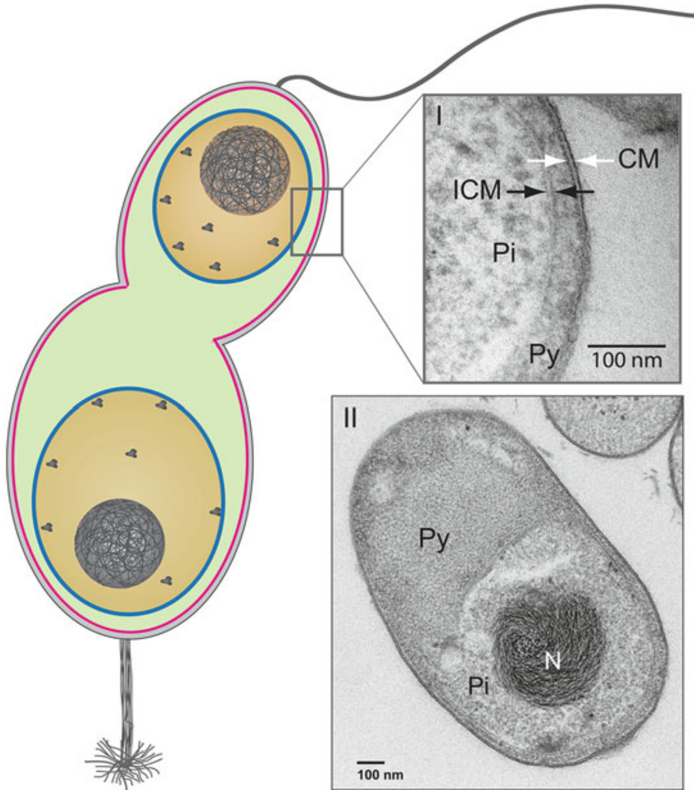

Fig. 6.1 Schematic drawing of a dividing *P. limnophilus* stalked cell. Cell compartmentalization is evident in TEM images of thin sections showing membrane organization (I) and the entire cell (II). The cell is enveloped by a proteinaceous cell wall (*gray*), beneath which lies the cytoplasmic membrane (*pink*/CM/bordered by *white arrows*) surrounding the paryphoplasm (*green*/Py). The pirellulosome (*orange*/Pi) is surrounded by the intracytoplasmic membrane (*blue*/ICM/bordered by *black arrows*) and harbors the ribosomes and the nucleoid (N). The stalk and the holdfast structure form at the pole opposite the reproductive pole where budding takes place and the flagellum forms

The complex developmental cycle of *P. limnophilus* resembles somewhat that of *Caulobacter crescentus* (Shapiro et al. 1971) (Fig. 6.2). Only stalked cells are able to divide. Division is initiated by the formation of a bud, the future daughter cell, at the pole of the mother cell opposite the stalk. When the bud reaches a certain size (Fig. 6.2IV), the two condensed chromosomes separate (Fig. 6.2V+VI) and a mature, flagellated daughter cell develops. These flagellated swimmer cells form a stalk with a holdfast and lose their flagellum before dividing. Although we examined thousands of cells using TEM, we never observed a flagellated swimmer cell undergoing division. While we refer to flagellated swimmer cells and stalked sessile cells, sometimes the term swarmer cell is used in the planctomycetal literature. This is misleading and we suggest to adopt instead the terminology based on the various mechanisms of bacterial surface translocation (Henrichsen 1972). Consequently we call the daughter cells "swimmers."

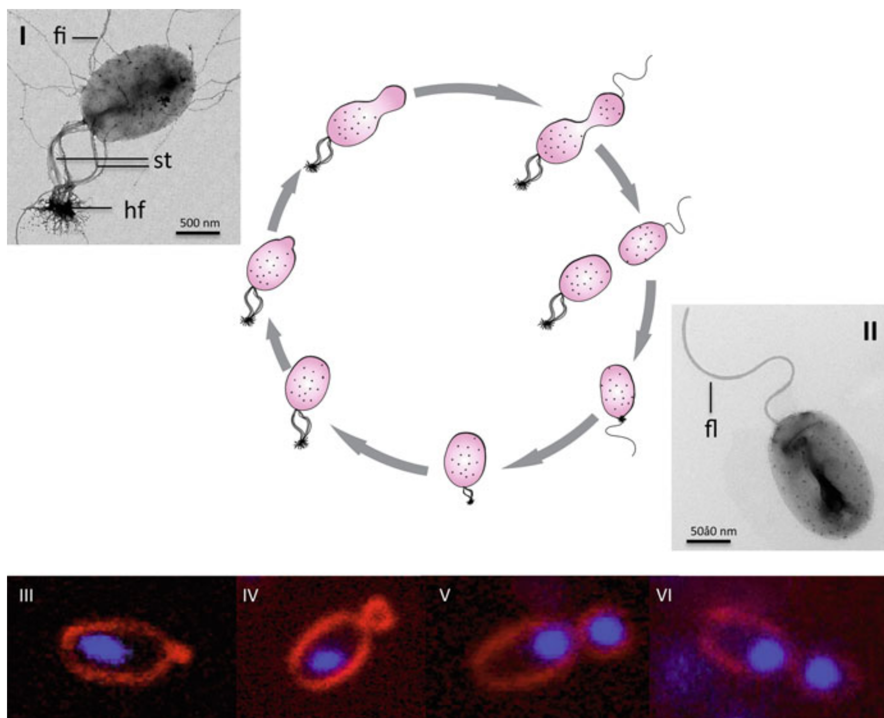

Fig. 6.2 Schematic drawing of the life cycle of *P. limnophilus*. Cells alternate between motile and sessile stages during their life cycle. Adult sessile stalked cells can divide through asymmetrical budding to produce flagellated swimmer daughter cells. TEM images show the structure of a stalked cell (I, st: stalk) and a swimmer cell (II, fl: flagellum). Note the use of fimbriae (I, fi) as well as the holdfast (I, hf) for attachment to surfaces (I) and the electron-dense crateriform structures dotting the cell surface. The daughter cell is formed prior to partitioning of the chromosome, as shown by spinning disk confocal microscopy (III–VI). When the daughter cell reaches a critical size, the two condensed copies of the chromosome separate (V–VI). Membranes are stained with FM4-64 (*red*) and DNA with DAPI (*blue*)

Although the genome sequence of *P. limnophilus* became available only recently (Labutti et al. 2010), its extrachromosomal element had been postulated earlier when the classical physical map of its genome was constructed (Ward-Rainey et al. 1996). This 37 kb plasmid is of great potential value as it might serve as a starting point for the development of planctomycetal shuttle vectors (Fig. 6.3). While the genome of *P. limnophilus* is the smallest of the planctomycetal genomes sequenced thus far (Table 6.1), its cell biology appears to be almost as complex as that of the other planctomycetes. Its smaller genome is an advantage provided it contains all the genes related to the typical planctomycetal life style and cell biology. Its lower G+C content (53.7 % vs. 67.2 % in *G. obscuriglobus*) suggests that some genetic experiments such as PCR amplifications might be more productive (Table 6.1). That functions could be assigned to the majority of the protein-coding genes (53.9 %) by automatic annotation may relate to its relatively small size (Labutti et al. 2010).

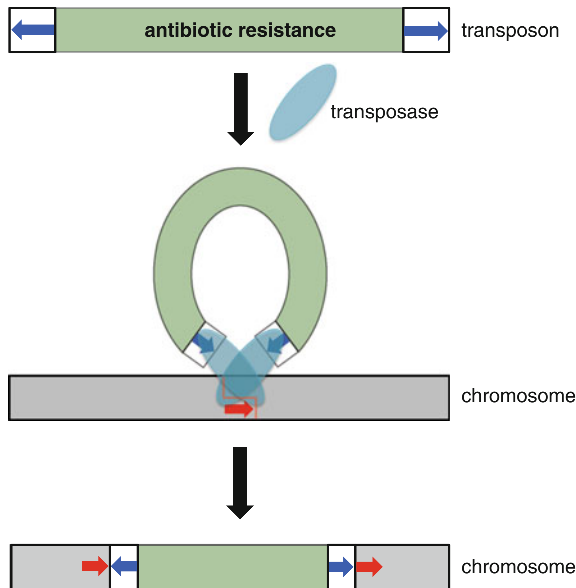

Fig. 6.3 The cut-and-paste mechanism of Tn5 transposition. So far, Tn5 transposition is the only proven method for generating planctomycetal mutants. The modified Tn5 transposon contains a kanamycin resistance gene and two terminal repeats (*blue arrows*). Preincubation with the transposase in vitro forms a stable enzyme-DNA complex at the termini. When transferred into *P. limnophilus* cells by electroporation, the transposase creates staggered double-strand breaks in the chromosomal DNA (*red arrow*) and the transposon is ligated to the target DNA. DNA polymerase fills the resulting gaps, generating short duplications of the target DNA at the transposon ends (*red arrows*)

Manual curation will undoubtedly yield additional assignments because, for example, the automated annotation predicted no cytoskeleton-associated genes (Labutti et al. 2010), while manual inspection easily revealed the presence of the bacterial actin homolog MreB (Jogler et al. 2012).

In addition an *rpoN* gene that encodes the alternative sigma factor σ^{54} was identified in *P. limnophilus* based on its ability to complement the $^{-}$Ntr phenotype of a *Salmonella typhimurium rpoN* mutant (Leary et al. 1998). The RpoN protein sequence encoded by *P. limnophilus* displayed all of the characteristic motifs found in members of this protein family, including the C-terminal helix-turn-helix motif and the well-conserved RpoN box (Leary et al. 1998). Importantly, this finding demonstrated that heterologous expression of *P. limnophilus* genes is possible. (For a detailed discussion of the implications, see Sect. 6.3, below.) It also shows that despite the many differences that have been observed, some regulatory aspects of the planctomycetes are shared with other bacteria.

An important recent advancement was the development of a gene transfer procedure for *P. limnophilus* that led to the generation of the first planctomycete mutants (Jogler et al. 2011). This opens the door for future genetic experiments, thus making possible the in-depth study of planctomycete biology as we describe next.

6.3 Toward a Genetic System Using *P. limnophilus*

Development of a useful genetic system requires not only the selection of a suitable model organism such as *P. limnophilus* but also the assembly of a panel of applicable tools and strategies. The most basic needs are (1) genetic markers for selection and counter-selection, (2) a transformation procedure, (3) both constitutive and inducible promoters, (4) a suitable cloning vector, and (5) the ability to generate chromosomal mutants. Most of these tools are still on the wish list of planctomycete researchers, but some have been recently developed. We will address each of them separately and give examples from other bacterial groups when a solution for planctomycetes has not yet been realized. Since our laboratory is currently working on all aspects of a genetic system for *P. limnophilus*, a substantial expansion of the available tools could be expected in the near future. As mentioned above, significantly more is known about the biology of *G. obscuriglobus* and *R. baltica* than *P. limnophilus*. Future experiments will be needed to determine whether these genetic tools will be easily transferred from *P. limnophilus* to other model planctomycetes or whether we will have to fall back on surrogate genetic experiments to provide a solution (see Sect. 6.3.5).

6.3.1 Selection Markers

Selection markers are required to ensure plasmid maintenance following experimental gene transfer and to force homologous recombination during mutagenesis (Fig. 6.4). Genes encoding proteins that confer resistance to antibiotics, other antimicrobials, or heavy metals have been successfully used for these purposes in other bacteria (Schweizer 2008). As a first step in assessing the applicability to this approach to planctomycetes, a comprehensive study determined the broad-spectrum antibiotic resistance of *P. maris*, *P. brasiliensis*, *B. marina*, *P. limnophilus*, *G. obscuriglobus*, and *R. baltica*. All tested planctomycete species were found to be resistant to penicillin G, imipenem, ampicillin, cefalotin, and ceftriaxone (Cayrou et al. 2010). This was not surprising since planctomycetes, lacking a peptidoglycan cell wall, are not susceptible to β-lactam antibiotics. However, this affects genetic tool development because it renders the many commercially available plasmids that use the *bla* gene encoding ampicillin resistance unsuitable for work with planctomycetes. All tested species were also found to be resistant to nalidixic acid and vancomycin, all were susceptible to tetracycline, and all, except *P. brasiliensis*, were susceptible to ciprofloxacin. Erythromycin and chloramphenicol, both frequently used for genetic experiments, showed a patchy distribution of resistance. *P. brasiliensis* and *P. maris* were resistant to chloramphenicol, while *R. baltica* and *G. obscuriglobus* displayed intermediate susceptibility. In contrast, all tested species, except *P. brasiliensis*, were susceptible to erythromycin, while *P. brasiliensis* showed intermediate susceptibility. All tested species were resistant to rifampicin except *R. baltica* that showed intermediate susceptibility (Cayrou et al. 2010).

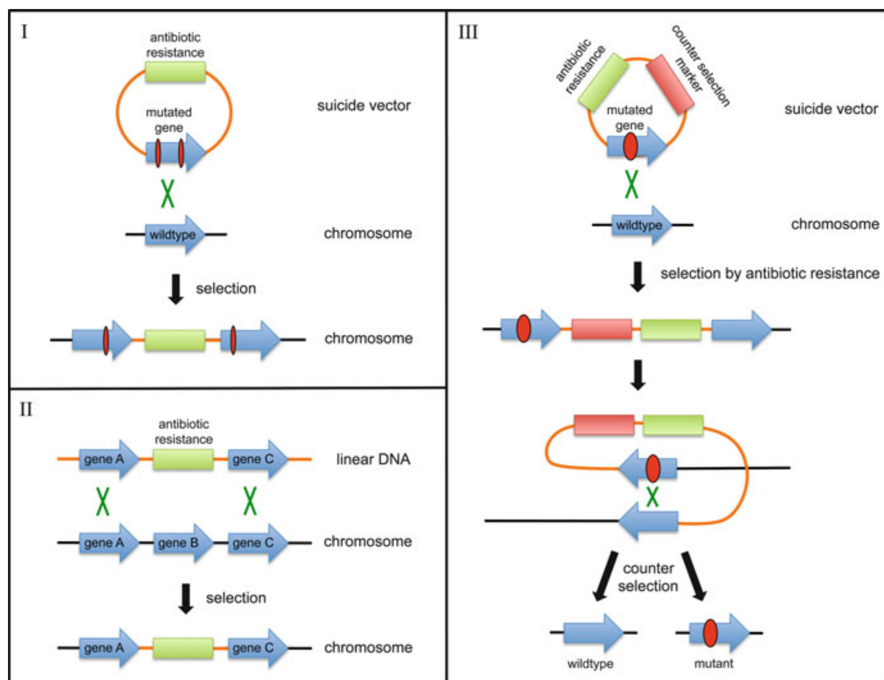

Fig. 6.4 Three standard mutagenesis strategies under development for planctomycetes. Stable insertion-duplication mutagenesis (I) requires a suicide vector that is incapable of replication in planctomycetes (*orange*). Such a plasmid would contain an antibiotic resistance gene (*green*), a segment similar to the central part of the target gene (*blue arrow*), and sometimes also introduced stop codons (*red*). Once transferred into the bacterium, the antibiotic selection pressure enforces homologous recombination and insertion of the suicide vector. Although the target gene is duplicated, introduced stop codons and/or careful design of the homologous region can ensure that both copies are dysfunctional. Linear DNA transferred into a planctomycete by electroporation can be incorporated into the chromosome by homologous recombination (II). As with suicide vectors, integration is enforced by the inability to replicate independently and by antibiotic-containing selection media (*green*). However, a double crossover event is also required to prevent double-strand breaks in the chromosome. The generation of unmarked mutants (III) is a two-step procedure. First a suicide vector is prepared containing segments homologous to the sequences both upstream and downstream of the chromosomal area to be mutated (*blue arrow*, mutation in *red*). Its insertion into the target chromosome is basically as shown above (I). In the second step, selection by a counter-selectable marker (*red*) enforces a second recombination event that eliminates the suicide vector from the bacterial genome leaving the unmarked mutation in the chromosome. All of these techniques are currently being adapted for use in planctomycetes in our laboratory and will be available soon

In our study, we focused on *P. limnophilus* and demonstrated that it was also susceptible to kanamycin. Taken together, three antibiotics frequently used for genetic experiments—tetracycline, chloramphenicol, and kanamycin—were found to be suitable selection markers for *P. limnophilus*, while only tetracycline has demonstrated effectiveness against *G. obscuriglobus* and *R. baltica*. However, kanamycin, which has not yet been tested, might provide a second option for these two

organisms, or alternatively selection markers conferring resistance to heavy metals such as tellurite (Sanchez-Romero et al. 1998) or mercury (Rochelle et al. 1989) might be efficacious. However, the intrinsic antibiotic resistance of these planctomycetes is not necessarily a disadvantage for the construction of genetic tools. Because of their resistance to β-lactam antibiotics, ampicillin could serve as a counter-selection marker in conjugation experiments to eliminate susceptible donor strains, such as *E. coli*. Due to the requirement for host mutations, the use of other metabolic markers is not feasible in wild-type bacteria (Schweizer 2008).

In contrast to selection markers, counter-selection markers such as *sacB* (Gay et al. 1983), *amiE* (Collier et al. 2001), and *pheS* (Kast 1994) eliminate unwanted genetic elements, for example, during the construction of unmarked deletion mutants (see below). Although helpful, they are not an absolute requirement for allelic exchanges (Schultheiss and Schuler 2003) and nothing is known at this point about their possible functionality in planctomycetes.

6.3.2 Gene Transfer

Development of a genetic system for the planctomycetes does require the ability to transfer recombinant DNA molecules into these organisms. In general, four methods are used: (1) natural transformation (Chen et al. 2005), (2) chemical transduction (Schweizer 2008), (3) electroporation (Schweizer 2008), and (4) conjugation (Chen et al. 2005). One report has described the successful transfer of the BF1 plasmid from *Pseudomonas putida* to *Planctomyces maris* via conjugation (Dahlberg et al. 1998). However, that study did not aim to develop a gene transfer system for *Planctomyces*, but rather to demonstrate conjugation. Thus, the authors did not determine whether the plasmid was maintained in *P. maris*. The pBF1 plasmid carried a *gfp* gene encoding the green fluorescent protein (GFP) under the control of the inducible *lacZ* promoter. Plasmid transfer was detected microscopically, thus confirming transcription and translation of this heterologous gene construct in *P. maris*. It therefore seems possible that many of the currently available tools such as inducible promoters might be functional in planctomycetes, as well. The potential for plasmid transfer via conjugation in other planctomycetes gained additional support from the identification of conjugation-related *tra* genes in *G. obscuriglobus* (Jenkins et al. 2002). Unfortunately, while pBF1 would have been the perfect starting point for genetic tool development, the plasmid was not available on request. Thus, neither we, nor other groups, have been able to reproduce these conjugation experiments.

Nevertheless encouraged by this report, we have performed an intensive series of conjugation experiments using *E. coli* S17 and *Pseudomonas putida* KT2442 as donor strains and *P. limnophilus* as the acceptor. Four different plasmids, pKT210 (R300B replicon, IncQ), RP4 (RK2 replicon, IncP), pLAFR-1 (RK2 replicon, IncP), and pBBR1MCS-3 (pBBR replicon) were evaluated under both liquid- and solid-surface mating conditions. Mating durations varied between 4 h and 4 days; post-mating recovery without selective pressure was allowed for 0–12 h in liquid culture. Despite literally hundreds of experiments, we failed to obtain true plasmid transfer

and replication. Occasional colonies were found on selection plates containing either tetracycline or chloramphenicol, suggesting successful plasmid transfer since negative controls (wild-type *P. limnophilus* cells on selection plates) never allowed growth of *P. limnophilus*, even when the plates were incubated for more than a month. However, we were not able to rescue the plasmid from these putative transformants and concluded that only resistance markers had somehow been integrated into the planctomycetal chromosome. Taken together, our attempts thus far to transfer plasmids via conjugation have failed. Whether this is due to problems related to gene transfer or to replication of the chosen broad-host-range plasmids, we cannot say at present.

Since conjugation turned out to be problematic, and being aware of a report of successful gene transfer into *Chlamydia* using electroporation, we directed our efforts toward establishing an electroporation protocol (Binet and Maurelli 2009). We had obtained our first positive results at the time when a similar strategy was published for *Verrucomicrobium spinosum* (Domman et al. 2011). We obtained 1.5×10^3 transformants per microgram of EZ-Tn5 transposon DNA using as our electroporation conditions a capacity of 25 μF, a resistance of 200 Ω, a voltage of 1.0 kV, and about 4×10^8 *P. limnophilus* cells (Jogler et al. 2011). Since this achievement still left room for improvement, we are currently optimizing electroporation conditions.

Despite this success with electroporation, we are also currently evaluating chemical methods that are known to induce competence for subsequent DNA uptake in other bacteria, methods such as prolonged incubation with calcium chloride or treatment with dimethyl sulfoxide, hexamminecobalt, or dithiothreitol (Hanahan et al. 1991). Transduction employing bacteriophages, which has proven to be a very efficient method for DNA transfer into other bacteria, also appears to be a possibility here as two bacteriophages of planctomycetes have been identified and it is likely that many more exist. Phage Pl-89 was isolated from *P. limnophilus*, phage Pi-57 from *Pirellula* sp. (Ward et al. 2006). Both contain linear dsDNA genomes and belong to the *Styloviridae* (Ackermann and Eisenstark 1974). In addition, a panel of broad-host-range bacteriophages is available for transduction experiments. For example, the "phagemid system" based on phage P1 is a potential candidate for DNA delivery into planctomycetes (Westwater et al. 2002). These factors bode well for the successful future development of a transduction system for planctomycetes.

6.3.3 Vectors

We have performed only preliminary electroporation experiments using the same four plasmids as were trialed in our unsuccessful conjugation experiments with *P. limnophilus* (pKT210, RP4, pLAFR-1, and pBBR1MCS-3; see above). So far we have no evidence that any of them replicate in *P. limnophilus*, perhaps due to incompatibility issues arising from the endogenous plasmid of our model organism. However, alternative broad-host-range replicons are available, such as pRO1600 (Olsen et al. 1982), RSF1010 (Scholz et al. 1989), and pVS1 (Itoh et al. 1984), all

of which we will evaluate in the near future. Alternatively, the endogenous plasmids identified in either *P. limnophilus* (Ward-Rainey et al. 1996) or *B. marina* (Ward-Rainey 1996) might serve as a starting point for the construction of a custom cloning vector, but this would most likely require their modification. To facilitate their genetic manipulation, ET recombination (Zhang et al. 1998) might be used to introduce multiple cloning sites and selectable genetic markers. Another possibility is construction of a shuttle vector, a strategy that was used successfully to develop plasmids for "difficult" bacteria including *Francisella tularensis* (LoVullo et al. 2006), *Capnocytophaga canimorsus* (Mally and Cornelis 2008), *Campylobacter fetus* (Kienesberger et al. 2007), *Helicobacter pylori* (Carpenter et al. 2007), *Xylella fastidiosa* (Qin and Hartung 2001), and *Lactobacillus delbrueckii* (Lee et al. 2007). Lastly, the plasmid pBF1, which was successfully transferred to *P. maris* via conjugation, warrants evaluation once it has been made available to the scientific community (Dahlberg et al. 1998).

6.3.4 Generation of Mutants

To advance our understanding of planctomycetal cell biology, including their endocytosis-like protein uptake, their complex developmental cycle, and their environmental importance, calls for gene function studies that, in turn, require the ability to generate chromosomal mutants and assess their phenotypes. This introduction of mutations can be either random or site specific. To generate random pools of chromosomal mutants, we implemented Tn5 transposon mutagenesis for *P. limnophilus* (Jogler et al. 2011) (Fig. 6.3). Transposon mutagenesis is arguably the most powerful method for the generation of such mutants (Jacobs et al. 2003). We developed an arbitrary PCR to easily characterize insertion sites of planctomycetal chromosomes using the commercially available EZ-Tn5™ <R6Kλ*ori*/KAN-2>Tnp Transposome™ Kit (Epicenter) (Jogler et al. 2011). Thus, random mutagenesis is well established for *P. limnophilus* and might be transferrable to the other model planctomycetes, *G. obscuriglobus* and *R. baltica*. However, given their larger genomes, a significantly greater number of mutants would need to be generated in order to achieve anything close to saturation. Thus, once again, the smaller genome of *P. limnophilus* is advantageous.

Site-specific mutagenesis has not yet been demonstrated for a planctomycete, but that will be a major step forward as it will allow the creation of defined mutants. In general, site-specific mutagenesis is achieved via gene replacement using either linear DNA fragments (Lauriano et al. 2003) or suicide vectors carrying a cloned mutated target gene (Schultheiss et al. 2004). Subsequent transfer of plasmids can be achieved by electroporation or conjugation (Schultheiss and Schuler 2003), while transfer of linear DNA requires electroporation. Figure 6.4 provides an overview of the most popular methods for site-directed mutagenesis and explains the technical details. All steps except for gene transfer still need to be established for planctomycetes and the required methods are currently under development in our laboratory.

6.3.5 Surrogate Genetics

Despite these recent advances in the development of a genetic system for planctomycetes, our efforts lag far behind the state of the art in commonly studied model organisms such as *E. coli*. Thus, surrogate genetic experiments might provide an expedient alternative strategy for studying planctomycetal gene functions. Success for this approach has been demonstrated even for distantly related organisms. A well-known example that has facilitated PCR analysis, a mainstay of research in molecular genetics, is the heterologous expression of the heat-stable DNA polymerase from *Thermus aquaticus* using *E. coli* as the surrogate host. Inducible expression was first realized using a lac promoter (Lawyer et al. 1989). Notably, the phylogenetic distance between *T. aquaticus* and *E. coli* is great, as they belong to the phyla *Deinococcus-Thermus* and *Proteobacteria*, respectively (Sayers et al. 2009). The first successful surrogate genetic experiments have now been reported for planctomycetes. For example, the trans-complementation of *Salmonella typhimurium* Δ*rpoN* mutants was achieved by expression of the *rpoN* gene from *P. limnophilus* (Jenkins et al. 2002), and the polysaccharide lyase RB5312 from *R. baltica* was expressed in *E. coli* and the enzyme subsequently crystallized for preliminary x-ray analysis (Dabin et al. 2008). These experiments demonstrate that the functional heterologous expression of genes from planctomycetes in very distantly related species is, in general, possible. Thus, surrogate genetic experiments will, for example, allow testing the biochemical function of proteins, localization studies employing GFP translational fusions to proteins of interest, the generation of mutants or truncations to characterize protein functions, or the complementation of host mutations to confirm the functionality of proteins.

6.4 Conclusions and Future Perspectives

While significant progress has been made recently in the development of genetic tools for planctomycetes, there is still room for further advancement (Jogler et al. 2011). From our point of view, development of unmarked mutagenesis strategies and translational fusions of planctomycetal proteins to fluorescence markers are the most urgent challenges (Schweizer 2008). In addition, the electroporation protocol requires further optimization. While these lines of research are close to fruition in our lab, we want to encourage others to become involved in tool development. There is also the attractive possibility of developing more efficient gene transfer methods employing bacteriophages. Lastly, it would be of great interest to see our protocols transferred to the other model planctomycetes, *G. obscuriglobus* and *R. baltica*. As already demonstrated by research on other model organisms with a compartmentalized cell plan, such as the magnetotactic bacteria, development of the basic genetic tools as outlined in this chapter can allow detailed studies that reveal stunning insights into their cell biology (Komeili et al. 2004, 2006; Scheffel et al. 2006). We eagerly anticipate that more genetic tools will soon be available for the planctomycetes, thus opening the door for in-depth analysis of this fascinating phylum.

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Chapter 7
Genomics and Bioinformatics of the PVC Superphylum

Olga K. Kamneva, Daniel H. Haft, Stormy J. Knight, David A. Liberles, and Naomi L. Ward

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Abbreviations

PVC Planctomycetes–Verrucomicrobia–Chlamydiae
HGT Horizontal gene transfer

7.1 Introduction

Whole-genome sequencing has become a powerful and informative approach for determining the genetic basis of known bacterial properties, predicting new properties, and enabling post-genomic tools such as transcriptomics and proteomics. It also shapes the choice of representative bacterial strains and species to serve as model organisms for experimental work. However, genome sequencing and annotation are most useful in the context of comparative genomic and evolutionary analysis, which allows the determination of phylogenetic relationships between extant organisms, provides insights into the evolution of different biological systems, and sheds light on processes accounting for organismal diversity. Classification of organisms based solely on their phenotypic characteristics is no longer recognized as useful for understanding the evolutionary relationships between organisms. Therefore, modern evolutionary studies are focused on DNA- and protein-based phylogenetics, generally using universally distributed genes such as ribosomal rRNA or informational protein-coding genes. However, the evolutionary relationships between species derived from different genes are rarely consistent with each other, due to incomplete incorporation of knowledge about complex processes of sequence evolution and limited evolutionary information available within a single gene. The availability of whole-genome sequences provides an opportunity to address these limitations and obtain a more objective and comprehensive understanding of evolutionary relationships among microbes and their genomes.

Recent and ongoing comparative genomics and ultrastructural (see Chaps. 2, 3 and 11) studies have generated fundamentally important and exciting insights into the evolution of biological systems within organisms of the PVC superphylum (a group named for three of its component phyla: *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae*). While some bacteria within this group (e.g., many of the *Chlamydiae*) have the simple cell structure common among bacteria, all the characterized planctomycetes, several verrucomicrobia, and the only cultured species in phylum *Lentisphaerae* have a common cell plan that features an additional intracellular membrane and is unique among bacteria (Fieseler et al. 2004; Fuerst 2005; Lee et al. 2009; Lindsay et al. 2001). It should also be noted that some PVC species currently considered to lack this plan (e.g., members of *Chlamydiae*) have not been examined by state-of-the-art cryotechniques and may yet be shown to possess it. Planctomycetes exhibit variations upon the common PVC plan, featuring additional membrane-enclosed compartments with functions and biological consequences that are either known (e.g., anammoxosome, anaerobic ammonia-oxidizing compartment, in anammox bacteria) or undetermined (e.g., double-layered membrane envelope surrounding the condensed genomic DNA of *Gemmata obscuriglobus*) (Fuerst 2005; Fuerst

and Webb 1991). The availability of genome sequences from members of the PVC superphylum allowed comparative/structural genomic analysis, followed by experimental immunogold localization demonstrating the presence of proteins structurally resembling eukaryotic membrane coat proteins (Santarella-Mellwig et al. 2010). While sterols are generally considered to be eukaryote-specific molecules, the *G. obscuriglobus* genome sequence established a foundation for the discovery of sterols and sterol biosynthesis genes in this organism (Pearson et al. 2003). The almost complete genome of *Candidatus* Kuenenia stuttgartiensis assembled from a community genome has permitted deduction of the biochemical pathway of anaerobic ammonium oxidation, a technologically important biochemical reaction (Strous et al. 2006). Identification of canonical pathogenicity determinants (encoding a putative type III secretion system) in the genome of *Verrucomicrobium spinosum* led to work suggesting that this species, not previously known to interact with eukaryotes, is capable of pathogenic or symbiotic relationships with invertebrates (Sait et al. 2011).

Accumulation of PVC genomes has also spurred genome-scale evolutionary analyses of individual genes to better understand the mechanisms of protein sequence evolution driving diversification of PVC organisms and underlying the emergence of unusual ecology, cell biology, and physiology within this group. The large phylogenetic distances separating members of the PVC superphylum challenge classical methods of evolutionary analysis and have stimulated development of novel sequence analysis approaches. Study of indel (insertion/deletion) evolution within PVC organisms has shown that indels evolve under differential selective pressure in different regions of protein-coding genes and on different lineages of the PVC superphylum (Kamneva et al. 2010). As well as providing insight into the emergence of new biological function, this study established a new framework for molecular evolutionary studies. The framework is generally applicable to any group of proteins, especially if available sequence data are sparse and separated by large evolutionary distances.

This chapter provides an overview of recent insights into the PVC superphylum that have emerged from whole-genome sequence comparisons and their implications for molecular, cellular, and evolutionary biology. The first section will review current knowledge of bacterial genome structure and the evolutionary processes contributing to this structure, followed by a summary of current progress in PVC genome sequencing, and several examples of genome-scale analysis of PVC organisms. Lastly, this chapter will introduce the PVC Genome Database—a resource for comparative genomics and evolutionary analysis of the PVC superphylum.

7.2 Structure and Evolution of Bacterial Genomes in Light of Comparative Genomics

In this section, we provide an overview of bacterial genome structure, its relevance for comparative genomics, and evolutionary processes shaping bacterial genomes. The main features of bacterial genome structure arise from fundamental molecular mechanisms facilitating either the flow of encoded genetic information from DNA to functional RNAs and proteins or vertical transfer of this information during

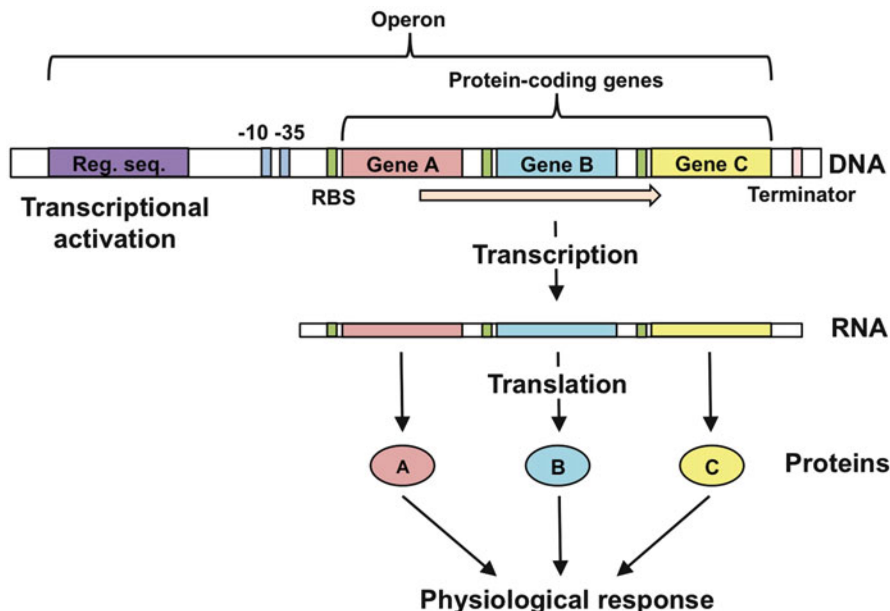

Fig. 7.1 General organization of bacterial operon and flow of genetic information in bacteria. RBS—ribosome binding site (*green rectangles*); −10, −35—TA-rich elements of a core promoter located at approximately −10 and −35 position relative to translation start site (*blue rectangles*)

reproduction. Bacterial genomes comprise single or multiple, circular or linear chromosomes, sometimes with the addition of extrachromosomal elements (plasmids). In addition to plasmids, bacterial genomes contain other mobile elements such as insertion elements, integrons, and different kinds of transposons. Bacterial genome size does not vary as much as that of eukaryotes, ranging from around 0.5 megabase pairs (Mbp) in intracellular pathogens and symbionts to 10 Mbp in developmentally complex free-living bacteria. A “typical” genome size for free-living organisms or those without obligate host associations is 4–5 Mbp.

Bacterial genomes contain stretches of DNA (usually uninterrupted by introns) encoding proteins and different types of RNAs. It is common in bacteria for protein-encoding open reading frames (ORFs) involved in a set of related processes to be organized into operons, which provide a simple general mechanism of coordinating gene expression (Fig. 7.1). Genes in an operon are regulated by a single set of regulatory elements (including promoter sequences) and are transcribed as a polycistronic RNA. The core of a single promoter generally consists of −10 and −35 (relative to the start site of transcription) AT-rich sequence segments, which are recognized by different sigma factors, accessory proteins to the RNA polymerase holoenzyme. This provides an additional mechanism of co-regulation of genes involved in producing certain physiological responses. Genetic information is further transmitted into proteins, in the case of protein-coding genes. Translation in bacteria is initiated when the 30S ribosomal subunit binds to the ribosome binding

site (RBS) preceding an ORF on the mRNA. Relative to eukaryotic genomes, bacterial genomes harbor very little noncoding DNA (generally made up of regulatory elements), explaining why genome size and gene number are strongly correlated. All the features of bacterial genomes described above are often conserved through evolution and therefore relevant to comparative and evolutionary genomics, influencing genome annotation and genome-based functional predictions.

A number of evolutionary processes shape bacterial genomes and affect coding and noncoding DNA sequences, allowing for different phenotypes to arise and creating an additional level of complexity for evolutionary analysis. The main events of genome content evolution are gene duplications and losses, and horizontal gene transfer (Mira et al. 2001; Ochman et al. 2000). Active mobile elements in bacteria are a major force for this kind of genome evolution, as they facilitate rapid genome rearrangements and acquisition of new genes. High numbers of mobile elements are known to be associated with high genome plasticity. Duplication and horizontal transfer of genes provide raw material for evolution and bring brand-new biological functions into bacterial genomes, potentially increasing an organism's fitness. However, there is an associated metabolic cost of propagating extra DNA. In the case of horizontal gene transfer, newly acquired genes might also be incompatible with the preexisting genetic background of the host and result in a fitness reduction. Mutation events (nucleotide substitutions, small insertions, and deletions) also shape the coding and noncoding regions of bacterial genomes, contributing to the evolution of novel biological functions. Within protein-coding genes, the need to maintain necessary biochemical functions often exerts considerable pressure of stabilizing selection on mutation events. Thus, the interplay between natural selection and rates of different evolutionary events allows bacterial genomes to diversify, while recombination among compatible lineages maintains the genetic integrity of the bacterial population.

7.3 History and Current Status of Genome Sequencing of PVC Organisms

The PVC superphylum includes bacteria that are interesting for diverse reasons (evolutionary, ecological, cell biological, biochemical, medical), and this has influenced the selection and timing of genome sequencing projects (Table 7.1). Organisms of phylum *Chlamydiae* are important to human health and possess genomes that are among the smallest known for cellular organisms. Therefore, genome sequencing projects for chlamydiae started rather early, with the genome of *Chlamydia trachomatis* D/UW-3/CX published in 1998 (Stephens et al. 1998) followed by that of *Chlamydophila pneumoniae* CWL029 in 1999 (Kalman et al. 1999). Further genome sequencing efforts established phylum *Chlamydiae* as a system for studying evolution of intracellular pathogens and symbionts in a comparative genomics framework, with reports on sequencing projects for *Protochlamydia amoebophila, Parachlamydia acanthamoebae,* and *Waddlia chondrophila* in 2004, 2009, and 2010 (Bertelli et al. 2010; Greub et al. 2009; Horn et al. 2004).

Table 7.1 PVC genome sequencing projects

Organism	Phylum[a]	Source	Date	Biotic relationships	Relevance
"*Candidatus* Protochlamydia amoebophila" UWE25	C	Univ of Vienna	12/1/06	Symbiotic	Medical, human pathogen
Chlamydia trachomatis D/UW-3/CX	C	UC, Berkeley	10/1/98	Human pathogen	Medical, human pathogen, animal pathogen
Chlamydophila pneumoniae CWL029	C	UC, Berkeley	4/1/99	Human pathogen	Human pathogen, medical
Parachlamydia acanthamoebae Hall's coccus	C	Univ of Lausanne	8/1/10	Symbiotic	Medical, evolutionary
Waddlia chondrophila WSU 86-1044	C	Univ of Lausanne	8/1/10	Cow pathogen	Human pathogen, animal pathogen
Lentisphaera araneosa HTCC2155	L	JCVI	12/1/07	Free-living	Marine microbial initiative (MMI), evolutionary, environmental
Victivallis vadensis ATCC BAA-548	L	DOE JGI	7/1/11	Human-associated	Medical, evolutionary, biotechnological
Blastopirellula marina SH 106T, DSM 3645	P	JCVI, MPI	12/1/06	Free-living	Marine microbial initiative (MMI), environmental
"*Candidatus* Kuenenia stuttgartiensis"	P	Genoscope	12/1/08	Free-living	Wastewater treatment, biotechnological
Gemmata obscuriglobus UQM 2246	P	JCVI	8/1/08	Free-living	Evolutionary, cellular morphology
Isosphaera pallida IS1B, ATCC 43644	P	DOE JGI	7/1/11	Free-living	GEBA[b]
Pirellula staleyi DSM 6068	P	DOE JGI	4/1/10	Free-living	GEBA
Planctomyces brasiliensis IFAM 1448, DSM 5305	P	DOE JGI	7/1/11	Free-living	Tree of life, GEBA
Planctomyces limnophilus Mu 290, DSM 3776	P	DOE JGI	8/1/10	Free-living	Tree of life, GEBA
Planctomyces maris DSM 8797	P	JCVI, MPI	12/1/07	Free-living	Marine microbial initiative (MMI), evolutionary, environmental, ecological, biotechnological
Rhodopirellula baltica SH 1	P	MPI	12/1/06	Free-living	Environmental, biotechnological
Akkermansia muciniphila ATCC BAA-835	V	DOE JGI	12/1/08	Human-associated	Medical, evolutionary
Chthoniobacter flavus Ellin428	V	DOE JGI	12/1/08	Free-living	Evolutionary
Coraliomargarita akajimensis DSM 45221	V	DOE JGI	8/1/10	Free-living	Evolutionary
Methylacidiphilum infernorum V4	V	Univ of Hawaii	12/1/08	Free-living	Physiology—extremely acidophilic methanotroph
Opitutaceae sp. TAV2	V	DOE JGI	12/1/07	Termite-associated	Energy production, biotechnological, biofuels
Opitutus terrae PB90-1	V	DOE JGI	8/1/08	Free-living	Evolutionary
Pedosphaera parvula Ellin514	V	DOE JGI	12/1/09	Free-living	Evolutionary
Verrucomicrobiales sp. DG1235	V	JCVI	8/1/10	Symbiotic	Marine microbial initiative (MMI), environmental
Verrucomicrobium spinosum DSM 4136	V	JCVI	8/1/08	Free-living	Evolutionary, unusual cellular morphology

Adapted from JGI IMG, as of March 2012

[a] *C Chlamydiae*; *L Lentisphaerae*; *P Planctomycetes*; *V Verrucomicrobia*

[b] Genome encyclopedia of bacteria and archaea (http://www.jgi.doe.gov/programs/GEBA/)

In contrast to the chlamydiae, the primary motivations for genome sequencing of planctomycetes and verrucomicrobia have been their unusual cell biology or biochemistry as well as their distinct evolutionary position on the bacterial phylogenetic tree. All characterized planctomycetes and verrucomicrobia have a common cell organization featuring an intracellular membrane, unique among the Bacteria (Fuerst 2005; Lee et al. 2009). Additionally some planctomycete bacteria exhibit variations on this common plan, featuring additional membrane-enclosed compartments of known or unknown function (Fuerst 2005). These cell biology properties helped to establish first planctomycetes, and then verrucomicrobia, as model systems for studying evolution of biological complexity and to launch several independent genome sequencing projects. The genome sequence of *Pirellula* st. 1 was the first published planctomycete genome (Glöckner et al. 2003). Discovery of the anammox reaction and marine "anammox" planctomycetes capable of this reaction established the biotechnological importance of planctomycete bacteria for the development of sustainable wastewater treatment systems and led to sequencing of the *Candidatus* K. stuttgartiensis genome (Strous et al. 2006). These initial projects laid the foundation for a new wave of experimental work in planctomycetes and verrucomicrobia, where genetic, biochemical, and functional studies have arisen from genomic information. In turn, these experimental studies have prompted new rounds of genome sequencing from additional strains, such that genomes currently available or in progress span the phylogenetic, metabolic, and lifestyle diversity of the PVC superphylum (Table 7.1).

7.4 Phylogenetic Position of PVC Organisms

The relative phylogenetic positions of organisms belonging to phyla *Planctomycetes*, *Verrucomicrobia*, *Lentisphaerae*, and *Chlamydiae* within the tree of life have been debated (Embley et al. 1994; Griffiths and Gupta 2007; Hedlund et al. 1997; Jenkins and Fuerst 2001; Van de Peer et al. 1994; Pilhofer et al. 2008; Roenner et al. 1991; Schloss and Handelsman 2004; Stackebrandt et al. 1984; Wagner and Horn 2006; Ward et al. 2000, 2006). Initially, planctomycetes were considered to be a deep-branching bacterial lineage (Roenner et al. 1991; Stackebrandt et al. 1984). This hypothesis was later rejected based on analysis of larger data sets with more sophisticated methods (Embley et al. 1994; Van de Peer et al. 1994). Some early studies suggested chlamydiae to be the closest relative of planctomycetes; later it was shown that verrucomicrobia are the closest living relatives of chlamydiae (Griffiths and Gupta 2007). More recently, it has been proposed that the four established phyla (*Planctomycetes, Verrucomicrobia, Chlamydia,* and *Lentisphaerae*) and two candidate phyla (*OP3* and *Poribacteria*) (the phylogenetic position of which has not been clearly established due to limited sequence availability for the representative species) form a coherent group of organisms named the PVC superphylum (Pilhofer et al. 2008; Schloss and Handelsman 2004; Wagner and Horn 2006). Since publication of these reports, the availability of genome sequences from additional PVC taxa has

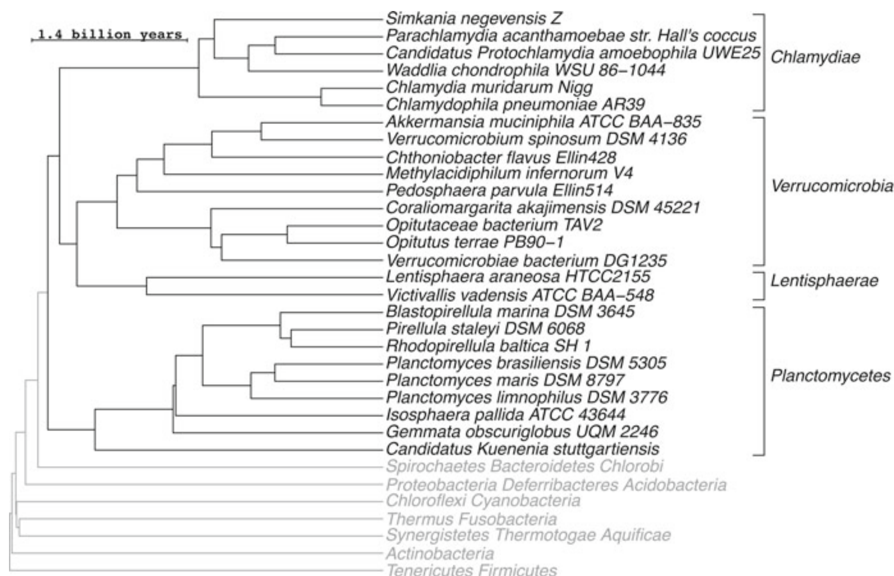

Fig. 7.2 Evolutionary relationships within the PVC superphylum. Species-tree topology was recovered for the entire set of 99 bacterial species from various bacterial phyla (including 26 PVC organisms), as a consensus tree averaging over gene trees of 41 phylogenetic markers. Divergence times were estimated using a concatenated alignment of all 41 phylogenetic markers (EngD, PrfA, SecY, MraW, NusG, ObgE, RRF, DNA primase, family 22 peptidase, 29 ribosomal proteins, and 3 tRNA synthases). Non-PVC clades were collapsed for clarity, and corresponding lineages are shown in *grey*. Names of PVC phyla are shown on the *right*. Phylum names are shown on the *right*

allowed us to evaluate the robustness and internal structure of the superphylum by including a larger number of phylogenetic markers and as many divergent PVC organisms as possible. Our large-scale phylogenetic study (Kamneva et al. 2012) used multiple protein families and a maximum-likelihood approach on concatenated phylogenetic markers to reveal the evolutionary history of 26 members of the PVC superphylum (Fig. 7.2). We observed species relationships that are largely consistent with most recently published 16S rRNA-, 23S rRNA-, and protein-based phylogenies (Hou et al. 2008; Pilhofer et al. 2008; Wagner and Horn 2006). Species within the four distinct phyla formed four well-supported monophyletic groups. Planctomycetes occupied a separate position from the rest of the superphylum, and *Kuenenia stuttgartiensis* appeared to be the most ancestral lineage among planctomycetes, as was observed in previous studies (Wagner and Horn 2006). Within the rest of the superphylum, *Lentisphaerae* formed a cluster with *Chlamydiae*, which contradicts previously published phylogenies where *Lentisphaerae* species were more closely related to phylum *Verrucomicrobia* (Hou et al. 2008). We also detected a hypothetical sister clade to the PVC superphylum containing phyla *Spirochaetes, Bacteroidetes,* and *Chlorobi* (Fig. 7.2). This relationship was also recovered in previous studies conducted using a different set of species and phylogenetic markers (Hou et al. 2008). This section is modified from reference (Kamneva et al. 2012), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.5 General Features of PVC Genomes

The major features of the sequenced PVC genomes are summarized in Table 7.2. The number of predicted protein-coding genes ranges from 940 in *Chlamydia muridarum* to 7,989 in the planctomycete *Gemmata obscuriglobus*. Gene number is highly correlated with lifestyle and suggests substantial variation in gene loss and gain rates among different evolutionary lineages. The genomes differ in total number of rRNA genes (16S, 5S, and 23S rRNA genes), which ranges from 3 to 4 in some *Planctomycetes* and *Verrucomicrobia* species to 12–15 in others. The underlying rRNA copy number variation somewhat correlates with the number of tRNA genes (Table 7.2). The number of mobile elements (approximated using the number of transposase genes) in a given genome was variable, ranging from none in the genomes of *C. muridarum* and *C. pneumoniae* to 3–4 % of all the protein-coding genes in *W. chondrophila*, *G. obscuriglobus*, and *L. araneosa*. This suggests different levels of genome plasticity in these organisms. Several chlamydial organisms harbor plasmids. Homologs of many plasmid-encoded genes are found on the main chromosomes of plasmid-free chlamydial strains, suggesting the presence of the plasmid in the ancestor of extant *Chlamydiae* (Collingro et al. 2011).

7.6 Analysis of Genome Properties

We investigated the genomic content of 24 PVC superphylum members through application of Genome Properties, a system that detects key biological properties encoded in prokaryotic genomes through the use of standardized computational methods and controlled vocabularies (Haft et al. 2005; Selengut et al. 2007). The output of this process consisted of more than 600 individual properties. Rendering and analysis of this large number of properties is beyond the scope of this chapter and will constitute a feature of the future PVC Genomics Database (see Sect. 7.11 below). The potential usefulness of the Genome Properties approach is illustrated here through comparative analysis of the distribution of 119 representative Genome Properties (Fig. 7.3). Visualization of the Genome Properties output through dual dendrogram heatmaps showed that, by and large, clustering of genomes according to shared genome properties (upper dendrogram) reflects established phylum-level relationships (Fig. 7.3). Minor discrepancies cannot be meaningfully interpreted due to the fact that the selection of properties for this analysis was somewhat arbitrary.

Other information that can be extracted from the heatmap analysis includes the prevalence of individual properties, with more universally distributed properties arrayed at the top of the heatmap, and less frequently occurring properties at the bottom. As might be expected, the majority of widely distributed properties are “housekeeping” functions such as the processing of the informational molecules DNA and RNA. For example, RNA polymerase (transcription) and ribosomal units (translation) are universally present, as are the GroEL/GroES and DnaK-DnaJ-GrpE chaperone systems. Other widely distributed properties include the metabolic

Table 7.2 Genome information summary

Genome name	Scaffold count[a]	CRISPR count[b]	GC (%)[c]	Genome size (bp)	CDS count[d]	Plasmid count	Genes on plasmid	rRNA count	tRNA count	Transposases and integrases count (%)	
Chlamydia muridarum MoPn/Nigg	2	0	0.4	1,080,434	911	1	7	8	57	2	0.22
Chlamydophila pneumoniae AR39	1	0	0.41	1,229,784	1,112	1	7	3	44	0	0
"*Candidatus* Protochlamydia amoebophila" UWE25	1	1	0.35	2,414,465	2,031	0	–	9	53	2	0.1
Parachlamydia acanthamoebae Hall's coccus	95	0	0.39	2,971,261	2,809	0	–	9	35	0	0
Waddlia chondrophila WSU 86-1044	2	0	0.44	2,131,905	1,956	1	22	3	38	90	4.6
Lentisphaera araneosa HTCC2155	81	1	0.41	6,023,180	5,104	0	–	4	58	164	3.21
Victivallis vadensis ATCC BAA-548	27	2	0.59	5,294,868	4,065	0	–	6	46	66	1.62
Blastopirellula marina SH 106T, DSM 3645	64	1	0.57	6,653,746	6,025	0	–	13	84	56	0.93
"*Candidatus* Kuenenia stuttgartiensis"	5	4	0.41	4,218,325	4,663	0	–	9	48	41	0.88
Gemmata obscuriglobus UQM 2246	922	10	0.67	9,161,841	7,989	0	–	14	55	263	3.29
Isosphaera pallida IS1B, ATCC 43644	2	2	0.62	5,529,304	3,763	1	32	3	46	1	0.03
Pirellula staleyi DSM 6068	1	2	0.57	6,196,199	4,773	0	–	3	66	11	0.23
Planctomyces brasiliensis IFAM 1448, DSM 5305	1	2	0.56	6,006,602	4,811	0	–	4	65	28	0.58
Planctomyces limnophilus Mu 290, DSM 3776	2	1	0.54	5,460,085	4,304	1	60	2	35	6	0.14
Planctomyces maris DSM 8797	125	0	0.5	7,777,997	6,480	0	–	4	59	23	0.35
Rhodopirellula baltica SH 1	1	0	0.55	7,145,576	7,325	0	–	3	46	90	1.23
Akkermansia muciniphila ATCC BAA-835	1	2	0.56	2,664,102	2,176	0	–	6	45	8	0.37
Chthoniobacter flavus Ellin428	62	0	0.61	7,848,700	6,716	0	–	4	61	33	0.49

Coraliomargarita akajimensis DSM 45221	1	0	0.54	3,750,771	3,136	0	–	4	58	8	0.26
Methylacidiphilum infernorum V4	1	3	0.45	2,287,145	2,472	0	–	3	76	6	0.24
Opitutaceae sp. TAV2	529	1	0.61	4,954,527	4,036	0	–	3	35	64	1.59
Opitutus terrae PB90-1	1	0	0.65	5,957,605	4,632	0	–	3	43	30	0.65
Pedosphaera parvula Ellin514	102	0	0.53	7,414,222	6,510	0	–	12	63	23	0.35
Verrucomicrobiales sp. DG1235	6	1	0.54	5,775,745	4,909	0	–	6	58	19	0.39
Verrucomicrobium spinosum DSM 4136	1	2	0.6	8,220,857	6,509	0	–	6	37	70	1.08

[a]Number of scaffolds containing assembled sequences
[b]*CRISPR* clustered regularly interspaced short palindromic repeats, a class of bacterial and archaeal repetitive elements
[c]mol% guanosine + cytosine
[d]*CDS* coding sequence

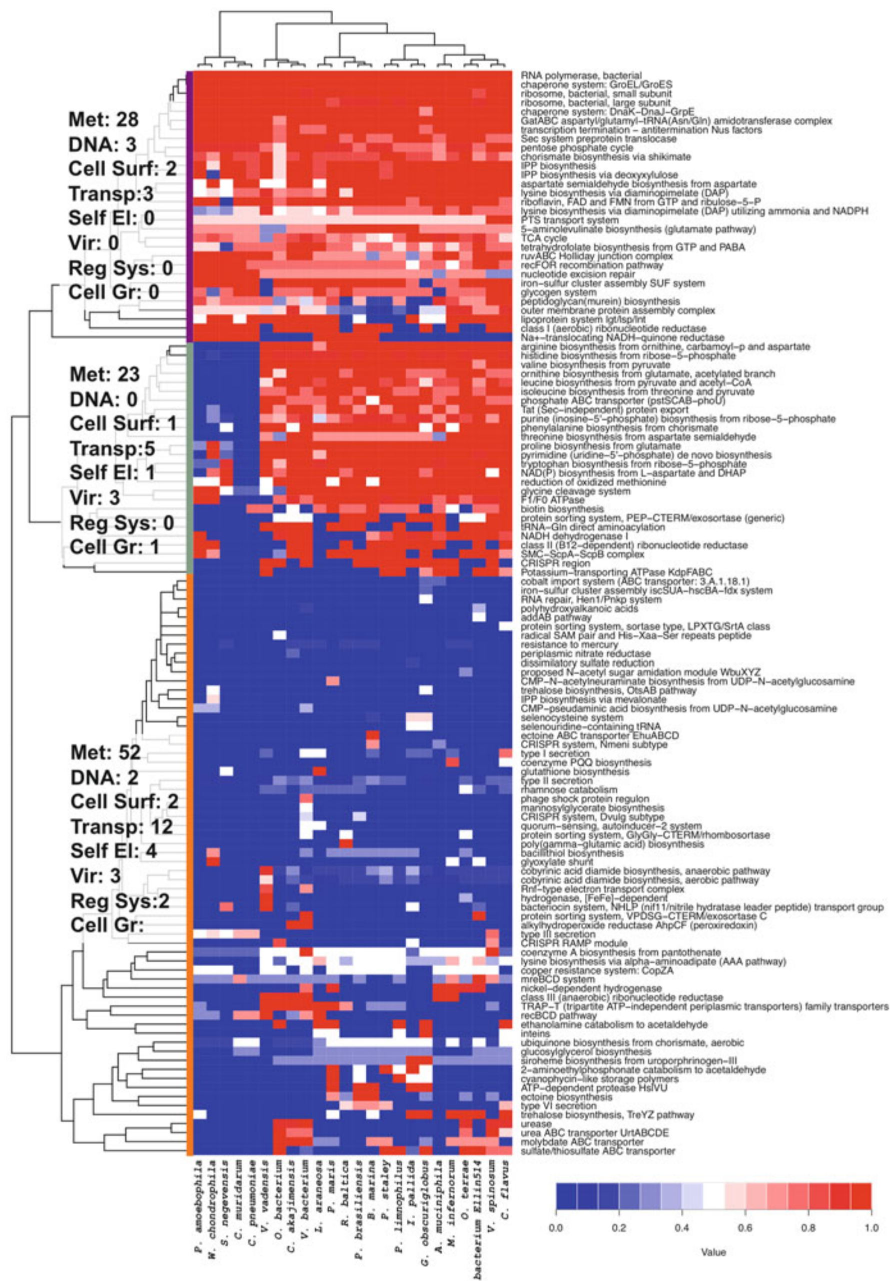

Fig. 7.3 Genome Properties analysis of PVC organisms. Colors on the heatmap represent the probability of any given genome possessing a certain property. The rows and columns of the heatmap are arranged according to hierarchical clustering of Euclidian distances for rows and columns, respectively. Names of the properties are shown on the *right*; species names are at the *bottom* of the figure. Three major clusters of the properties are denoted by *purple*, *grey*, and *orange* bars on the *left* of the heatmap. The counts of properties for major functional categories are shown for every

pathways such as the TCA and pentose phosphate cycles, as well as glycogen storage. DNA repair pathways are also represented in this group of “universal” or “almost universal” properties, which form the top cluster in the left-hand dendrogram. At the bottom of this cluster are five properties which show less universal distribution, including peptidoglycan (murein) biosynthesis. As expected, the planctomycetes lack (all or part of) peptidoglycan biosynthesis, but it is interesting to note that the verrucomicrobial termite symbiont *Opitutaceae* bacterium TAV2 also appears to lack peptidoglycan (based on failure to detect the required components: D-alanine–D-alanine ligase, murABCEFGI, and peptidoglycan biosynthetic transglycosylases). While the presence/absence of peptidoglycan in the cell wall of *Opitutaceae* bacterium TAV2 is not established, peptidoglycan-less verrucomicrobia have been previously reported (Yoon et al. 2010), but genomes are not available for these *Cerasicoccus* species and thus could not be included in our Genome Properties analysis.

Two additional clusters of properties can be observed. The upper part of the middle cluster on the figure displays a strong segregation of phylum *Chlamydiae* from the other phyla, based primarily on their known lack of many amino acid synthesis pathways. Other properties separating the *Chlamydiae* from other PVC phyla include the lack of phosphate ABC transporters, Tat (Sec-independent) protein export, and the synthesis of inosine-5-phosphate from ribose-5-phosphate. In the lower part of the middle cluster, properties that differ in distribution within the *Chlamydiae* can also be seen. These include the presence in *Waddlia chondrophila* (uniquely among the *Chlamydiae* examined) of proline biosynthesis from glutamate and de novo uridine-5-phosphate biosynthesis, as previously reported by Bertelli et al. (2010). As also found in *P. amoebophila*, *W. chondrophila* possesses a glycine cleavage system, class II (B12-dependent) ribonucleotide reductase, and F1/F0 ATPase (Bertelli et al. 2010). Other variable properties within the *Chlamydiae*

Fig. 7.3 (continued) cluster of the properties (Met = metabolism; DNA = DNA handling; Cell Surf = cell surface component; Transp = transport; Self El = selfish genetic elements; Vir = virulence; Reg Sys = regulatory systems; Cell Gr = cell growth, organization, and division). Organism names are abbreviated as follows: *S. negevensis* (*Simkania negevensis* Z), *P. acanthamoebae* (*Parachlamydia acanthamoebae* str. Hall’s coccus), *P. amoebophila* (*Candidatus* Protochlamydia amoebophila UWE25), *W. chondrophila* (*Waddlia chondrophila* WSU 86-1044), *C. muridarum* (*Chlamydia muridarum* Nigg), *C. pneumoniae* (*Chlamydophila pneumoniae* AR39), *A. muciniphila* (*Akkermansia muciniphila* ATCC BAA−835), *V. spinosum* (*Verrucomicrobium spinosum* DSM 4136), *C. flavus* (*Chthoniobacter flavus* Ellin428), *M. infernorum* (*Methylacidiphilum infernorum* V4), *P. parvula* (*Pedosphaera parvula* Ellin514), *C. akajimensis* (*Coraliomargarita akajimensis* DSM 45221), *O. bacterium* (Opitutaceae bacterium TAV2), *O. terrae* (*Opitutus terrae* PB90−1), *V. bacterium* (Verrucomicrobiae bacterium DG1235), *L. araneosa* (*Lentisphaera araneosa* HTCC2155), *V. vadensis* (*Victivallis vadensis* ATCC BAA−548), *B. marina* (*Blastopirellula marina* DSM 3645), *P. staleyi* (*Pirellula staleyi* DSM 6068), *R. baltica* (*Rhodopirellula baltica* SH 1), *P. brasiliensis* (*Planctomyces brasiliensis* DSM 5305), *P. maris* (*Planctomyces maris* DSM 8797), *P. limnophilus* (*Planctomyces limnophilus* DSM 3776), *I. pallida* (*Isosphaera pallida* ATCC 43644), *G. obscuriglobus* (*Gemmata obscuriglobus* UQM 2246), *K. stuttgartiensis* (*Candidatus* Kuenenia stuttgartiensis)

separate the amoebal symbionts from the mammalian chlamydiae *C. pneumoniae* and *C. muridarum*.

The last property cluster (lower part of diagram) contains properties that occur infrequently, and in some cases, in only one genome of the PVC representatives analyzed. Unique occurrences include an ectoine ABC transporter in *B. marina*. Ectoine is a compatible solute, suggesting a mechanism for salt homeostasis in *B. marina*, which is quite halotolerant (Schlesner and Stackebrandt 1986). Genes required for the production of a biosynthetically related compatible solute, 5-hydroxyectoine, have been previously reported in *B. marina* (Bursy et al. 2007). Glutathione, another compound that protects against cellular stresses (Masip et al. 2006), appears to be uniquely synthesized in *L. araneosa*. A third stress compound, polygammaglutamate (which also has a role for pathogenesis), is predicted only for *R. baltica*, and the genomic potential for this synthesis has been previously reported (Candela et al. 2010). These examples, and others which will be revealed by full comparative analysis of Genome Properties for the PVC superphylum, provide interesting starting points and testable hypotheses for future experimental work.

7.7 Genes Encoded in PVC Genomes

Robust identification of a gene family set (genes derived from the same ancestral gene) is a necessary first step for reliable evolutionary/comparative genomic analysis of any group of organisms. By using the computational procedure described and implemented previously within OrthoMCL software (Li et al. 2003), an initial set of protein families was constructed. Many clusters obtained included paralogous genes and xenologs that evolved via duplication and HGT events at different stages of evolution. On the other hand, many gene families contained just one member (singletons). This can be attributed to the fact that construction of gene families for distantly related taxa, such as different bacteria of the PVC superphylum, leads to smaller groups and a greater fraction of one-member clusters. Altogether, we identified 17,608 gene families that included two or more sequences from PVC genomes and the outgroup genome, leaving 63,679 singletons with no detected close homologs within other PVC genomes.

The conserved core of genes, present in all 25 PVC species under consideration and 74 outgroup genomes, consisted of 44 gene families. Analysis of functional distribution of these core gene families showed that the majority encode components of the information-processing systems (translation, transcription, and replication) and some enzymes from core biosynthetic pathways. Furthermore, 13, 35, 64, and 2 gene families were exclusively detected within the PVC phyla *Planctomycetes, Lentisphaerae, Chlamydiae,* or *Verrucomicrobia*, respectively. One of these genomic markers for planctomycete bacteria encodes proteins containing a cytochrome C assembly protein (PF01578) domain. In all planctomycete species, these genes were located next to the glutamyl-tRNA reductase gene and might be co-regulated with this housekeeping gene. A second planctomycete genomic marker was a gene family encoding proteins containing the domain found at the N-terminus of the

chaperone SurA (PF13624). These genes were located in close proximity to those encoding DNA topoisomerase I.

Another interesting genetic module not associated with a particular phylum, but rather conserved across a number of *Planctomycetes, Lentisphaerae,* and *Verrucomicrobia* species, features the domain of unknown function DUF1501. A large number of genetic clusters containing PSCyt1/PSCyt2/PSD1 and DUF1501 (*Planctomycetes*-specific cytochromes and *Planctomycetes*-specific domain of unknown function) containing proteins of varying domain composition and structure are preferentially encoded in the genomes of *I. pallida, G. obscuriglobus, Planctomyces* and *Pirellula* species, *P. parvula, V. spinosum, C. flavus, C. akajimensis,* and *L. araneosa*. The most complex gene clusters included four genes: (1) DUF1501, sometimes with twin-arginine signal peptide, (2) protein with weak support for one or several PPC domains normally found in secreted bacterial peptidases (Yeats et al. 2003) and conserved regions without characterized signatures, (3) PSCyt1/Big_2/PSCyt2/PSD1 protein, and (4) PSCyt1/WD40 protein. The three former proteins also contain predicted type I signal peptides. Domains Big_2 and WD40 are known to be involved in protein–protein interaction (Kelly et al. 1999; Xu and Min 2011) and probably are responsible for protein complex assembly or substrate recognition. Twin-arginine signal peptide is often found in proteins transported through the membrane in the folded state because of prosthetic groups acquired in the cytoplasm. *Planctomycetes*-specific cytochrome domains contain a highly conserved CxxCH motif responsible for heme binding within other cytochrome domains. All these suggest that these proteins form a complex either outside the cell or within the periplasm and carry out undetermined enzymatic reactions.

7.8 Influence of Indel Substitutions on Evolution of Protein-Coding Genes in PVC Genomes

Indel substitutions represent a common type of sequence variation contributing to the evolution of both coding and regulatory/noncoding sequences (Brandstrom and Ellegren 2007; Britten et al. 2003; Britten 2002; Chan et al. 2007, 2010; Osterberg et al. 2002; Podlaha et al. 2005; Podlaha and Zhang 2003; Schully and Hellberg 2006). As with amino acid replacement substitutions, interplay between natural selection and other factors results in the differential fixation of indels. In case studies of individual genes, including the Catsper1 calcium ion channel genes in mammals (Podlaha et al. 2005; Podlaha and Zhang 2003) and the Acp26Aa gene in *Drosophila* species (Schully and Hellberg 2006), it has been found that positive diversifying selection acts upon indels. In Kamneva et al. (2010), we have expanded on this general concept in a genome-wide study of indel substitutions. To investigate the patterns of selective constraints on indel substitutions in a genome-wide manner, we estimated secondary structure-specific insertion and deletion rates for every lineage of every gene family in the data set using gapped ancestral sequence reconstruction (Edwards and Shields 2004) and then compared the observed

distribution of insertion/deletion rates with the expected distributions obtained using simulations under neutral conditions. We applied this approach to a data set of 17 genomes from members of the PVC superphylum to evaluate how insertions and deletions have affected the evolution of this group (Kamneva et al. 2010). This section is modified from reference (Kamneva et al. 2010), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.8.1 Rates of Indel Substitutions in Proteins from PVC Genomes

It has been previously shown that different types of secondary structure have different susceptibility to insertions and deletions (Benner and Gerloff 1991). Loops or coils accommodate indels more easily than alpha-helices or beta-strands. To evaluate secondary structure-specific patterns of indel substitutions, alignments in every gene family were split based on predicted secondary structure. Branch lengths of gene trees were reevaluated using generated alignment partitions, and insertion/deletion rates were recalculated for every branch of every gene phylogeny for each type of secondary structure (loops, alpha-helices, and beta-strands). As expected, most gene tree lineages show no insertion or deletion events. However, in full-length proteins and in loops, there is a more pronounced local maximum of density at about five insertions/deletions per unit of sequence divergence per unit of alignment length (Fig. 7.4). This section is modified from reference (Kamneva et al. 2010), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.8.2 Indel Size Distribution

This study represents the first analysis of indel substitutions in the genomes of distantly related organisms, providing insights into the general characteristics of insertions and deletions in the set of divergent protein sequences as well as into their patterns of selective constraints. We identified 37,365 insertion and 53,557 deletion events along the branches of the gene trees in full-length alignments. Observing larger number of deletions than insertions is consistent with what has been shown in other studies of protein-coding sequences from nematodes (Wang et al. 2009) and in a rat/mouse comparison (Taylor et al. 2004). It seems that the presence of small genomes from chlamydial species might have influenced our results for insertion/deletion frequency; it has been shown in eukaryotes that DNA loss is one of the underlying mechanisms of genome shrinkage (Petrov 2002). However, we examined the evolution of individual genes, whereas processes associated with dramatic genome size changes in pathogenic bacteria occur on a larger scale with loss of whole genes or large parts of genomes containing several open reading frames (Gregory 2004; Mira et al. 2001; Moran and Mira 2001; Nilsson et al. 2005).

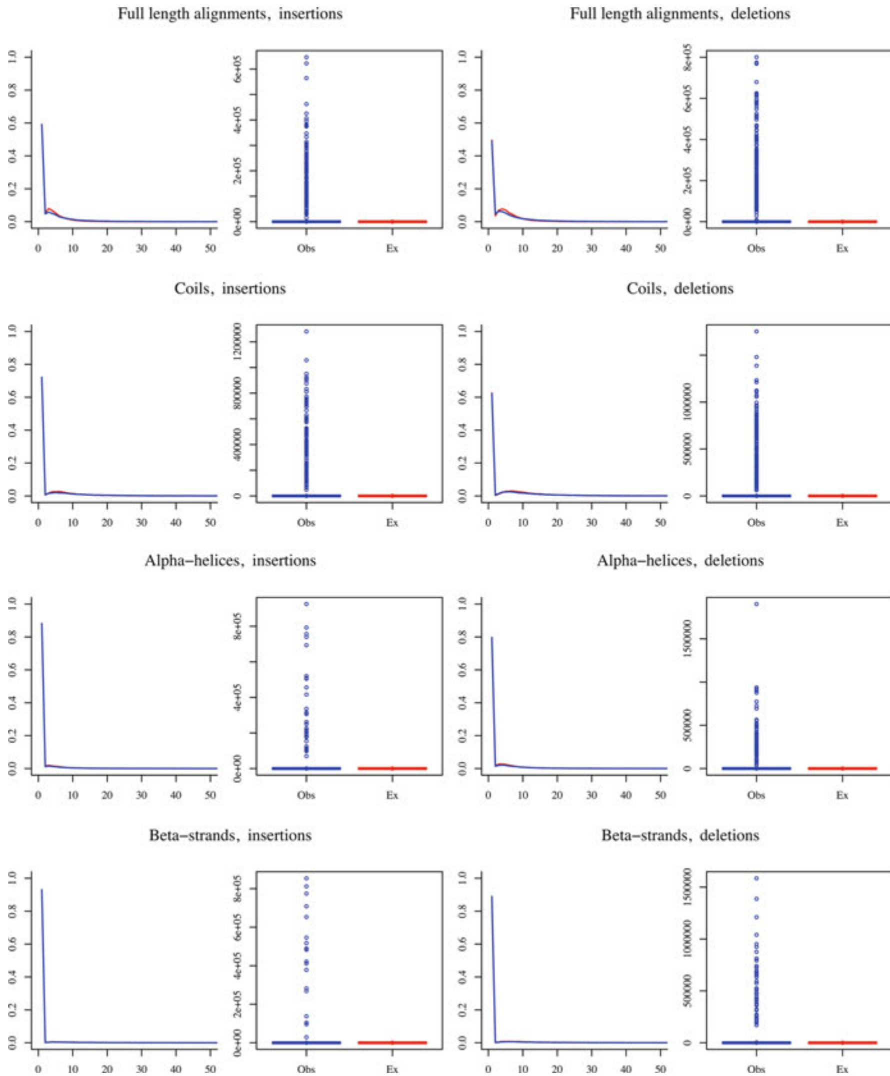

Fig. 7.4 Expected and observed insertion/deletion rate distributions derived from gene families encoded in PVC genomes. Adapted from Kamneva et al. (2010). Rate distributions are shown for every type of secondary structure (coils, alpha-helices, and beta-strands) and for full-length alignments. For every type of event (insertions and deletions), distributions are depicted with a histogram (*x*-axes: event rates, number of events from 0 to 50 per unit of evolutionary distance, per unit of alignment length; *y*-axes: density) and a boxplot of the entire data set (*x*-axes: class of the data; *y*-axes: event rates, number of events per unit of evolutionary distance, per unit of alignment length). In both cases, *blue* and *red* colors denote observed (Obs) and expected (Ex) distributions, respectively (Adapted from Kamneva et al. 2010)

The longest insertion identified in our data set was 217 amino acids, whereas the longest deletion was 190 amino acids. The most common insertion or deletion event was a one amino acid-long substitution, independent of the type of secondary structure under consideration. The mean length value of observed insertions/deletions was 3.77/3.22 amino acids for full-length proteins. Observed insertions generally tended to be longer than deletions in all the types of structural elements. This section is modified from reference (Kamneva et al. 2010), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.8.3 Detecting Strength of Natural Selection on Indels

In order to be able to differentiate between varying strengths of selective pressure on indel substitutions, respective null distributions were generated for every observed distribution using randomization. Our results showed that specific branches of many gene trees possess significantly higher number of insertions/deletions than would be expected by chance. For many partitions, the maximum observed event rate is several orders of magnitude higher than the maximum rate in randomized data. An insertion/deletion rate value significantly higher than that expected by chance on a branch of the gene phylogeny is consistent with positive Darwinian selection on insertion/deletion substitutions on that particular branch. The magnitude of the indel influence on the overall evolutionary trend might be estimated as a percentage of the branches where it was possible to detect positive selection on insertions or deletions (Table 7.3). Insertions and deletions on up to 12 % of all the branches in the data set evolved under positive selection. This section is modified from reference (Kamneva et al. 2010), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.8.4 Indels in Proteins of Different Biological Functions

Selection is observed at the level of the individual gene/protein but actually occurs in the context of broader cellular biology. We used KEGG metabolic pathways (Kanehisa et al. 2010) to classify gene families in the data set and systematically identify molecular pathways affected by indel processes. We linked every gene family with information from the KEGG Molecular Pathway Database using a Blast search against the database. We were able to map all full-length gene families onto 106 groups of cellular pathways. However, the total number of pathways obtained varied depending on the specific types of secondary structure in which indels occurred. We employed a binomial test to identify pathways with positive selection on insertions/deletions of different length in varying secondary structural elements consistently overrepresented among gene families. Different types of transporters (ABC transporters, pore ion channels) as well as several pathways related to general

Table 7.3 Number (#) and percentage of branches showing evidence for positive selection on insertions (ins) and deletions (del) in different length groups and secondary structural units

Full-length	# Total	52,018			
	Length	≥1	≥2	≥3	≥4
	# Sig ins	6,466	4,858	3,059	2,018
	% Sig ins	12.43	9.34	5.88	3.88
	# Sig del	6,683	5,130	3,607	2,455
	% Sig del	12.85	9.86	6.93	4.72
Coils	# Total	47,875			
	Length	≥1	≥2	≥3	≥4
	# Sig ins	4,484	1,936	1,166	611
	% Sig ins	9.37	4.04	2.44	1.28
	# Sig del	4,802	2,740	1,631	1,216
	% Sig del	10.03	5.72	3.41	2.54
α-Helices	# Total	47,896			
	Length	≥1	≥2	≥3	≥4
	# Sig ins	1,412	611	316	197
	% Sig ins	2.95	1.28	0.66	0.41
	# Sig del	2,341	1,386	929	675
	% Sig del	4.89	2.89	1.94	1.41
β-Strands	# Total	31,962			
	Length	≥1	≥2	≥3	≥4
	# Sig ins	473	152	78	56
	% Sig ins	1.48	0.48	0.24	0.18
	# Sig del	754	488	277	216
	% Sig del	2.36	1.53	0.87	0.68

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metabolism (cysteine and methionine, thiamine, selenoamino acid, phenylalanine, sphingolipid metabolism, base excision repair, glycosaminoglycan degradation, terpenoid backbone biosynthesis, ribosome, bacterial secretion systems, and protein export) were consistently overrepresented among gene families with positive selection on insertions/deletions of different length. Noticeably, ABC-type transporters and ion-coupled transporters show elevated rates of deletions and insertions in coils. This may suggest a general pattern of evolution for these types of proteins. Insertions (deletions) in coiled regions might change the structural composition of the protein by introducing (eliminating) structural elements, in the case of long indels containing alpha-helices or beta-strands. In the case of indels that do not affect structural composition of the protein, they may alter flexibility of the existing protein fold in terms of positioning of structural elements relative to each other or to binding partners. In some cases, this might also change the thermodynamic stability of proper protein folding (Meenan et al. 2010; Viguera and Serrano 1997). As described below, we examined the structural and functional consequences of indel events in example gene families that exhibited evidence for positive selection on indel substitutions. This section is modified from reference (Kamneva et al. 2010), with permission of Oxford University Press, Society for Molecular biology and Evolution.

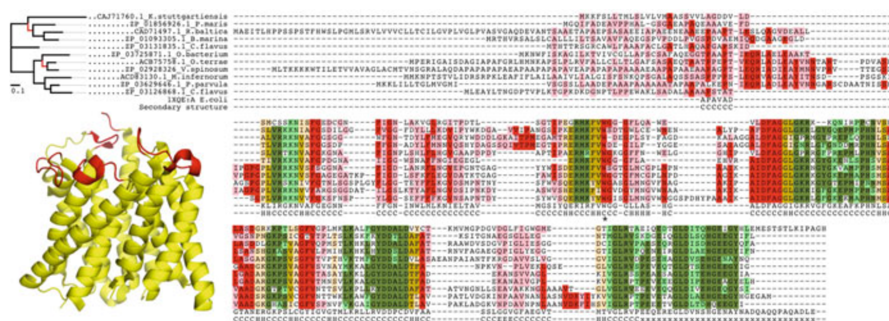

Fig. 7.5 Ammonium transporter protein family and representatives in the PVC organisms. Gene family with insertions in coils under positive selection. Adapted from (Kamneva et al. 2010). (**a**) Phylogenetic tree of the ammonium transporter protein family (ion-coupled transporter, according to KEGG), individual sequences are designated by GenBank accession numbers and species name. Branches with significantly high level of insertions in coils are shown in *red*. Two additional names correspond to PDB accession numbers for the homologous sequence with determined tertiary structure and to the corresponding secondary structural elements identified based on tertiary structure. (**b**) Corresponding multiple sequence alignment of the members of the protein family and *E. coli* AmtB sequence from PDB (the parts of alignment corresponding to transmembrane helices have been trimmed). *Bright* and *light shadings* correspond to 50 % identical or similar (based on PAM250) residues in the sequences of the protein family. *Red* palette—periplasmic parts; *yellow* palette—trimmed transmembrane parts; *green* palette—cytoplasmic parts. *Last line* represents types of secondary structure elements, based on tertiary structure of *E. coli* AmtB 1XQE:A (Zheng et al. 2004). Residue W148 at the beginning of periplasmic coil between transmembrane helices four and five is marked with *asterisk*. (**c**) Structural model 1XQE:A of *E. coli* AmtB ammonium transporter was used to show periplasmic side coiled and **α**-helical regions with an unexpectedly high number of insertions (marked in *red*) (Adapted from Kamneva et al. 2010)

7.8.5 Insertions in Ammonium Transporter Proteins in Planctomycetes and Verrucomicrobia

One of the ion-coupled transporters with an unexpectedly high number of insertions in loop regions is the ammonium transporter from planctomycete and verrucomicrobia species (Fig. 7.5). Several branches of the gene phylogeny for this protein family exhibit elevated levels of insertions in coils. Additionally, mapping of insertions on the tertiary structure of *E. coli* AmtB showed clustering of otherwise conserved insertions in periplasmic loops. There are no known binding partners that would interact with the periplasmic domain of AmtB. However, a previous study of the *E. coli* protein allowed identification of several mutations in the periplasmic domain of the pore entrance that significantly increased ammonium uptake (Javelle et al. 2008). W148A is particularly interesting as it is located in the periplasmic coil between the fourth and fifth transmembrane helices, adjacent to a small periplasmic helical element. In the proteins of the planctomycete and verrucomicrobia clade, the periplasmic helix contained several small indels. Furthermore, part of the loop adjacent to the fifth transmembrane helix contained an additional protein segment conserved among members of the family. Ammonium has been found to induce surface attachment and

biofilm formation in *R. baltica* (Frank et al. 2011); therefore, the observed evolutionary changes in AmtB might have led to emergence of new regulatory interactions with other proteins within the periplasmic domain. An alternative explanation might be that changes in AmtB structure created a more efficient ammonium transporter, which would be a beneficial trait for organisms living in generally low-nutrient conditions, as many planctomycetes and verrucomicrobia do. This section is modified from reference (Kamneva et al. 2010), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.9 Genome Content Evolution in PVC

It is commonly acknowledged that genome dynamics (gene family acquisition, expansion, and contraction) contribute significantly to the general evolution of bacterial species as well as to the emergence of particular ecological and physiological properties of microorganisms. Genome content can vary a great deal, even between closely related bacterial species, in terms of the presence and size of particular gene families. This is mostly attributed to horizontal gene transfer and rapid loss of non-beneficial genes, due to large population sizes and high pressure of natural selection in bacteria.

Reconstruction of gene family evolution is fundamental for understanding the evolution of living organisms; a number of methods for studying genome content evolution have been developed over the years. These methods can be broadly divided into two major classes: gene-tree species-tree reconciliation-based and phyletic pattern-based methods grouped by the type of input information and parsimony-based and likelihood-based methods grouped by the utilized statistical framework. The majority of existing approaches take into account at least two major types of evolutionary events contributing to gene family evolution, i.e., gene duplication and loss. Some of them consider horizontal gene transfer as well, which makes such methods especially useful for characterizing evolution of bacterial species. This section is modified from reference (Kamneva et al. 2012), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.9.1 Gene Family Dynamics in PVC Genomes

In order to evaluate rates and patterns of gene family gain, loss, expansion, and contraction, we performed analysis of all the gene families inferred as described in Sect. 7.6 above, using a parsimonious gene-tree species-tree reconciliation procedure implemented in the AnGST program (David and Alm 2010). The algorithm identifies evolutionary events (HGT, gene duplication, and loss) necessary to explain discrepancy between gene and species phylogeny. We performed gene-tree species-tree reconciliation for every gene family which allowed us to explicitly infer the evolutionary history of every gene family in the PVC superphylum and to evaluate genome size for every ancestral genome on the PVC species tree (Kamneva et al. 2012). The summarized results of this analysis are depicted in Fig. 7.6.

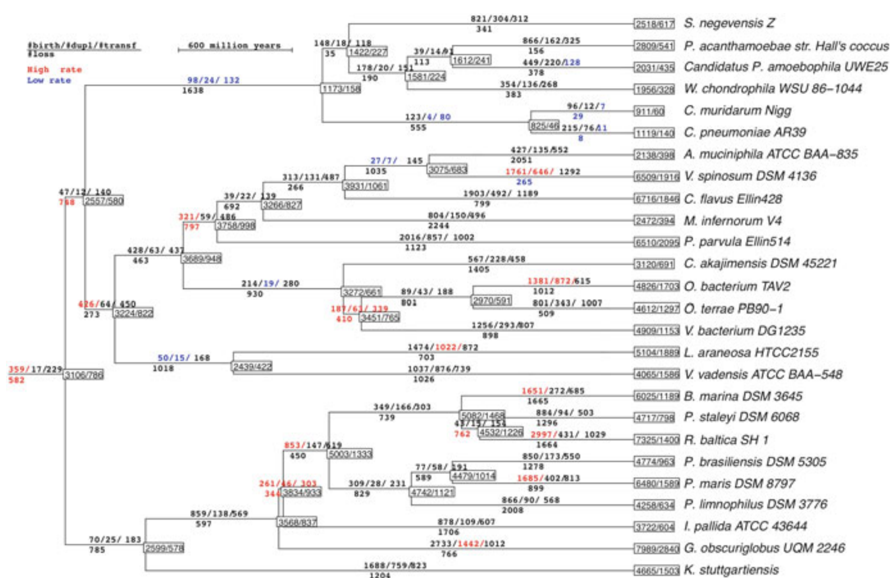

Fig. 7.6 Genome content evolution in the PVC superphylum. Adapted from Kamneva et al. (2012). Events of genome content evolution were mapped onto lineages of the species tree; only the PVC clade is shown here. Numbers at every node, either ancestral or extant, represent genome size and number of genes in multigene families (for instance, the *I. pallida* genome contains 3,722 genes, out of which 604 genes are predicted to be members of multigene families). Numbers above and below every lineage represent the number of birth/duplication/transfer and loss events, respectively, predicted to occur on the branch. Numbers shown in *red* or *blue* correspond to accelerated or decelerated rates of events on the branch (for instance, on the lineage leading to *V. spinosum*, 1,761 births, 646 duplications, 1,292 transfers, and 265 loss events occurred. This observed event count implies elevated gene birth and duplication rates on this lineage and low gene loss rate). Species names are abbreviated as in Fig. 7.3 (Adapted from Kamneva et al. 2012)

Our analysis suggested that the common ancestor of all PVC organisms had a genome containing 3,106 genes, of which 786 were predicted to be in multigene families. Thus, the origin of the four PVC phyla involved extensive loss of ancestral genes on some lineages and acquisition of novel genes through various mechanisms on other lineages. After the *Planctomycetes* split from the rest of the superphylum, a number of planctomycete lineages underwent acquisition of gene families and expansion of existing families. This process resulted in the largest genomes in the group, seen in the extant species *G. obscuriglobus* and *R. baltica,* where many new families appeared. The ancestor of *Verrucomicrobia*, *Chlamydiae,* and *Lentisphaerae* is predicted to have possessed a relatively small genome containing 2,557 genes. This ancestral gene set was shaped primarily by gene loss and gene family contraction on the lineage leading to the common ancestor of *Lentisphaerae* and *Chlamydiae* and by gene gain and gene family expansion on the lineage leading to the ancestor of *Verrucomicrobia*. The ancestral genome of *Chlamydiae* and *Lentisphaerae* was further minimized by gene loss and gene family contraction on the lineage leading to the ancestor of all *Chlamydiae* and even further on the lineage leading to obligate intracellular pathogens belonging to the genera *Chlamydia* and *Chlamydophila*.

Table 7.4 Non-PVC organisms (extant or ancestral) frequently acting as donors in lateral transfer events

Recipient	Donor (# transfer events); only organisms frequently acting as donors are shown ($p < 1e-8$)
K. stuttgartiensis	Deferribacteres (18); *D. vulgaris* Miyazaki F (25); *G. lovleyi* SZ (39); *Synergistetes* (15); *Hydrogenobacter/Persephonella/Sulfurihydrogenibium* (15)
Gemmata/Isosphaera/Pirellulaceae/Planctomyces	Candidatus *S. usitatus* Ellin6076 (45)
G. obscuriglobus UQM 2246	Candidatus *S. usitatus* Ellin6076 (49)
I. pallida ATCC 43644	*C. aggregans* DSM 9485 (22)
V. vadensis ATCC BAA-548	*Spirochaeta* sp. Buddy (21); *T. azotonutricium* ZAS-9 (22); *D. vulgaris* Miyazaki F (25)
M. infernorum V4	α-Proteobacteria (22)
P. parvula Ellin514	*T. saanensis* SP1PR4 (34); Candidatus *S. usitatus* Ellin6076 (78)
Opitutaceae/Opitutus/Verrucomicrobiae	Candidatus *S. usitatus* Ellin6076 (29)
V. bacterium DG1235	Candidatus *S. usitatus* Ellin6076 (38)
O. terrae PB90-1	Candidatus *S. usitatus* Ellin6076 (81); *D. vulgaris* Miyazaki F (24)
C. flavus Ellin428	*S. cellulosum* So ce 56 (60); Candidatus *S. usitatus* Ellin6076 (63)
A. muciniphila ATCC BAA-835	*B. fragilis* NCTC 9343 (47); *D. vulgaris* Miyazaki F (17)

Conversely, gene gain and gene duplication contributed significantly to the evolution of genomes on many lineages of phyla *Lentisphaerae* and *Verrucomicrobia*, with the exception of *A. muciniphila* and *M. infernorum* lineages. This section is modified from reference (Kamneva et al. 2012), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.9.2 Horizontal Gene Transfer Among PVC Organisms and from Members of Other Bacterial Groups

Horizontal (lateral) gene transfer (HGT) is a major source of diversity in bacterial genomes (Jain et al. 2003). The frequency of HGT between different organisms depends on a number of factors such as genome size and similarity in GC content, as well as ecological factors such as carbon source utilization and oxygen tolerance (probably pointing to similarity in ecological habitat) (Jain et al. 2003; Smillie et al. 2011). Other factors which intuitively should affect frequency of HGT between organisms include differences in codon usage and divergence of regulatory motifs between donor and recipient organisms. Modes of interaction in an ecosystem must also be critical to HGT. A number of transfer events between different organisms were detected in various gene families within our data set, asserted on the basis of gene-tree species-tree reconciliation data (Table 7.4). Several genes were predicted to be acquired from the Candidatus *Solibacter usitatus* Ellin6076 lineage on different

branches of *Planctomycetes*- and *Verrucomicrobia*-specific clades as well as from Deltaproteobacteria (*D. vulgaris* Miyazaki F and *S. cellulosum* So ce 56 lineages) on various superphylum lineages but excluding the chlamydial clade. We also detected a large number of genes transferred laterally to *A. muciniphila* from the *B. fragilis* NCTC 9343 lineage. These findings suggest previous ecological contexts shared between the recipient PVC lineages and donor lineages outside the superphylum. This section is modified from reference (Kamneva et al. 2012), with permission of Oxford University Press, Society for Molecular biology and Evolution.

7.10 Large Outer Membrane Autotransporter Barrel Domain Protein Family in Verrucomicrobia

A number of protein-sorting systems acting within bacterial cells are known. The PEP-CTERM/EpsH system has been recently proposed to facilitate outer membrane/cell wall directed trafficking of proteins in environmental microorganisms (Haft et al. 2006). The system includes two main components, the first being EpsH (exopolysaccharide locus protein H). It is predicted to act as a signal peptidase upon proteins containing a conserved carboxy-terminal PEP motif, followed by a stretch of hydrophobic residues and a short segment of positively charged amino acids (PEP-CTERM, TIGR02595) (Haft et al. 2006). *V. spinosum* has the largest known number of proteins bearing the PEP-CTERM motif. One of the proteins containing a PEP-CTERM domain is a divergent multimember family of large outer membrane autotransporter barrel domain proteins. These large proteins, in addition to PEP-CTERM, are predicted to contain an autotransporter-associated beta-strand repeat domain (PF12951) and type I signal sequences, along with a putative lipid attachment site. However, we lack exact functional predictions (and experimental validation) for these protein domains.

Sixteen autotransporter barrel domain proteins have been detected within *V. spinosum*, and two within *C. flavus*. Analysis of the genomic neighborhood revealed the presence of four hypothetical genes in proximity to the autotransporter genes. These genes are organized in one or two operons and are present only within these genomic clusters. The structure of one representative region is shown in Fig. 7.7.

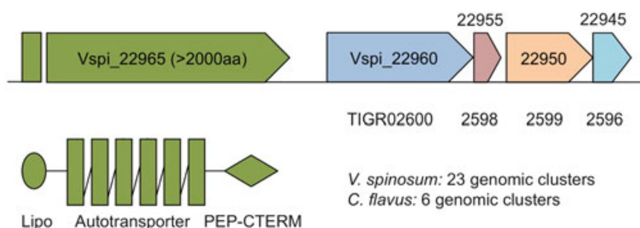

Fig. 7.7 Schematic of one representative genomic region containing large outer membrane autotransporter barrel domain protein gene, and four co-located hypothetical genes arranged in an operon. Locus names are shown for all the loci (Vspi), domain structure shown schematically for Vspi_22965, names of domains detected in four genes in the operon are shown under the map, number of genomic clusters found in *V. spinosum* and *C. flavus* genomes is indicated

While these four genes lack explicit functional assignment, they possess conserved domains TIGR02596, TIGR02600, TIGR02599, and TIGR02598, respectively. The highly organized structure of genomic regions containing these genes, along with PEP-CTERM bearing autotransporter-encoding genes, suggests functional relatedness of these proteins and EpsH. While the exact molecular mechanisms and functional importance of this association are yet to be uncovered, it is an exciting example of a gene family related to protein sorting, considering the distinctive cell plan of PVC organisms.

7.11 PVC Genomics Database

In order to facilitate comparative genomic analysis of distantly related PVC organisms, we are currently constructing the PVC Genome Database (anticipated location: http://www.pvcgenomics.org) (Kamneva et al. unpublished). The database will include all *Planctomycetes, Verrucomicrobia,* and *Lentisphaerae* species with publicly available genome sequences and representative species from phylum *Chlamydiae*. The database will contain information on organisms (including taxonomy, isolation site, culture collection accessibility, cellular structure), their genomes (status of genome project, genome structure, number of features in a genome), genes and gene products (including protein annotation, predicted PFAM protein domains, hydrophobicity patterns, signal peptides, subcellular localization, and functional sites), orthologous protein families (including high-quality orthologous groups, alignments, gene phylogeny, and history of gene families in terms of duplication, loss, and transfer events), predicted operons, orthologous groups of operons, and putative upstream genomic regions for every operon in the group (including predicted conserved DNA motifs, representing transcription factor binding sites). The database will be integrated with BLAST (Altschul et al. 1990) and Psi-Square programs (Glazko et al. 2006). BLAST will be used to search for sequences similar to a query sequence and sequences in the PVC Genome Database. The Psi-Square program will be used to search for features with specific phyletic patterns. The PVC Genome Database will aim to provide a high-quality genomics and evolutionary biology resource for the PVC research community and will be freely available for use.

7.12 Concluding Remarks

The field of PVC superphylum genomics and bioinformatics is currently at an exciting stage. The PVC research community, together with sequencing center partners, has generated genome data and analysis from organisms representing the breadth and diversity of the superphylum. These data are invaluable for understanding the genomic basis for various intriguing properties of PVC superphylum members, predicting previously unknown properties, and formulating hypotheses

for experimental testing. They also allow us to begin to unravel the evolutionary history of these fascinating organisms and their genomes. Lastly, the data challenge us to develop new tools for optimally extracting biologically significant information from the genomes. We hope that this brief survey of PVC comparative genomics and bioinformatics provides a useful resource for the PVC research community and stimulates both deeper bioinformatic analysis and new experimental studies.

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Chapter 8
The Distribution and Evolution of C1 Transfer Enzymes and Evolution of the Planctomycetes

Ludmila Chistoserdova

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Abbreviations

H_4MPT	Tetrahydromethanopterin
C1	Single carbon atom as in C1 compound, organic compound containing a single carbon atom
MtdB	NAD(P)-linked methylene-H_4MPT dehydrogenase
MFR	Methanofuran
H_4MPT	Tetrahydromethanopterin
F_{420}	Coenzyme F_{420}
CoM	Coenzyme M
CoB	Coenzyme B. Fae formaldehyde-activating enzyme
MtdB MtdC	Methylene-H_4MPT dehydrogenases

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Mch	Methenyl-H_4MPT cyclohydrolase
FhcABCD	Formyltransferase/hydrolase complex
FdwD	Is homologous to the D subunit of formyl-MFR dehydrogenase
LUCA	Last universal common ancestor

8.1 Introduction: History and Significance of the Question

Since their discovery, Planctomycetes have continued to be a fascinating group of organisms as they possess properties not typical of other bacteria, such as a cell wall lacking peptidoglycan reminiscent of Archaea, intricate cell compartmentalization reminiscent of Eukaryotes, division by budding reminiscent of yeasts, and unique metabolites such as sterols produced by the *Gemmata* species and ladderane lipids produced by autotrophic ammonia-oxidizing planctomycetes (Fuerst and Sagulenko 2011). When the first genomic sequence of a Planctomycete, of *Pirellula* sp. strain 1 (since renamed as *Rhodopirellula baltica*; Schlesner et al. 2004) was sequenced in 2003, it revealed another unusual feature, the presence of genes for C1 metabolism and more specifically genes encoding tetrahydromethanopterin (H_4MPT)-linked reactions for C1 transfers (Glöckner et al. 2003). Why was this discovery so significant? It was significant because it identified Planctomycetes as the third major phylum to possess genes for reactions requiring H_4MPT as a cofactor and a second phylum within the bacterial domain. Only a few years before, such genes were serendipitously discovered in a methylotrophic bacterium, *Methylobacterium extorquens*, and demonstrated to be indispensable for growth on C1 substrates such as methanol (Chistoserdova et al. 1998), which was followed by the identification of these genes in a number of other methylotrophic Proteobacteria (Vorholt et al. 1999), for the first time suggesting that functions thought to be unique to a limited group of Archaea, specifically methanogenic and sulfate-reducing Archaea, all classed within the kingdom Euryarchaeotes, may be in fact more widespread. However, gene/protein homologs from the Archaea and the Proteobacteria were only distantly related. In addition, the pathways they encoded were parts of distinctly different biochemical processes, i.e., methanogenesis (reducing CO_2 to methyl) and methylotrophy (oxidizing methyl to CO_2; Fig. 8.1). As the involvement of the H_4MPT-linked functions in methanogenesis and methylotrophy established the common root of the two bioconversions, the question arose of their evolutionary history. Which pathway evolved first? Did the methanogenesis precede the methylotrophy or vice versa? The possibility of lateral gene transfer between Euryarchaea and Proteobacteria has been discussed in this context, and the most probable direction of the transfer was assumed to be from Archaea into Bacteria (Chistoserdova et al. 1998; Vorholt et al. 1999; DeLong 2000; Gogarten et al. 2002; Boucher et al. 2003; Martin and Russell 2003). A scenario of lateral transfer of these genes from a (aerobic) proteobacterial methylotroph into a euryarchaeon was also suggested (Cavalier-Smith 2002), but this scenario would necessitate aerobic methylotrophy preceding anaerobic methanogenesis, which contradicts the current understanding of the history of Earth's atmosphere (Kasting and Siefert 2002). The discovery of the H_4MPT-linked C1 transfer function in the Planctomycetes, a deeply

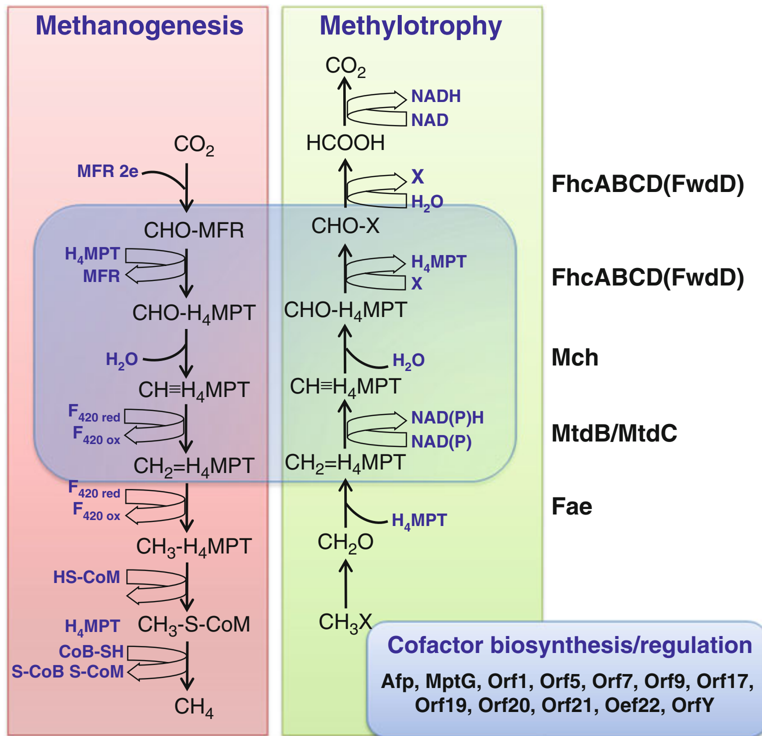

Fig. 8.1 The commonality of methanogenesis and methylotrophy. Analogous reactions are highlighted by a *blue box*. *MFR* methanofuran, *H_4MPT* tetrahydromethanopterin, *F_{420}* coenzyme F_{420}, *CoM coenzyme M*, *CoB* coenzyme B, *Fae* formaldehyde-activating enzyme, *MtdB*, *MtdC* methylene-H_4MPT dehydrogenases, *Mch* methenyl-H_4MPT cyclohydrolase, *FhcABCD* formyltransferase/hydrolase complex, FwdD is homologous to the D subunit of formyl-MFR dehydrogenase

branching division of Bacteria (Brochier and Philippe 2002; Di Giulio 2003), provided a missing link in understanding the history of metabolism and offered an opportunity for refining the picture of the evolution of methanogenesis and methylotrophy, as well as for a better understanding of the evolution of C1 metabolism on Earth.

8.2 Phylogenetic Analysis of C1 Transfer Enzymes in Planctomycetes

One of the major outcomes of discovering the third deeply branching microbial group possessing H_4MPT-linked functions was the potential to obtain new insights into the possible scenarios for the evolution of these functions and to test the then currently prevalent hypothesis of lateral transfer of these genes from Euryarchaeota to Proteobacteria (Gogarten et al. 2002; Boucher et al. 2003; Martin and Russell

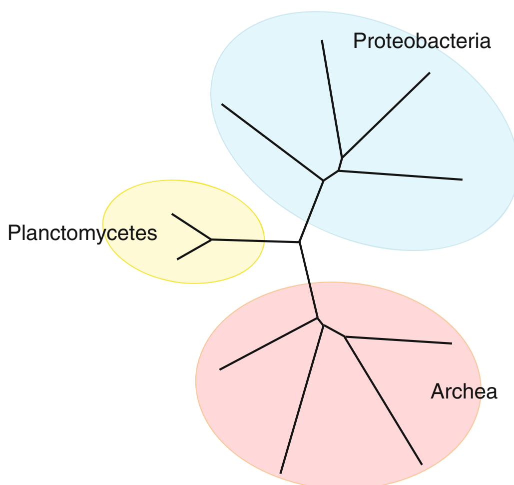

Fig. 8.2 A cartoon depicting a typical topology of phylogenetic trees resolving positions of Planctomycete C1 transfer pathway peptides with respect to the counterparts from Euryarchaea and Proteobacteria

2003). Two groups independently carried out phylogenetic analysis of the polypeptides translated from the C1 genes shared between Planctomycetes (at the time represented by two genera, *Rhodopirellula* and *Gemmata*), Proteobacteria (both methylotrophs and non-methylotroph species), and Archaea (both methanogens and sulfate-reducing species). Chistoserdova and colleagues analyzed a total 16 polypeptides (Chistoserdova et al. 2004), and Bauer and colleagues analyzed a total of seven polypeptides (Bauer et al. 2004). In both cases, phylogenetic analyses showed that, in general, the polypeptide counterparts from Planctomycetes appeared to be distant from both their archaeal homologs and from their proteobacterial homologs, in most cases forming a distinct third group on phylogenetic trees, with significant bootstrap confidence for the node defining the group's monophyly (Fig. 8.2). Notably, this pattern was revealed by the methenyl-H_4MPT cyclohydrolase (Mch) polypeptides that were previously assumed to be some of the most reliable enzymes for following the evolutionary history of methanogenesis (and likely of C1 transfers in bacteria) based on the criteria of its essential function, the lack of duplication in any known organism (at least at that time), and the absence of substitution by functionally equivalent enzymes (Reeve et al. 1997). Mch phylogeny also seemed to agree with the 16S rRNA phylogeny in both Euryarchaeota (Reeve et al. 1997) and Proteobacteria (Vorholt et al. 1999). The data from the phylogenetic analyses suggested that a single event was responsible for the emergence of the functions in question for each major phylum possessing them. However, some of the trees built in these studies showed deviations from this common pattern. In some cases, tree topologies were complicated by the presence of multiple gene homologs, potentially reflecting a more complex evolution of these genes, which suggested early duplications, as well as early and recent gene transfers for some of the genes (Chistoserdova et al. 2004). Another deviation from the common pattern was noted for some of the genes/enzymes in *Rhodopirellula baltica*, specifically for the polypeptides involved in the formyltransferase/hydrolase (Fhc) complex, which tended to cluster within the proteobacterial branch instead of clustering with the *Gemmata* sequences

(Chistoserdova et al. 2004; Bauer et al. 2004), such clustering potentially reflecting either specific selective pressures or results of lateral transfers from Proteobacteria.

As many as seven alternative scenarios were invoked to explain the presence of the H_4MPT-linked C1 transfer genes and their evolution in the three phylogenetically separated prokaryotic divisions: Euryarchaeota, Planctomycetes, and Proteobacteria. These included (a) their presence in the last universal common ancestor (LUCA) followed by gene loss in most of the divisions, as well as most of the Proteobacteria (Chistoserdova et al. 2004; Bauer et al. 2004); (b) lateral transfer from Euryarchaeota into the bacterial domain before the separation of Planctomycetes and Proteobacteria (Chistoserdova et al. 2004; Bauer et al. 2004); (c) independent transfers from Euryarchaeota into Planctomycetes and into Proteobacteria after the latter groups separated (Chistoserdova et al. 2004; Bauer et al. 2004); (d) transfer from Euryarchaeota into Proteobacteria and later from Proteobacteria into Planctomycetes; (e) transfer into Planctomycetes and later from Planctomycetes into Proteobacteria (Bauer et al. 2004); (f) emergence of the genes in Proteobacteria with subsequent independent lateral transfers into Euryarchaeota and Planctomycetes; and (g) emergence in Planctomycetes followed by independent transfers into Proteobacteria and Euryarchaeota (Chistoserdova et al. 2004). While Bauer et al. (2004) have postulated that in all scenarios Euryarchaeotes had to be the ancestral carrier of the H_4MPT-linked C1 transfer genes, Chistoserdova et al. (2004) favored scenarios in which the genes in question were either present in the LUCA or have emerged in Planctomycetes, the conclusion mainly based on the topology of the phylogenetic trees and based on the presumed antiquity of the Planctomycetes (Brochier and Philippe 2002; Di Giulio 2003). It was concluded that, in both scenarios, a selective pressure would be required to prevent the loss of the entire complement of the genes. Thus, for early life on Earth, a fitness advantage corresponded by this pathway could be predicted. Formaldehyde is thought to have been abundant on early Earth (Arrhenius et al. 1994). Therefore, it was argued that early cells could benefit from a system to reduce the toxic effect of formaldehyde, the role that could have been carried out by the H_4MPT-linked C1 transfer pathway in the early Planctomycetes. At later stages, an additional fitness could be derived from the ability to draw energy from these reactions. Whichever scenario was true, it appeared that the H_4MPT-linked C1 transfer pathway between the oxidation levels of formaldehyde and formate was likely an early, important function for life, which provided the essential building block in the formation of both methanogenesis and methylotrophy pathways (Chistoserdova et al. 2004).

8.3 MtdC: A Novel Methylene-H_4MPT Dehydrogenase Found in Planctomycetes

While most of the enzymes involved in the H_4MPT-linked C1 transfer pathway are shared between Bacteria and Archaea, some are Bacteria specific. One of these enzymes, the NAD(P)-linked methylene-H_4MPT dehydrogenase (MtdB), that is unique to Bacteria operates in the pathway in place of its functional counterparts,

that are linked to H_2 or cofactor F_{420} (Fig. 8.1). Based on the lack of sequence similarity, MtdB must have evolved independently of the archaeal functional counterparts. Enzyme properties and mutant analyses demonstrated that MtdB fulfills a dual physiological role in methylotrophic metabolism, in energy generation (in the form of NADH), and in formaldehyde detoxification (Hagemeier et al. 2000). In some methylotrophs, a paralog of MtdB is present, named MtdA, and this has been characterized as a bifunctional methylene-H_4MPT/methylene-tetrahydrofolate (H_4F) dehydrogenase (Vorholt et al. 1998). While their specificities overlap and both can oxidize methylene-H_4MPT, the main function of MtdA is believed to be in reducing methenyl-H_4F to methylene-H_4F (Chistoserdova 2011). In addition, MtdA has a function in general metabolism (e.g., purine biosynthesis) in organisms that do not possess the traditional enzyme, FolD, which is an enzyme that fulfills this function in most bacteria and eukaryotes (Chistoserdova 2011). The origin and evolutionary history of MtdA and MtdB remained poorly understood. While MtdA reveals low levels of sequence similarity to FolD enzymes (15 % identity at the amino acid level), MtdB shares no similarity with FolD (Hagemeier et al. 2000). However, the two paralogs reveal a significant level of similarity to each other (about 30 % at the amino acid level), pointing to their common origin (Vorholt et al. 1998). In the Planctomycete genomes, a single ortholog was identified through BLASTP analysis using either MtdA or MtdB sequences as queries, and these revealed higher similarity to the former (43–53 %) than to the latter (28–32 % at the amino acid level; Vorholt et al. 2005). This finding was unexpected considering the established functions for MtdA and MtdB (i.e., reduction of methenyl-H_4F and oxidation of methylene-H_4MPT, respectively), especially given the fact that Planctomycetes encode FolD (Vorholt et al. 2005). These considerations suggested that the function of Mtd protein orthologs in Planctomycetes could be more similar to the function of MtdB than to the function of MtdA. This hypothesis was tested by expressing the *mtd* gene homolog from *Gemmata* sp. in the mutants of *M. extorquens* containing lesions in either *mtdA* or *mtdB*, which are both negative for growth on methanol (Chistoserdova 2011). Neither of the mutants could be complemented by the *mtd* gene from *Gemmata* sp., suggesting that its product may possess substrate specificities differing from the ones of MtdA or MtdB. Indeed, the purified enzyme, while highly active in catalyzing the methylene-H_4MPT dehydrogenase reaction using NADP as a cofactor, revealed low efficiency in catalyzing the dehydrogenation of either methylene-H_4MPT using NAD as a cofactor, in contrast to the characterized MtdB (Hagemeier et al. 2000), or methylene-H_4F with NADP as a cofactor, in contrast to the characterized MtdA (Vorholt et al. 1998). However, compared to MtdA and MtdB, the new enzyme, named MtdC, was shown to possess a broader substrate range, revealing affinities for NAD, NADP, H_4F, and H_4MPT, with the highest affinity for the H_4MPT/NADP couple. This substrate combination likely represents the physiological activity of this enzyme. Thus, while phylogenetically more related to MtdA, MtdC must be a functional homolog of MtdB, which acts as a part of the oxidative pathway linked to H_4MPT (Fig. 8.1). This conclusion is also supported by the chromosomal location of *mtdC* genes in physical proximity of other genes involved in the pathway (Vorholt et al. 2005), which is also the case with the *mtdB*

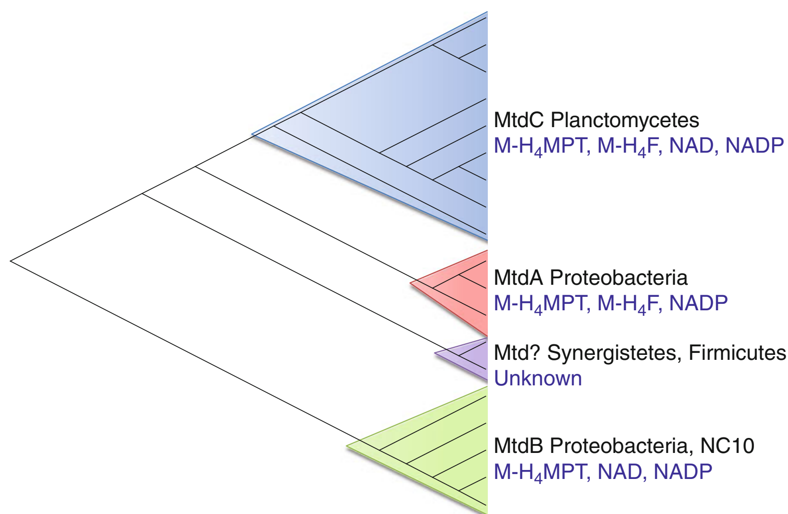

Fig. 8.3 Phylogeny of relevant Mtd proteins, Mtd protein affiliations with specific phyla, and substrate/cofactor specificities

gene (Kalyuzhnaya et al. 2005). Both the substrate "promiscuity" of MtdC and its phylogenetic separation from its homologs (Fig. 8.3) were used as arguments for its ancestral role with respect to MtdA and MtdB (Vorholt et al. 2005).

8.4 Recent Genomic Insights into the Distribution of the H_4MPT-Linked C1 Transfer Functions

Over the past few years, microbial genomic databases expanded substantially, including novel and phylogenetically divergent organisms (Wu et al. 2009; Pagani et al. 2012). The Planctomycete genomic database remains very limited, with a total count of 11 publically available genomes, providing genetic blueprints for *Blastopirellula marina* (5.7 Mb; http://genome.jgi.doe.gov/), *Candidatus* Kuenenia stuttgartiensis (4.2 Mb; Strous et al. 2006), *Gemmata obscuriglobus* (9.1 Mb; http://genome.jgi.doe.gov/), *Isosphaera pallida* (5.5 Mb; Göker et al. 2011), *Pirellula staleyi* (6.2 Mb; Clum et al. 2009), *Planctomyces brasiliensis* (6.0 Mb; http://genome.jgi.doe.gov/), *Planctomyces limnophilus* (5.5 Mb; LaButti et al. 2010), *Planctomyces maris* (7.8 Mb; http://genome.jgi.doe.gov/), *Rhodopirellula baltica* (7.1 Mb; Glöckner et al. 2003), *Singulisphaera acidiphila* (9.7 Mb; http://genome.jgi.doe.gov/), and an unclassified strain, an endophyte of *Porphyra umbilicalis* that is most closely related to *R. baltica* (7.3 Mb; http://genome.jgi.doe.gov/). Analysis of these genomes reconfirms that genes for the H_4MPT-linked C1 transfer reactions are some of the most conserved genes among the diverse

Planctomycetes, one exception being the metabolic specialist *Candidatus* Kuenenia stuttgartiensis, an anaerobic ammonia oxidizer (anammox) that lacks these genes (Strous et al. 2006). As this organism possesses a genome of significantly smaller size compared to other Planctomycetes, it is not unlikely that these genes were lost through genome reduction, which is typical of organisms evolved to specialize in a specific mode of metabolism, and the anammox Planctomycetes are an example of such specialization (Strous et al. 2006). The remaining ten genomes encode complete or nearly complete complements of genes implicated in the C1 transfer pathway. Single exceptions, such as no recognizable homolog for *fhcB* gene in *R. baltica* or *I. pallida* (Glöckner et al. 2003; Göker et al. 2011) or no recognizable *orf21* in *P. limnophilus* (LaButti et al. 2010) in BLAST analyses, are likely results of gene divergence beyond recognition by the BLAST tool or of functional gene replacement. Indeed, extreme divergence of C1 transfer genes in Planctomycetes is one insight resulting from the availability of new genomes, as well as from the growing databases of genes belonging to yet uncultivated Planctomycete species (Kalyuzhnaya and Chistoserdova 2005; Elshahed et al. 2007; Woebken et al. 2007). Despite such sequence divergence (e.g., some proteins involved in C1 transfer functions are less than 30 % identical among different Planctomycetes), phylogenetic analyses typically result in the outcomes similar to the ones presented in Fig. 8.2. The Planctomycete sequences tend to cluster together on phylogenetic trees, forming branches separated from the branches representing other phyla, which reinforces the notion of a monophyletic origin for most of the C1 genes in Planctomycetes. The degree of divergence for these genes, obvious even from the analysis of a very limited set of data, must further support the notion of a long history of the Planctomycetes after their separation from other lineages. In terms of gene clustering, a trend previously noted for the early genomes (Chistoserdova et al. 2004; Kalyuzhnaya et al. 2005; Woebken et al. 2007) maintains: the C1 genes are less clustered in the Planctomycetes than in other Bacteria but more clustered than in Archaea. However, many Planctomycete gene clustering signatures are shared with the signatures found in other phyla. For example, cluster *orf19-mptG* that is conserved in the Planctomycete genomes is not typical of Proteobacteria but is found in the genomes of Synergistetes, Firmicutes, and Division NC10 (Fig. 8.4), cluster *fae-mtdC* is shared between the Planctomycetes and Division NC10, cluster *orf17-orf1-orf9-orf21* is shared with Beta- and Gamma- but not Alphaproteobacteria (Kalyuzhnaya et al. 2005), and cluster *mch-orf5-orf7* is typical (so far) of all Bacteria. The fact that the H_4MPT-linked C1 transfer pathway genes were maintained in the majority of the extant Planctomycetes, likely through vertical inheritance, must further suggest that, despite its enigmatic role, this pathway must be of great physiological and environmental importance to the Planctomycetes. The role proposed originally was the detoxification of formaldehyde (Chistoserdova et al. 2004; Fig. 8.1). The argument for this role is the persistent presence of the (true) *fae* gene (i.e., encoding formaldehyde-activating enzyme that condenses formaldehyde with H_4MPT; Vorholt et al. 2000) for all the Planctomycetes possessing the pathway. Interestingly, *fae*

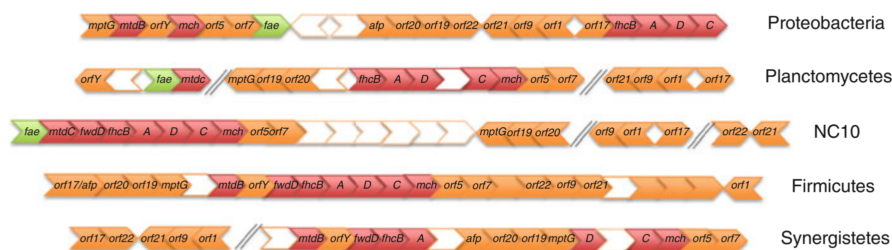

Fig. 8.4 Clustering of the C1 genes on the chromosomes of major phyla. Proteobacteria are represented by *Methylibium petroleiphilum* (Kane et al. 2007); Planctomycetes are represented by *Singulisphaera acidiphila* (note that this organism reveals more clustering than other Planctomycetes; http://genome.jgi.doe.gov/); Division NC10 is represented by *Candidatus* Methylomirabilis oxyfera (Ettwig et al. 2010); Firmicutes are represented by *Halanaerobium hydrogeniformans* (Brown et al. 2011); and Synergistetes are represented by *Anaerobaculum hydrogeniformans* (http://genome.jgi.doe.gov/). Genes for H_4MPT-linked C1 transfer enzymes are in *red*; genes for cofactor biosynthesis/regulation are in *orange* (genes without designation are not conserved among clusters); *fae* genes are in *green* (note their absence on the chromosomes of Firmicutes and Synergistetes); genes not relevant to discussion are *colorless*. *orfY* is not part of a cluster and is not shown for NC10. *Parallel lines* indicate that clusters are not contiguous on the chromosomes

genes are missing (likely through gene loss; Fig. 8.4) from the genomes of Firmicutes and Synergistetes. The lack of Fae potentially suggests that these organisms may employ the pathway as part of a metabolic scheme not involving free formaldehyde. Instead, they would employ (unknown) reactions that would transfer a methyl group directly onto H_4MPT. However, the nature and the metabolic purpose of such variants remain unknown. In contrast to the *true fae* genes, for the ones that are phylogenetically Planctomycete specific (Chistoserdova et al. 2004), some Planctomycetes (6 out of 9) encode distant homologs named Fae3 (Kalyuzhnaya et al. 2005; Chistoserdova 2011). These do not follow the typical Planctomycete phylogenetic pattern (shown in Fig. 8.2). Instead, they cluster together with the proteobacterial sequences, all the known sequences revealing over 80 % identity at the amino acid level, suggesting that recent evolution for these genes involved both intra- and inter-domain transfers. The function of Fae3 remains unknown (Chistoserdova 2011).

Overall, comparisons of genes/enzymes involved in H_4MPT-linked C1 transfers in Planctomycetes with their counterparts in other phyla, in terms of both sequence conservation/divergence and gene clustering, suggest a long evolutionary history for each lineage. While the genes from the newly identified lineages such as Synergistetes, Firmicutes, and Division NC10 show a high degree of divergence with any previously described C1 transfer genes/proteins (Ettwig et al. 2010 and unpublished observations by the author), conservation in gene clustering between different lineages (Fig. 8.4) suggests a common origin.

8.5 New Insights into the Evolution of Microbial C1 Metabolism

Alternative scenarios for the evolution of C1 transfer pathways in the microbial world that remained unresolved due to the limited distribution of the pathway (Chistoserdova et al. 2004; Bauer et al. 2004) can now be revisited. Based on the analysis of recently sequenced genomes, the pathway appears to be much more widespread in both bacterial and archaeal phyla than previously thought. Complete or partial sets of genes have now been identified, besides methanogenic and sulfate-reducing Archaea, Proteobacteria, and Planctomycetes, in the genomes of anaerobic methane oxidizers (which are related to the methanogens; Knittel and Boetius 2009), genomes representing Crenarchaeota (e.g., *Ignisphaera*; incomplete pathway, no confirmed metabolic function); the yet unclassified Division NC10 (complete pathway and confirmed methylotrophy metabolism); phylum Synergistetes (complete pathway with no confirmed metabolic function); Firmicutes (complete pathway, no confirmed metabolic function); and in Actinobacteria (incomplete pathway, no confirmed metabolic function). This broad distribution of the pathway and further expansion of the phylogenetic diversity of the respective genes/enzymes clearly point to the likelihood of the presence of this pathway in the last universal common ancestor (LUCA). Remarkably, some of the new members of the bacterial domain possessing this pathway are obligate anaerobes (e.g., members of the NC10 division, Synergitetes, Firmicutes), supporting the hypothesis of the early emergence of the pathway (Chistoserdova et al. 2004), possibly prior to the emergence of oxygenic photosynthesis. Likely, this pathway, potentially in its formaldehyde-oxidizing capacity, has evolved before any of the primary C1 oxidation (such as methane monooxygenase and methanol dehydrogenase) or C1 reduction modules (such as methyl-CoM reductase) have emerged.

While the existence of the deeply diverging genes in the major microbial lineages reflect the long history of this pathway, it is certain that more recent lateral transfers played a role, which is demonstrated by the *fae3* genes that are shared among Planctomycetes and Alpha-, Beta-, and Gammaproteobacteria (Chistoserdova et al. 2004; Chistoserdova 2011). At least in Betaproteobacteria, genes encoding the H_4MPT-linked pathway appear to be of polyphyletic origin with the sequences of *Burkholderiaceae* separating from the sequences of *Methylophilaceae* (Kalyuzhnaya et al. 2005; Chistoserdova et al. 2007), with both types emerging from a common ancestor of Proteobacteria.

The recent genomic data also argue against the previous assumption that the pathway could not have been lost in many lineages of the Prokaryotes. To the contrary, the pathway in question appears to be a currency easily gained and lost. For example, recent deletion events could be noted by comparing genomes of two closely related *Nitrosococcus* species: while gene synteny and high gene identity are maintained between the C1 transfer gene clusters in *Nitrosococcus halophilus* and *Nitrosococcus oceani,* in the latter, key genes are missing from the cluster

(and from the genome), suggesting that the pathway is no longer operational in this species (Chistoserdova 2011). Another example is the (extensively sampled) Burkholderiales species, many of which encode the entire pathway, but many lack the entire pathway, and some possess multiple (phylogenetically distinct) gene clusters, suggesting that both gene losses and lateral transfers must be taking place (Chistoserdova 2011). More ancient, lineage-specific gene loss is also apparent based on comparisons of the gene complements present. One example is the *fwdD* gene homolog (that would likely be a subunit of the formyltransferase/hydrolase complex) that is maintained in Archaea, Synergistetes, Firmicutes, and Division NC10 (Fig. 8.4) but is missing from most of the known genomes of Planctomycetes or Proteobacteria. Another example is the Afp protein that is encoded in the genomes of Archaea, Synergistetes, Firmicutes, and most Proteobacteria. However, the respective gene is not recognized in the Planctomycete genomes and was shown to be substituted by a nonhomologous gene in the *Methylobacterium* species of Alphaproteobacteria (Marx et al. 2003; Vuilleumier et al. 2009).

8.6 Conclusions: Changing Trees

While the Planctomycetes are ubiquitous in the environment, their lifestyles and their environmental functions remain enigmatic, except for the established function of the anammox Planctomycetes (Strous et al. 2006). Excluding those species, from genomics of the cultivated species and based on culture-independent detection in a variety of environments (Kalyuzhnaya and Chistoserdova 2005; Elshahed et al. 2007; Woebken et al. 2007), the C1 transfer genes appear to be persistently present in diverse Planctomycetes, both aerobes and anaerobes, suggesting their importance for species survival/fitness. Currently, no methylotrophy capability has been documented for a Planctomycete, and no obvious source of formaldehyde in their habitats has been established. Thus, the selective pressure for maintaining C1 metabolism functions, as well as the exact nature of the metabolism involving these functions, remains a mystery. However, Planctomycetes are often detected in environments with high rates of C1 metabolism (Lösekann et al. 2007; Kalyuzhnaya et al. 2008; Webster et al. 2011; Sauter et al. 2012), suggesting that they may be somehow involved, potentially through synergistic relationships in which C1 transfer capabilities provide an advantage. At the same time, as the tree of life is becoming better sampled through genomic approaches, the tree of the organisms encoding H_4MPT-linked C1 transfer functions is also expanding. Based on the genomic information available in 2004, we favored two alternative hypotheses for the evolution of these genes: their emergence in the LUCA with subsequent losses from most microbes or their emergence in the Planctomycetes with subsequent transfers into Euryarchaeota and Proteobacteria (Fig. 8.5a, b). At this time, based on the newly established presence of these genes in a number of deeply branching phyla, such as Firmicutes,

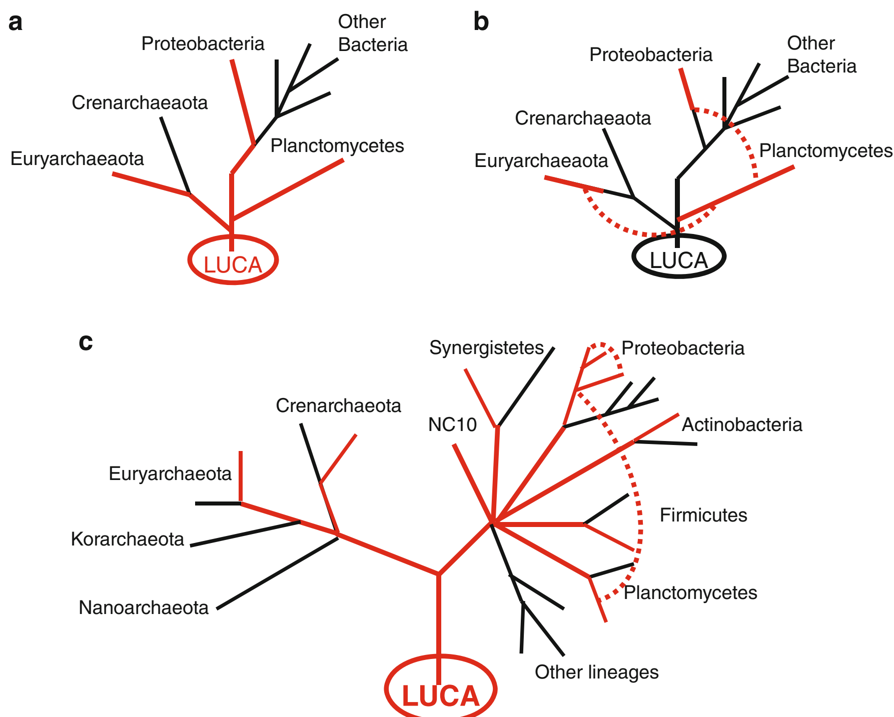

Fig. 8.5 A cartoon depicting the probable scenarios of the evolution of H_4MPT-linked C1 transfer genes in the microbial world. *Red branches* indicate (partial) presence, *black branches* indicate absence, and *dotted lines* indicate lateral transfers. (**a**, **b**) Scenarios proposed in 2004 (Chistoserdova et al.), based on limited sets of genomic data. (**c**) A scenario proposed here based on the newly available genomic data

Synergistetes, Division NC10, and based on the remarkable divergence of the genes within both Bacteria and Archaea, the former scenario appears to be more plausible (Fig. 8.5c), which further suggests that the pathway indeed must be very ancient. Currently, the involvement of the pathway has been established in four physiological processes: methanogenesis and anaerobic methane oxidation, both in Euryarchaeota, aerobic methylotrophy/formaldehyde detoxification in Proteobacteria, and anaerobic methane oxidation in Division NC10. It is entirely possible that the C1 transfer reactions encoded by Planctomycetes and by other phyla may be involved in or linked to metabolic processes that are neither methanogenesis nor methylotrophy. Further sampling of the diversity of the Planctomycetes and delineation of their relationships with other members of microbial communities will be instrumental in exploring such an intriguing possibility.

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Chapter 9
Unusual Members of the PVC Superphylum: The Methanotrophic *Verrucomicrobia* Genus "*Methylacidiphilum*"

Christine E. Sharp, Huub J.M. Op den Camp, Ivica Tamas, and Peter F. Dunfield

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9.1 Introduction

Methane is an important molecule in the environment. From a societal perspective, it is a clean alternative fuel as well as a greenhouse gas contributing 18 % of total atmospheric radiative forcing (IPCC). From an ecological point of view it is a key intermediate in the global carbon cycle. In the absence of primary electron acceptors such as O_2, SO_4^{2-}, and NO_3^-, methanogenic archaea couple the oxidation of small molecules like acetate and H_2 to the reduction of CO_2 or a methyl group to form methane. An estimated 2 Gt of methane is produced each year through anaerobic degradation of organic matter (Thauer et al. 2008). Methane can also be formed

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by nonmicrobial processes in the deep subsurface and efflux to the atmosphere from seeps and geothermal zones.

Methanogenic environments are therefore usually subsurface environments, either shallow flooded sediments for archaeal methanogenesis or deeper thermal zones for geochemical processes. As methane diffuses upwards from its subsurface origins towards surface oxic zones, it becomes an energy source for methanotrophs. These microbes couple the oxidation of methane to the reduction of O_2, SO_4^{2-}, or NO_2^-. The SO_4^{2-}-driven methanotrophic consortia are particularly important in ocean seeps and methane hydrate deposits (Knittel and Boetius 2009). The only known NO_2^--using methanotroph belongs to the bacterial candidate phylum NC10 and has been tentatively named "*Candidatus Methylomirabilis oxyfera*" (Ettwig et al. 2009, 2010). It was demonstrated that this bacterium has the unique ability to produce intracellular O_2 through an alternative denitrification pathway (Ettwig et al. 2010). The importance of this process in the environment is currently being investigated. However, of the three physiological types of methanotrophs, the O_2-using or aerobic methanotrophs are the best-studied group.

The taxonomic and ecological diversity of aerobic methanotrophs was once thought to be extremely limited. Until recently all known species belonged to the bacterial phylum *Proteobacteria*, specifically to the classes *Gammaproteobacteria* and *Alphaproteobacteria*. It was considered that gammaproteobacterial and alphaproteobacterial methanotrophs could be easily distinguished on the basis of a few physiological and biochemical characteristics (Hanson and Hanson 1996), and they were therefore often treated as two coherent groups called type I and type II methanotrophs, respectively. However with ongoing study the known physiological and ecological diversity within each group has expanded. There are now 18 described genera of proteobacterial methanotrophs (Table 9.1), and many do not conform to the simple characteristics once used to delineate the two types (Op den Camp et al. 2009; Stein et al. 2012). Even more interesting from a biodiversity perspective is that non-proteobacterial methanotrophs have now also been found. These include some members of the phylum *Verrucomicrobia* (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008) and a member of the candidate division NC10 (Ettwig et al. 2009, 2010).

9.2 Methanotrophic *Verrucomicrobia*

Methanotrophic *Verrucomicrobia* have to date only been found in geothermal environments. Geothermal gas typically contains 0.1–1 % CH_4, with a $\delta^{13}C$ signature of −20 ‰ to −30 ‰, strongly indicating a nonmicrobial source (Etiope and Klusman 2002; Giggenbach 1994). This nonmicrobial source may be thermogenic, in which methane is formed from the thermal cracking of buried organic matter, or abiotic, in which H_2 and CO_2 react to form methane in Fischer-Tropsch-type reactions (Xu 2010). Methane is emitted from geothermal systems via degassing of spring water and diffuse gas seepage from underground reservoirs through soil cover. As in non-geothermal systems this release may be constrained by the activity of

Table 9.1 Comparison of the three main phylogenetic groups of aerobic methanotrophic bacteria

Phylum and class	*Proteobacteria* (*Gammaproteobacteria*)	*Proteobacteria* (*Alphaproteobacteria*)	*Verrucomicrobia* subphylum 6
Genera	*Methylococcus, Methylocaldum, Methylohalobius, Methylothermus, Methylobacter, Methylomicrobium, Methylomonas, Methylosarcina, Methylosoma, Crenothrix, Clonothrix, Methylosphaera, Methylovulum*	*Methylocystis, Methylosinus, Methylocella, Methylocapsa, Methyloferula*	"*Methylacidiphilum*"
Internal membranes or compartments	Type I: membrane bundles perpendicular to the cell envelope	Type II: membrane stacks along the cell periphery, parallel to the cell envelope (except *Methylocella*)	Uncharacterized inclusion bodies
Lowest reported growth pH	5.0 (many species)	4.2 (*Methylocapsa acidiphila*)	0.8 (*M. fumariolicum* SolV)
Highest reported growth pH	11 (*Methylomicrobium buryatense*)	9.5 (*Methylocystis* sp. strain B3)	6.0 (*M. infernorum* V4)
Lowest reported growth T (°C)	0 (*Methylosphaera hansonii*)	4 (*Methylocella silvestris*)	37 (*M. kamchatkense* Kam1)
Highest reported growth T (°C)	72 (*Methylothermus* sp. strain HB)	40 (many species)	65 (*M. fumariolicum* SolV)
Major PLFAs (more than 15 % of total in any species)	14:0 (1–24 %) 16:1ω8c (0–41 %) 16:0 (4–63 %) 18:1ω7c (0–60 %) 16:1ω5t (0–30 %)··· 18:1ω9c (0–35 %) 16:1ω7c (8–57 %)	16:1ω8c (0–29 %) 18:1ω7c (10–83 %) 18:1ω8c (32–74 %)	i14:0 (7–22 %) a15:0 (13–31 %) 18:0 (14–42 %)
Carbon fixation pathway	Ribulose monophosphate pathway, Calvin-Benson-Bassham cycle	Serine cycle, Calvin-Benson-Bassham cycle	Calvin-Benson-Bassham cycle
G+C mol%	43–65	60–67	40.8–45.5
N_2 fixation	±	±	±
sMMO	±	±	−
pMMO	+	±	+

For detailed references see Op den Camp et al. (2009) and Dunfield (2009)

methanotrophic bacteria. In 2005, aerobic methane oxidation activity was reported in laboratory incubations of soils from the Solfatara volcano (Italy) with pH levels as low as 1.8 and temperatures as high as 70 °C (Castaldi and Tedesco 2005). A later study of steaming soil in Hell's Gate (Tikitere), New Zealand (pH 3–5, 60 °C), suggested that methanotrophic bacteria at this site were also actively consuming methane in geothermal gas (Dunfield et al. 2007). The acidic conditions in these environments were too harsh to allow the survival of any known methanotroph.

The microorganisms responsible for methane oxidation in extremely acidic geothermal environments were identified in three independent publications in 2007–2008 (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008). Remarkably, they were not alpha- or gammaproteobacteria but rather members of a distinct phylum within the domain *Bacteria*, the *Verrucomicrobia*. The three isolates described probably represent a single genus based on the high level of similarity among their 16S ribosomal RNA (rRNA) gene sequences (>98.4 %). There have been difficulties in getting these bacteria preserved in a viable state in public culture collections, and therefore, they are still not taxonomically validated. However, Op den Camp et al. (2009) suggested that they be all considered members of a single genus called "*Methylacidiphilum*," with three proposed species: "*M. kamchatkense*" Kam1, "*M. infernorum*" V4, and "*M. fumariolicum*" SolV. The three isolates of "*Methylacidiphilum*" are remarkably similar (see comparison table in Op den Camp et al. 2009), and they will be treated as a single entity in this chapter.

Hedlund (2010) classifies all 16S rRNA sequences from cultured and uncultured *Verrucomicrobia* into 7 subphyla or classes, of which only three are named: *Verrucomicrobiae, Opitutae*, and "*Spartobacteria*." "*Methylacidiphilum*" is the only cultured representative of subphylum 6 (Fig. 9.1). Most sequences in this group have been recovered from geothermal habitats, although a few are from soils or invertebrate guts (Hedlund 2010). It is unknown whether other bacteria in subphylum 6 are methanotrophic.

Published physiological studies of "*Methylacidiphilum*" are still quite few. However, a complete genome is available for strain V4 (Hou et al. 2008) and draft genomes for SolV (Khadem et al. 2012a) and Kam 1 (unpublished). These have allowed detailed comparisons of metabolic potential and evolutionary histories to be made to model proteobacterial methanotrophs, for which several genomes are now available. An interesting element of the genome analysis of strain V4 by Hou et al. (2008) was a correspondence analysis of its gene content compared to 58 other bacteria. Orthologous groups were defined based on the eggNOG database. This analysis suggested that the gene content of V4 was more similar to species of *Proteobacteria* than to other *Verrucomicrobia*. This may indicate a history of promiscuous lateral gene transfer (LGT). However, given that the analysis was more a comparison of metabolic lifestyle than a phylogenetic analysis, this conclusion is not certain. Based on the closest BLAST hits, the JGI IMG site for strain V4 predicts 970 genes (38.5 %) as putatively laterally transferred. Again, this is likely to some extent an artifact of the few verrucomicrobial genomes in the database (9), but it is also suggestive of a fluid genome. Alien Hunter, a program that uses nucleotide dimer to octamer frequencies to identify large islands of LGT (Vernikos and Parkhill 2006),

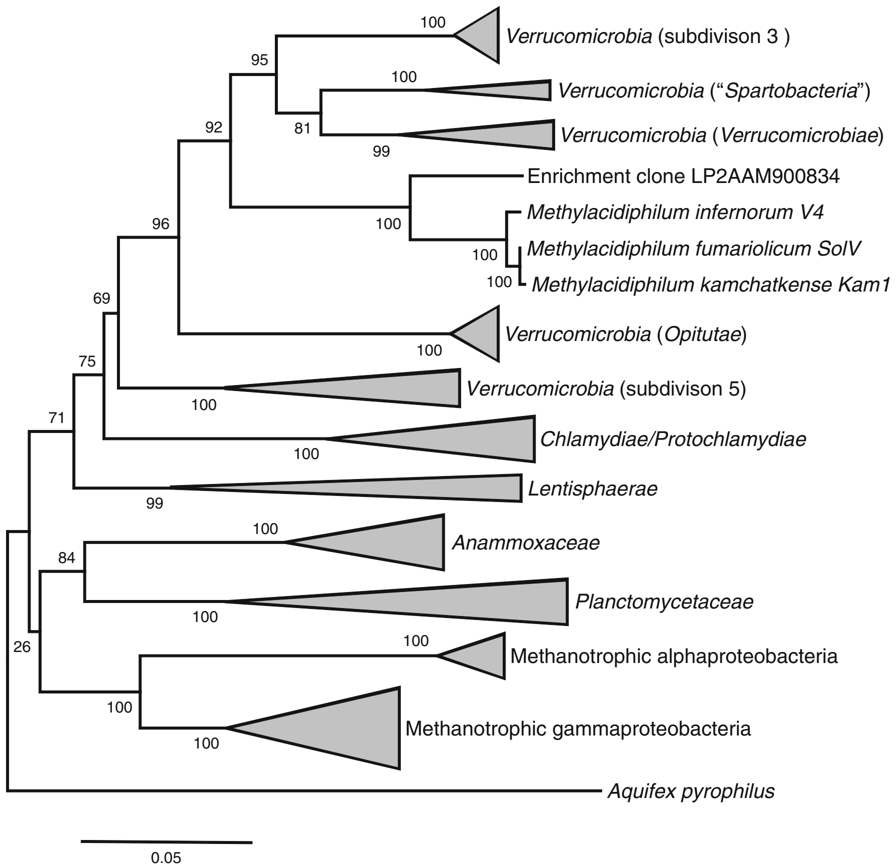

Fig. 9.1 Neighbor-joining 16S rRNA gene-based phylogeny showing 6 of the 7 main subdivisions of *Verrucomicrobia,* including "*Methylacidiphilum*" in subdivision 6, in relation to other phyla of the domain *Bacteria.* The other aerobic methanotrophs within the phylum *Proteobacteria* are included for reference. Node support values are based on 500 bootstrap replicates. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). The scale bar represents 0.05 changes per nucleotide position

identifies 29 regions of the V4 genome as putatively foreign DNA, summing to a total of 270,000 nt (11.80 % of the genome). The largest region is 32,500 nt. This program is conservative in that it can only identify large regions of LGT (5,000 nt windows), as only these provide enough data to estimate octamer frequencies.

A close phylogenetic examination of key methane metabolism genes shows some evidence for lateral transfer. Formate dehydrogenase and formate hydrogen lyase genes associate most closely with genes from *Proteobacteria* and *Aquificae.* Several genes involved in nitrogen metabolism, which are intimately linked to methane oxidation (Stein et al. 2012), show phylogenetic evidence of lateral transfer as well. These include the nitric oxide reductase-encoding genes *norB* and *norC,*

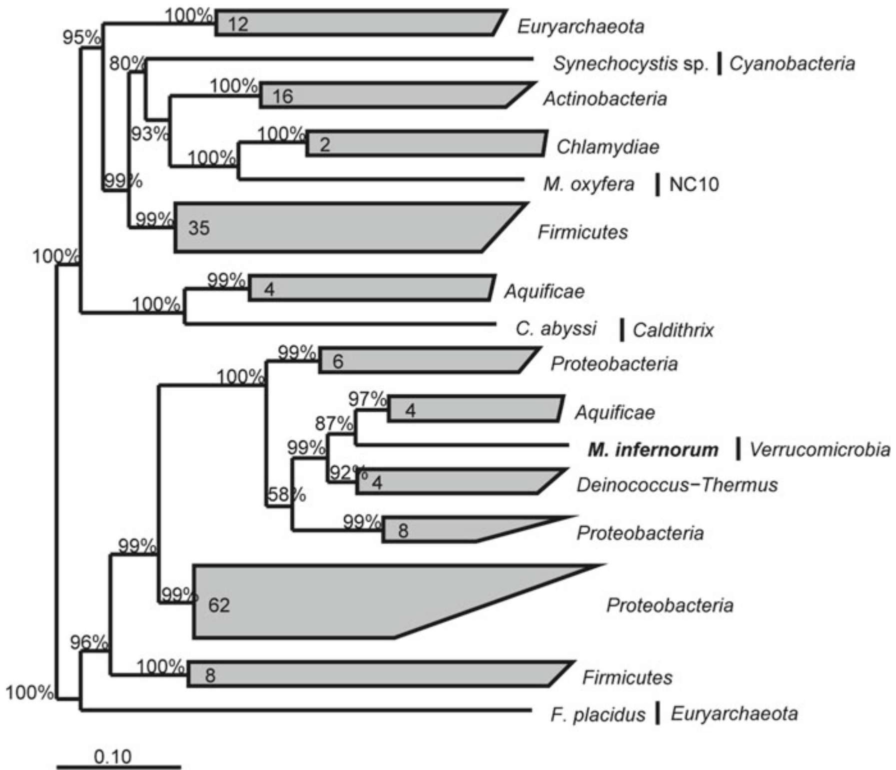

Fig. 9.2 Neighbor-joining phylogenetic tree of 166 derived amino acid sequences (length 474) of nitric oxide reductase subunit B (NorB). The tree was constructed using the ARB software environment (Ludwig et al. 2004). Node support values are based on 5,000 bootstrap replicates. The scale bar represents 0.1 changes per amino acid position

which form a phylogenetic cluster together with sequences from *Aquificae* and *Thermus/Deinococcus,* nested within a large group of genes from *Proteobacteria* (Fig. 9.2). The hydroxylamine oxidoreductase-encoding *haoA* was possibly obtained via LGT from *Proteobacteria* (see the phylogeny in Klotz et al. 2008). Nitrogen fixation genes such as *nifHDK* are most closely related to genes from *Proteobacteria* as well (Khadem et al. 2010), and the entire gene set encoding nitrogen fixation is likely to have been acquired via lateral transfer. *Methylacidiphilum* is also highly unusual in possessing two distinct H^+ pumping F_1F_0 ATPases, one of which appears to be ancestral to *Verrucomicrobia* and the other obtained from a gammaproteobacterium (Hou et al. 2008). We present more evidence of LGT later.

A unique acidic and thermostable bacterial hemoglobin has been characterized from "*Methylacidiphilum infernorum*," named Hell's Gate globin I (HGbl). HGbl is unusual due to an extremely high resistance to oxidation at low pH and high temperature. It also has high structural resemblance to the vertebrate neuroglobins, providing a strong evolutionary link between the bacterial flavohemoglobins and eukaryotic neuroglobins (Teh et al. 2011).

9.3 Physiological Comparison with Proteobacterial Methanotrophs

Species of proteobacterial methanotrophs differ from each other in their dominant phospholipid fatty acids, type of methane monooxygenase (MMO) present, nitrogen fixation ability, geometric arrangement of intracellular membranes, ecological tolerances (to temperature, salt, and acid stresses), genetic systems available to deal with nitrification stress, and carbon assimilation pathways. C-assimilation pathways include the ribulose monophosphate pathway (RuMP), the serine cycle, and the Calvin-Benson-Bassham cycle (Hanson and Hanson 1996). The characteristics that most distinguish the verrucomicrobial genus "*Methylacidiphilum*" from its proteobacterial counterparts are an extremely acidophilic phenotype, a prevalence of saturated fatty acids, a general lack of intracytoplasmic membrane stacks but presence of other inclusion bodies, at least as deduced from thin sections of chemically fixed cells, and an inability to fix C at the level of formaldehyde. Most of the other properties of "*Methylacidiphilum,*" particularly its central enzymatic machinery for processing methane, are common to several proteobacterial methanotrophs.

Most proteobacterial methanotrophs are neutrophilic (pH range 5.5–8.5) and mesophilic (temperature range 20–40 °C), although some moderately acidophilic and some thermophilic isolates have been identified (Dunfield 2009) (Table 9.1). The alphaproteobacteria *Methylocapsa* and *Methylocella* are mild acidophiles growing between pH 4.2 and 7.2. These genera, along with the alphaproteobacterium *Methylocystis* and occasionally some gammaproteobacterial methanotrophs like *Methylomonas*, are commonly detected via cultivation-independent methods in acidic peats at pH 3.5–5.0 (Dunfield and Dedysh 2010; Kip et al. 2011). However, the lower limit of pH 0.8 for "*Methylacidiphilum*" is well below this range. The upper temperature limit for growth of "*Methylacidiphilum*" falls just short of that reported (67 °C) for the most thermophilic proteobacterial methanotroph, *Methylothermus thermalis* (Tsubota et al. 2005).

Proteobacterial methanotrophs contain mainly 14C, 16C, and 18C phospholipid fatty acids (PLFAs), usually monounsaturated. Some are signature compounds found exclusively in proteobacterial methanotrophs and not in any other known organism: C16:1ω8c and C16:1ω5t (usually in the gammaproteobacterial methanotrophs) and C18:1ω8c (usually in the alphaproteobacterial methanotrophs) (Bodelier et al. 2009). The PLFA profiles of "*Methylacidiphilum*" are very different and show distinctive characteristics of extreme acidophiles. They contain predominantly (>96 %) saturated fatty acids i14:0, a15:0, and 18:0, consistent with the minimization of membrane permeability to protons (Op den Camp et al. 2009). This is in contrast to even the moderately acidophilic proteobacterial methanotrophs, which contain predominantly unsaturated PLFAs. However, the bacteriohopanepolyol compounds in "*Methylacidiphilum*" are not unique compared to other methanotrophs. The main bacteriohopanepolyol is aminotriol, which is common to many other bacteria, including *Methylocella palustris* (van Winden et al. 2012).

Most proteobacterial methanotrophs oxidize methane via a membrane-bound particulate methane monooxygenase (pMMO). In order to increase the membrane

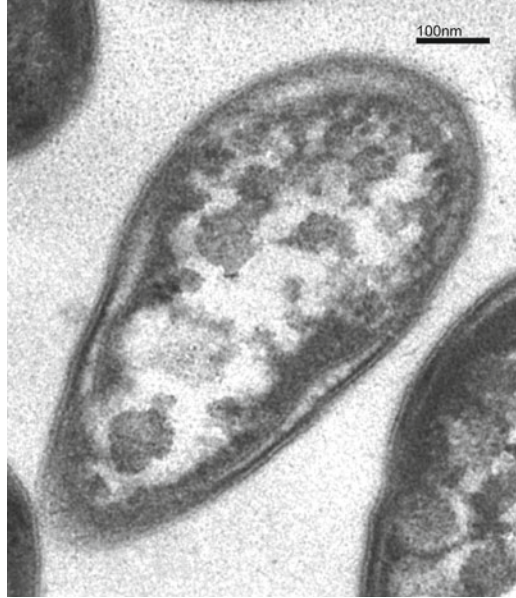

Fig. 9.3 Transmission electron micrograph of a thin-sectioned chemically fixed cell of "*Methylacidiphilum infernorum*" V4 showing a large putative periplasmic space and internal inclusion bodies. Scale bar, 100 nm

surface area to hold this enzyme, they produce internal cytoplasmic membrane stacks. Although "*Methylacidiphilum*" also possesses pMMO, it does not typically display internal membranes. Internal membrane systems were sometimes observed in strain V4 but were not reported in SolV or Kam1. Instead, multiple electron dense inclusion bodies are seen (Fig. 9.3). Although these internal compartments were originally proposed to be carboxysomes, Khadem et al. (2011) determined that the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in the SolV genome is a non-carboxysomal type and that carboxysome shell proteins are missing. The exact structure and function of these inclusion bodies has been difficult to resolve as chemical fixation of these bacteria lacks resolution, a phenomenon also seen with members of the *Planctomycetes*. Whether the cells of these strains possess a cell plan like many non-methanotrophic verrucomicrobia and planctomycetes including a major intracytoplasmic membrane also will depend on future application of cryo-techniques alternative to chemical fixation.

9.4 Methane Metabolism

Most methanotrophs are highly specialized. They are incapable of growing on substrates with C-C bonds but rather survive solely on methane, methanol, and a few other 1-carbon compounds (Stein et al. 2012). There are exceptions, notably the facultative methanotroph *Methylocella* (Dedysh et al. 2005). When strain V4 was tested on alternative growth substrates used by *Methylocella,* none supported growth, suggesting it is an obligate methylotroph like most known species

(Dunfield et al. 2007). However, genome data provides interesting clues to a possible facultative metabolism in strain SolV. There is a hydrogenase gene cluster in the draft genome (Khadem et al. 2012a) and the strain was shown to oxidize H_2, although it did not support growth when used as a sole substrate (Pol et al. 2007). Interestingly, the draft genome of SolV also contains acetate kinase and acetyl-coenzyme A synthase (Khadem et al. 2012a), which in theory might confer the ability to grow on acetate. The genome of V4 also contains acetate kinase, but no acetyl-coenzyme A synthase or other phosphotransacetylase could be identified (Hou et al. 2008).

Methanotrophs convert methane to carbon dioxide in the overall reaction: $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$. The first step is catalyzed by the enzyme methane monooxygenase (MMO). In this step, one atom of oxygen is incorporated into methane to generate methanol (CH_3OH). There are two forms of the methane monooxygenase enzyme, a soluble form (sMMO) and particulate form (pMMO). The pMMO form is an enzyme of the copper-containing membrane-bound monooxygenase superfamily that also includes ammonia monooxygenase (AMO). pMMO is universally found in all methanotrophic genera with the exception of *Methylocella,* while the sMMO is found in a few genera. Multiple copies of pMMO genes were detected in each "*Methylacidiphilum*" strain, but no genes for sMMO. Each gene *pmoC*, *pmoA,* and *pmoB* is a distant homologue of its counterpart in proteobacteria, and a similar *pmoCAB* operon structure as in most proteobacterial operons was observed. All "*Methylacidiphilum*" isolates contain three complete *pmoCAB* operons, with V4 possessing an orphan fourth *pmoC* copy and Kam1 an additional *pmoCA* operon (Op den Camp et al. 2009). Interestingly, within each strain the three orthologous operons differ by as much as 50 % at the amino acid level. The most divergent of the three operons is that dubbed *pmoCAB3* (as opposed to *pmoCAB1* and *pmoCAB2*). It has been speculated that each ortholog may have evolved different kinetic properties and thus is differentially expressed under distinct environmental conditions. Baani and Liesack (2008) demonstrated a similar phenomenon in *Methylocystis* sp. strain SC2. This isolate has two copies of the *pmoCAB* operon that exhibit a low degree of amino acid sequence similarity (59.3–65.6 %) between the respective *pmoA*, *pmoB,* and *pmoC* genes of each operon. The two operons have different kinetic affinities to methane and are differentially expressed in response to methane concentration (Baani and Liesack 2008).

Recently, RNA-seq transcriptome analysis of "*M. fumariolicum*" SolV showed that the *pmoCAB*2 operon was highly expressed in cells growing at μmax with excess oxygen and ammonium (Khadem et al. 2012b). The other two *pmoCAB* operons were hardly expressed under these culture conditions. The expression pattern of cells from CH_4-limited, N_2-fixing, and O_2-limited chemostat cultures (dO_2 of 0.5 % and 0.03 % oxygen saturation, respectively) was remarkably different. Under these conditions, the *pmoCAB*2 operon was downregulated 40 times, while the *pmoCAB*1 operon was highly upregulated. The *pmoCAB*3 operon was hardly expressed in these cells. The results point to a regulation of the *pmoCAB*1/*pmoCAB*2 expression by oxygen concentration. In addition, qPCR analysis of the four *pmoA*

genes of "*Methylacidiphilum kamchatkense*" Kam1 showed that the *pmoA*2 gene was 35-fold more strongly expressed than the other copies during batch cultivation (Erikstad et al. 2012). Suboptimal pH and temperature during growth did not change this pattern, but growth on methanol resulted in a tenfold decreased expression of *pmoA*2.

9.5 Evolutionary History of *pmo*

Phylogenetic analysis of all three *pmoA* gene copies relative to *pmoA/amoA* genes of other prokaryotes revealed that all *pmoA* genes from *Verrucomicrobia* fell into a monophyletic cluster, suggesting that the three orthologues are derived via lineage-specific duplication rather than lateral gene transfer (Dunfield et al. 2007) (Fig. 9.4). Phylogenetic analysis of *pmoB* and *pmoC* genes revealed similar topologies (Op den Camp et al. 2009). However, the placement of the *pmoCAB3* genes was often quite distant and sensitive to the particular sequence set and tree-building algorithm used. One phylogenetic conclusion at least is obvious: proteobacterial and verrucomicrobial *pmo* operons form distinct lineages without recent lateral gene transfer between the two groups. However, the relationship of the *pmoCAB3* genes to the other two operons in "*Methylacidiphilum*" is not clear.

A Bayesian phylogenetic analysis done by Tavormina et al. (2010) led to some interesting speculation about the deepest nodes of the trees. The topologies of

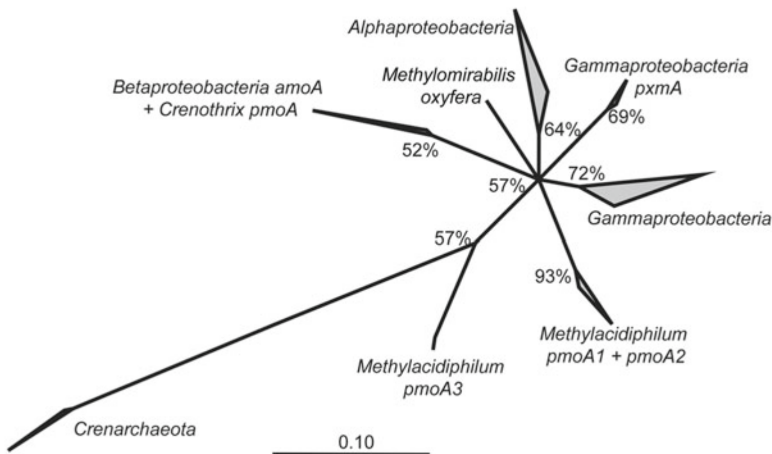

Fig. 9.4 Phylogenetic tree of partial (495 nucleotides) *pmoA/amoA* gene sequences of major groups of ammonia oxidizers and methanotrophs. The tree was constructed with TREE_PUZZLE, a quartet maximum-likelihood method, using a Schoeniger-von Hasseler distance calculation (Schmidt et al. 2002) and 50,000 iterations. Support values for the major nodes are given. The scale bar represents 0.1 changes per nucleotide position

amoA/pmoA and *amoB/pmoB* in their Bayesian trees support the hypothesis that "*Methylacidiphilum*" *pmoA3* genes are related to the *amoA* genes of ammonia-oxidizing *Crenarchaeota*. Nodes connecting verrucomicrobial *pmoA3/pmoB3* with *amoA/amoB* from *Crenarchaeota* were highly supported. Unfortunately, Bayesian phylogenies are notorious for delivering misleadingly high support values for deep nodes (e.g., Yang and Rannala 2005). In addition, the extremely deep branch to the *Crenarchaeota amoA* cluster may cause long-branch-attraction artifacts, a known hazard in any phylogenetic construction. Quartet-puzzling phylogenies constructed by Dunfield et al. (2007) and Stein et al. (2012) also include crenarchaeal *amoA*. These sometimes support a monophyletic *Verrucomicrobia* clade, but sometimes also support an affiliation of the verrucomicrobial *pmoA3* with crenarchaeal *amoA*. As is often the case, the conclusions are sensitive to different phylogenetic construction methods and datasets. A phylogeny constructed with a quartet-puzzling method and a modified dataset is shown in Fig. 9.4. The support for the *pmoA3* and crenarchaeal *amoA* node is present but weak at 57 %. Although the speculations of Tavormina et al. (2010) on the evolutionary connection of the *pmoA3* with *amoA* from *Crenarchaeota* are intriguing, they will require more data for confirmation.

Tavormina et al. (2010) also noted that the *pmoA1* and *pmoA2* genes in V4 also have a similar GC content as the overall genome (47 % vs. 45 %), but the *pmoA3* genes consist of only 37 % GC. They concluded that the *pmoA3* gene may be foreign to the genome. The Alien Hunter analysis noted earlier predicts the same, supporting the speculation of Tavormina et al. (2010) about the separate origin of the *pmoCAB3* operon compared to the other operons.

Conserved residues in derived PmoA and PmoC amino acid sequences of "*Methylacidiphilum*" were identical to those in the sequences from *Proteobacteria*. However, most of the conserved residues in proteobacterial PmoB were different in the *Verrucomicrobia*. These residues are involved in copper binding in proteobacterial pMMO, and their variability suggests a different metal coordination in the verrucomicrobial patterns (Op den Camp et al. 2009).

9.6 Pathways for C1 Metabolism and CO_2 Fixation

Aerobic methanotrophs first convert methane to methanol, which is oxidized to formaldehyde, formate, and CO_2. Methanotrophs employ a modular design for these metabolic steps (see Chistoserdova 2011) and Chap. 8 in this volume), with different species combining different modules for each step. The systems in "*Methylacidiphilum*" are not unique, although the particular combination of them is not seen in any known proteobacterial methanotroph. "*Methylacidiphilum*" has a gene encoding the large subunit (*mxaF*/*xoxF*) of a pyrroloquinoline quinone-dependent alcohol dehydrogenase of the methanol/ethanol family. No genes encoding the small subunit (*mxaI)* of methanol dehydrogenase or genes for several accessory proteins were found (Hou et al. 2008). Formaldehyde is probably oxidized via an enzymatic pathway using the C1-carrying cofactor tetrahydrofolate.

"*Methylacidiphilum*" does not have the methylene-H_4F dehydrogenase and methenyl-H4F cyclohydrolase enzymes that have been found in other methylotrophs, but does have a bifunctional *fol*D gene product that may perform these functions. A more common pathway in proteobacterial methanotrophs uses the C1-carrier tetrahydromethanopterin, but this is absent in "*Methylacidiphilum*." Formate is oxidized by a simple formate dehydrogenase.

Formaldehyde is a key intermediate in methane oxidation. It can be oxidized to CO_2 for energy generation or directly assimilated into cell material. Methanotrophs assimilate formaldehyde via either the serine cycle, in which formaldehyde and CO_2 are utilized in a one-to-one ratio to produce acetyl-CoA for biosynthesis, or via the RuMP pathway, in which formaldehyde is added to ribulose-5-monophosphate, which via sugar rearrangements produces glyceraldehyde-3-phosphate as a biosynthetic building block. Therefore, although there is some anapleurotic CO_2 assimilation, particularly in methanotrophs employing the serine cycle, these bacteria are considered heterotrophs that assimilate organic carbon. An estimated 85–95 % of biomass C in gammaproteobacterial methanotrophs and 50 % of the biomass C in alphaproteobacterial methanotrophs is derived from methane rather than CO_2 (Trotsenko and Murrell 2008).

Key enzymes of the RuMP pathway are absent in "*Methylacidiphilum*." Two key enzymes of the serine cycle, malyl-CoA lyase (*mcl*) and glycerate kinase (*glc*), could not be identified in the V4 genome, and other putative serine cycle enzymes were not arranged in genomic islands as they are in other methanotrophs, suggesting that their function may not be in the serine cycle. Hou et al. (2008) suggested that a modified serine cycle may operate, but further evidence would be required. The only clear pathway for C fixation identified in the genome of V4 was the Calvin-Benson-Bassham cycle.

RuBisCO, the key enzyme in the Calvin-Benson-Bassham (CBB) cycle, is present in both V4 and SolV. A number of methanotrophs possess the enzymes of the CBB, most in addition to other C1 assimilation pathways (Chistoserdova 2011). The CBB cycle is associated with a large energy use per mole of CO_2 fixed and was not considered a likely way to support growth on CH_4. However, Khadem et al. (2011) applied both $^{13}CH_4$ and $^{13}CO_2$ during growth experiments to verify the genome prediction that CO_2 is in fact the only carbon source for strain SolV. Genome and transcriptome analyses verified that all the genes necessary for the CBB cycle are present and expressed, most prominently the two genes encoding RuBisCO. Carboxysomes are thought to enhance the concentration of CO_2 for RuBisCO as it has a low affinity for CO_2, but strain SolV uses a non-carboxysome-associated form of RuBisCO, in agreement with the high concentrations of CO_2 required for growth (>0.3 %). The verrucomicrobial methanotrophs have been isolated from volcanic regions with high concentrations of CO_2, suggesting that there is no need to sequester CO_2 for growth (Khadem et al. 2011). Phylogenetic analysis of the *rbcL* gene suggests an LGT event from the *Actinobacteria*; however, this divergence must be ancient given that the *Verrucomicrobia rbcL* gene clade is clearly distinct from the *Actinobacteria*.

The autotrophic nature of the methanotrophic verrucomicrobia has recently been confirmed in strain V4 by Sharp et al. (2012) by performing a modified stable-isotope probing (SIP) technique labeling with both $^{13}CH_4$ and $^{13}CO_2$, individually and in combination. Strain V4 only assimilated $^{13}CO_2$, not $^{13}CH_4$, confirming the source of carbon for the verrucomicrobial methanotrophs is CO_2. In combination with quantitative PCR (qPCR) of verrucomicrobial-*pmoA* genes, this technique was then applied to geothermal soils from New Zealand, providing the first demonstration of the autotrophic nature of verrucomicrobial methanotrophs in situ. This study also demonstrated that there are several diverse clades of *Verrucomicrobia* methanotrophs present at the study site. Further application of the technique should allow an assessment of the overall diversity of *Verrucomicrobia* methanotrophs in nature.

9.7 Nitrogen Metabolism

The ability to fix gaseous nitrogen confers an advantage in environments where nitrogen is limiting. Genomic analyses of strains V4 and SolV suggest that these strains should be able to fix gaseous nitrogen, as they possess complete sets of genes for nitrogen fixation. Most of these genes have their organization in putative operons resembling those of *Methylococcus capsulatus* Bath (Ward et al. 2004), a proteobacterial methanotroph that has been shown to fix nitrogen. Khadem et al. (2010) demonstrated that the verrucomicrobial methanotroph strain SolV was able to fix nitrogen under low O_2 concentrations. Based on acetylene reduction assays and growth experiments, the nitrogenase enzyme of strain SolV appeared to be extremely O_2 sensitive compared to that of proteobacterial methanotrophs. Phylogenetic analysis of a concatenated set of the derived amino acid sequences of the *nifH*, *nifD,* and *nifK* genes showed that the verrucomicrobial nitrogenases group with those of the *Proteobacteria* and acidophilic *Leptospirillum* species that inhabit acid mine drainage. The tree is supportive of an LGT scenario with *Proteobacteria* (Khadem et al. 2010).

Ammonia is a strong competitive inhibitor of pMMO. Many methanotrophs oxidize ammonia to nitrite in two steps. In the first step, ammonia is oxidized to hydroxylamine by pMMO. In the second step, hydroxylamine is oxidized to nitrite. Many methanotrophs possess hydroxylamine cytochrome *c* oxidoreductase (HAO) activity to prevent accumulation of toxic hydroxylamine (Ward et al. 2004; Klotz et al. 2008). Recently, ammonia-responsive transcription of the *haoAB* genes encoding HAO have been reported for the methanotroph *Methylococcus capsulatus* Bath (Poret-Peterson et al. 2008). The finding of the *haoAB* gene on a plasmid in *Silicibacter pomeroyi* suggests a high mobility of these genes (Klotz et al. 2008). Both strains V4 and SolV of *Methylacidiphilum* possess an *haoA* gene encoding the large subunit of HAO. Phylogenetically, these groups with HAOs from methanotrophic *Proteobacteria* form a separate clade from the nitrifying bacteria *Nitrosococcus, Nitrosospira,* and *Nitrosomonas*. This may indicate lateral transfer of *haoA* from *Proteobacteria* to the *Verrucomicrobia* (Klotz et al. 2008), although in theory it may instead indicate transfer in the opposite direction.

The oxidation of hydroxylamine by HAO is accompanied by the production of small amounts of nitric oxide (NO). In "*Methylacidiphilum*," NO is then reduced to N_2O via a *norCB*-encoded nitric oxide reductase. This is found in some but not all proteobacterial methanotrophs (Stein and Klotz 2011). Phylogenetic analysis of *norC* and *norB* suggests that these are other strong candidates for lateral transfer. A small cluster of *norB* from *Aquificae, Thermus/Deinococcus,* and *Verrucomicrobia* cluster within a much larger clade of genes from *Proteobacteria* (Fig. 9.2). This phylogenetic clade may represent a thermotolerant form of the enzyme that was originally obtained via transfer from *Proteobacteria* and then shared among several thermophiles.

9.8 Conclusions

We are only beginning to understand the physiology of the "*Methylacidiphilum*"-type methanotrophs. They share many enzymatic modules with proteobacterial methanotrophs, but have a unique combination of these. Particularly special is their reliance on autotrophic C-assimilation, which seems counterintuitive for a bacterium growing on a reduced carbon substrate. Methanotrophs have often been defined in the literature as bacteria that can use methane as their sole energy and carbon source, but in fact "carbon" should be removed from this definition. Studies into the ecology and physiology of these bacteria are ongoing.

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Chapter 10
Phyla Related to *Planctomycetes*: Members of Phylum *Chlamydiae* and Their Implications for *Planctomycetes* Cell Biology

Claire Bertelli and Gilbert Greub

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10.1 Introduction

Widespread in the environment, members of phylum *Planctomycetes* raised the interest of microbiologists as they present interesting biological characteristics such as the potential ability to metabolize or detoxify formaldehyde thanks to specific enzymes also present in methylotrophs and in some archaea (Bauer et al. 2004; Chistoserdova et al. 2004), or to oxidize ammonium in an anoxic atmosphere (anammox bacteria reviewed in Jetten et al. 2005 and Chap. 4 of this volume). The phylum *Planctomycetes* has recently been grouped with phylum *Chlamydiae* in a so-called PVC superphylum, which has also included *Verrucomicrobia, Lentisphaerae,* and *Poribacteria* (Wagner and Horn 2006). Progresses in

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understanding the biology of these clades are limited by the small community of researchers focusing on these bacteria and the relatively fastidious growth of some of their members. *Planctomycetes* share several interesting characteristics with *Chlamydiae,* and knowledge gathered on this latter phylum may illuminate the biology of the whole superphylum.

The order *Chlamydiales* initially comprised a single family, the *Chlamydiaceae,* which has been actively studied given the role of several *Chlamydia* spp. as human and animal pathogens (Everett et al. 1999; Longbottom and Coulter 2003; Senn et al. 2005). During the last decade, the identification of several additional members, the so-called *Chlamydia*-related bacteria, raised the curiosity of fundamental microbiologists given their widespread occurrence in the environment and clinicians given the growing evidence of their role as emerging human and animal pathogens (reviewed in Greub and Raoult 2002a; Friedman et al. 2003; Corsaro and Greub 2006). These new lineages are currently classified within the families *Parachlamydiaceae, Waddliaceae, Criblamydiaceae, Rhabdochlamydiaceae,* and *Simkaniaceae* (Everett et al. 1999; Rurangirwa et al. 1999; Thomas et al. 2006).

Members of this phylum are obligate intracellular bacteria and exhibit two main developmental stages: reticulate bodies (RBs) and elementary bodies (EBs). The metabolically active RBs are able to divide by binary fission, whereas EBs are infectious particles able to survive outside cells (Thomas et al. 2006; Greub and Raoult 2002b; Kahane et al. 2002). They all encode a type III secretion system that translocates effector proteins in the host cell cytosol or in the membrane of the bacteria-containing vacuole (Peters et al. 2007). However, the new clades of *Chlamydia*-related bacteria, which diverged from *Chlamydiaceae* more than 700 million years ago (Greub and Raoult 2003), all exhibit specific characteristics in terms of ecology and intracellular trafficking. Classical *Chlamydia* are commonly found in mammals and multicellular eukaryotes (Longbottom and Coulter 2003; Abdelrahman and Belland 2005), whereas *Waddlia* and *Simkania* are associated with mammalian cell lines (Friedman et al. 2003; Corsaro and Greub 2006; Baud et al. 2008), and *Parachlamydiaceae* and *Criblamydiaceae* are associated with free-living amoebae (Thomas et al. 2006; Lienard et al. 2011; Corsaro et al. 2009).

In this chapter, we summarize the main features supporting a common origin of *Chlamydiae* and *Planctomycetes* and present recent knowledge gathered on the cell biology and genomics of *Chlamydia* and *Chlamydia*-related bacteria, especially focusing on specific features shared with *Planctomycetes* (Table 10.1).

10.2 Common Origin of *Chlamydiales* and *Planctomycetes*

In 1986, an early report based on the analysis of the 16S rRNA-encoding gene suggested a common origin of *Planctomycetes, Verrucomicrobia,* and *Chlamydiae* (Weisburg et al. 1986). The monophyletic relationship of *Planctomycetes* and *Chlamydiales* was confirmed by a phylogenetic analysis of the 23S rRNA-encoding

Table 10.1 Selected ultrastructural and genomic features of *Chlamydiae* and *Planctomycetes*

	Chlamydiae	*Planctomycetes*
Ultrastructure		
Intracellular compartmentalization	–	Membrane-bound compartment surrounding DNA
Intracellular organelles	Oblong lamellar structures of unknown function in some *Chlamydia*-related species	Anammoxosome in anammox *Planctomycetes*
Peptidoglycan	Absent	Absent
Proteic cell wall	Present	Present
Sterols	Present	Present
DNA compaction		
DNA condensation	Present in EBs	Present
Histone-like proteins	Hc1 and Hc2 in classical *Chlamydia*	–
Histone methyltransferase	SET-domain protein	–
Histone protease	EUO protein	–
Chromatin remodelling	SWI/SNF family helicase SWIB domain-containing protein	SWI/SNF family helicase
Genomic features		
Size	1.1–3 Mb	3.8–9.6 Mb
Nucleotide scavenging	ADP/ATP translocase	–
DnaA	Two copies	Two copies
FtsZ	Absence (candidate functional homolog MreB)	Absence (functional homolog in anammox Planctomycetes)

gene (Weisburg et al. 1986; Ward et al. 2000). On the contrary, phylogenetic reconstructions based on elongation factor Tu did not support this relationship (Jenkins and Fuerst 2001). However, single gene sequences often do not provide sufficient information to infer a robust phylogeny for such distantly related clades due to the saturation of amino acid substitutions, and variable gene evolution among clades reveals discordant phylogenies.

With the growing availability of whole genome sequences, the use of genome-wide studies has evolved as a method of choice to reconstruct phylogenetic relationships. A recent analysis using concatenated ribosomal genes convincingly supported the creation of a common *Planctomycetes, Verrucomicrobia,* and *Chlamydiae* (PVC) superphylum (Teeling et al. 2004), as illustrated here with a Bayesian tree based on concatenated proteins of 60 COGs (Fig. 10.1). More recently, the superphylum was enlarged with two new candidate phyla (Wagner and Horn 2006): *Lentisphaerae,* new marine bacteria isolated using very low nutrient concentrations (Cho et al. 2004), and *Poribacteria,* a new *Candidatus* genus recently identified in marine sponges (Fieseler et al. 2004)

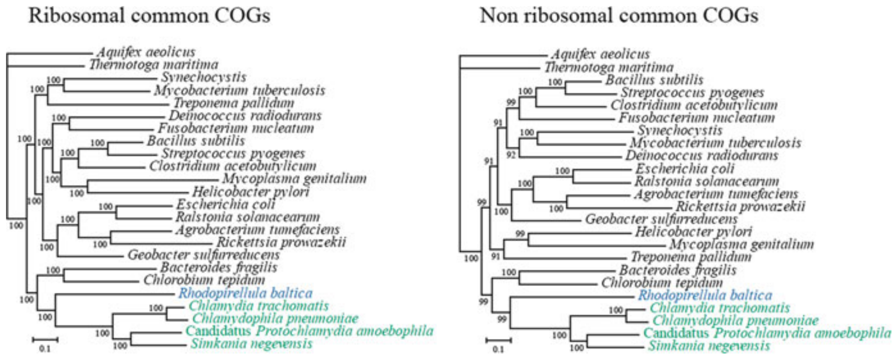

Fig. 10.1 Bayesian tree showing the phylogenetic relatedness of *Planctomycetes* and *Chlamydiales.* This tree was inferred from concatenated aligned sequences of 60 conserved orthologous proteins (COGs) retrieved from 24 distantly related bacterial species. Support values are shown at each node

10.3 Ultrastructure of *Chlamydiales*

As observed by electron microscopy, members of the *Chlamydiales* order generally have round-shaped RBs, the intracellular developmental form, and smaller EBs with more variable shapes (Fig. 10.2a). The cell wall of *Chlamydiales* lacks detectable peptidoglycan (Fox et al. 1990; Ghuysen and Goffin 1999; McCoy and Maurelli 2006), but the cell structure is constrained by a proteinaceous layer highly linked with disulfide bridges (McCoy and Maurelli 2006). The major constituents of this protein network are the outer membrane porins OmpA and PorB as well as OmcA and OmcB outer membrane proteins. Once internalized into the host cell, disulfide bridges at the EB surface are reduced to allow the cell to swell in size and form RBs. Thus, the extracellular infectious form, EBs, is more resistant to osmotic pressure than RBs. Moreover, under stress conditions chlamydial cells can enter a third stage called aberrant bodies characterized by enlarged cell size and associated with persistence (Hogan et al. 2004; Kebbi-Beghdadi et al. 2011; Lambden et al. 2006).

Interestingly, RBs appear as Gram-negative cocci, whereas EBs appear as Gram-positive cocci (Lambden et al. 2006) (Fig. 10.2b) despite (1) the presence of LPS or truncated LPS (Horn et al. 2004) and (2) the presence of inner and outer membrane similar to the cell wall of Gram-negative bacteria. This difference in Gram staining clearly reflects the significant modification of the cell wall during the maturation from RBs to EBs (Fig. 10.3).

Similar to the case when cells of the *Planctomycetes* member *Gemmata obscuriglobus* with the protein-rich walls are chemically fixed (Lindsay et al. 1995, 2001), the low amount of peptidoglycan likely explains the modified shape of some *Chlamydiales* that may be observed after exposure to various buffer and fixative solutions used for electron microscopy. The crescent-like shape observed by electron microscopy for *Parachlamydia acanthamoebae* (Greub and Raoult 2002b)

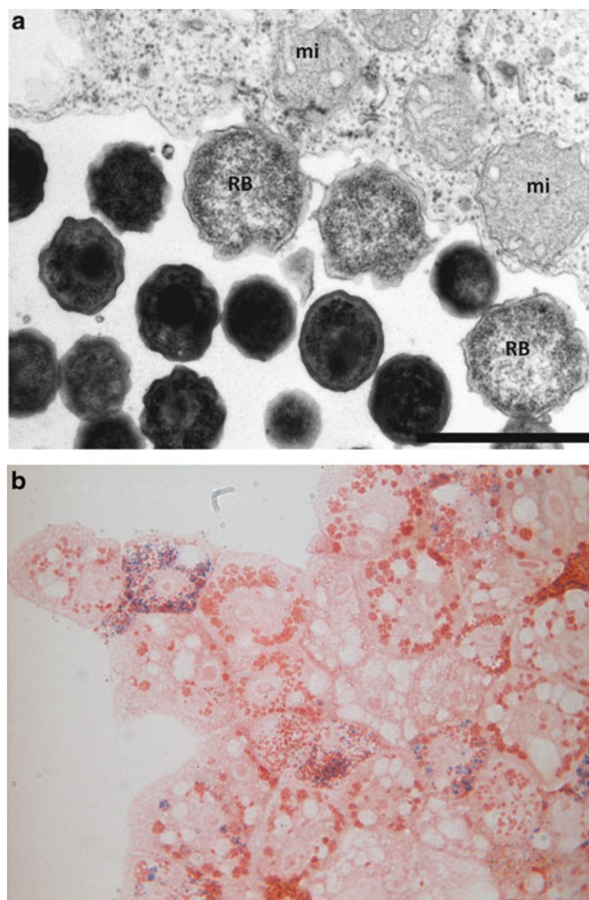

Fig. 10.2 (**a**) *Parachlamydia acanthamoebae* in the vacuole of an *Acanthamoeba* amoeba, as seen by electron microscopy. Note the presence of several electron dense infectious elementary bodies (EBs) with condensed nucleoids, as well as three larger reticulate bodies (RBs), the metabolically active replicative stage. RBs are similar in size to the amoebal mitochondria (mi). Magnification 30,000×; bar = 1 µm. (**b**) *Parachlamydia acanthamoebae* in *Acanthamoeba* amoebae, as revealed by Gram staining. EBs are stained in *blue violet* by the Gram stain, whereas RBs appear as Gram-negative cocci. Magnification 1,000×

(Fig. 10.3c), which is similar to that seen in the chemically fixed planctomycete *Gemmata,* may also be observed among several other *Chlamydia*-related bacteria such as *Protochlamydia naegleriophila* (Borel et al. 2007), *Simkania negevensis* (Lamoth and Greub 2010), and *Criblamydiaceae* (Lienard et al. 2011). The two members of the *Criblamydiaceae* family commonly exhibit star-shaped EBs, which suggested the name of the *Estrella* genus (Lienard et al. 2011). Such peculiar shapes may result from the intrinsic resistance to osmotic pressure at play in EBs. Crescent and star shapes are observed in different proportions for various *Chlamydia*-related bacteria, depending on fixative buffers used (Rusconi et al. submitted). Although these shapes are artifact of fixation and not additional developmental stages, they reflect underlying differences in cell wall or membrane structure and organisation. Interestingly, a multilamellar oblong structure may be seen in most EBs of *Criblamydia sequanensis* (Fig. 10.3d). The function of this putative organelle is unknown but similar structures may also be observed in EBs of *Estrella lausannensis* (Lienard et al. 2011) and *Rhabdochlamydia crassificans* (Corsaro et al. 2007).

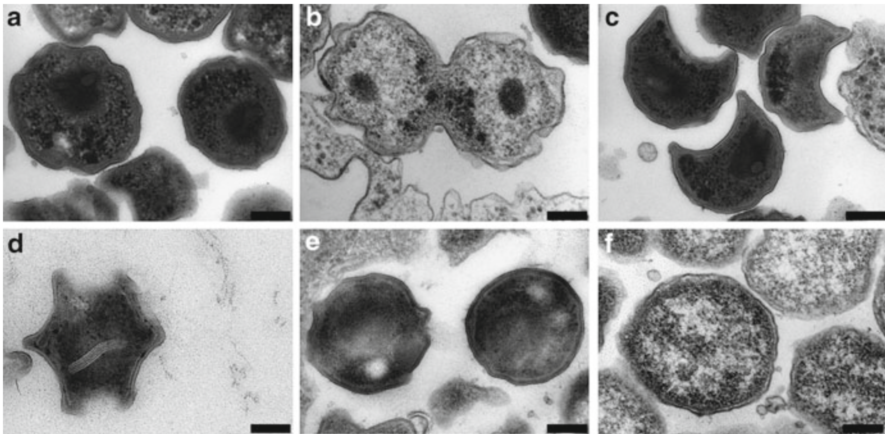

Fig. 10.3 (**a**–**c**) *Parachlamydia acanthamoebae*, as seen by electron microscopy. Note the condensation of the elementary bodies (panel **a**) and the division by binary fission of reticulate bodies (panel **b**). Some electron dense EB-like infectious particles exhibit a crescent shape and are called "crescent bodies" (panel **c**). (**d**) *Criblamydia sequanensis,* as seen by electron microscopy. Note the typical *star shape* of elementary bodies of this member of the *Criblamydiaceae* family as well as the presence of a multilamellar oblong structure of unknown function. (**e**) Elementary bodies of *Simkania negevensis,* as seen by electron microscopy. (**f**) Reticulate bodies of *Waddlia chondrophila.* Magnification of all six panels: 70,000×; bar length corresponds to 0.2 μm

10.4 DNA Condensation

Maturation between replicative and infectious states generally implies condensation and decondensation of the chlamydial nucleoid (Barry et al. 1992, 1993). An example of such nucleoid condensation may be seen in EBs of *P. acanthamoebae* (Fig. 10.3a), whereas decondensation is observed in the RBs of *Waddlia chondrophila* (Fig. 10.3f). In *Chlamydia,* so-called eukaryotic histone-like proteins (Hc1 and Hc2) encoded by *hctA* and *hctB*, respectively (Hackstadt 1991), may be implicated in both condensation and decondensation processes (Barry et al. 1992) and thus regulate chlamydial development (Barry et al. 1993). McInerney et al. (2011) recently pointed out that the name "histone-like" of these latter proteins originates from spurious similarities and eukaryotic histone H1 and Hc1 do not share homology but only a functional analogy.

Although nucleoid condensation is also a key feature of the *Planctomycetes* cell plan (Fuerst 2005), other members of the PVC superphylum do not encode homologs to Hc1 and Hc2 (McInerney et al. 2011). Therefore, nucleoid condensation is likely to take place in *Chlamydiales* and in *Planctomycetes* by different mechanisms and may only reflect an example of convergent evolution, as proposed by McInerney et al. (2011). The elucidation of mechanisms for DNA compaction in other members of the PVC superphylum is a key in evaluating the evolutionary origin of such probable convergent functions.

In addition to a structural function and a reduction of DNA packaging size, nucleoid condensation may provide regulatory functions. Indeed, HctA interaction with DNA and RNA affects transcription and translation, respectively (Pedersen et al. 1994). Recently, the translation of HctA protein was shown to be regulated by the small RNA lhtA consistently across the different Chlamydiae species (Grieshaber et al. 2006a, b; Tattersall et al. 2012). The investigation of histone-like protein presence and function in *Chlamydia*-related bacteria is now a critical issue in understanding the evolutionary history of these interesting proteins.

Besides Hct proteins, several additional eukaryote-like proteins might also be involved in nucleoid condensation/decondensation (Barry et al. 1992; Stephens et al. 1998). The EUO protein was described as a specific protease of Hc1 that may lead to nucleoid decondensation (Kaul et al. 1997). Furthermore, *Chlamydiae* encode a SET domain-containing protein that serves as a histone methyltransferase (Murata et al. 2007). Less well studied are the two SWI/SNF and the SWIB domain-containing proteins that may participate to chromatin remodelling processes by analogy to the SWIB complex in eukaryotes (Bennett-Lovsey et al. 2002).

10.5 *Chlamydiales* Genomes

High throughput sequencing boosted full bacterial genome-sequencing projects, and more than 30 *Chlamydiales* members have been sequenced so far. As expected for free-living organisms, in opposition to obligate intracellular bacteria, genomes of *Planctomycetes* range from 3.8 Mb for *Phycisphaera mikurensis* to 9.6 Mb for *Singulisphaera acidiphila* and are thus much larger than genomes of *Chlamydiales* which range from 1.1 Mb for *C. trachomatis* to 3 Mb for *P. acanthamoebae* (Table 10.2).

Interestingly, as many as 5 % of the proteins encoded on the small *C. trachomatis* genome exhibit a best BLAST hit (BBH) against a *Planctomycetes* member, when excluding other *Chlamydiales* (Stephens et al. 1998). The larger genomes of *Chlamydia*-related bacteria such as *Protochlamydia amoebophila* (2.4 Mb) exhibit similar proportions of BBHs against *Planctomycetes*, highlighting the common evolutionary history of *Planctomycetes* and *Chlamydiales*. More importantly, most of these genes were not horizontally transferred but belong to the core set of genes. Indeed, we observed that among genes present in both *Planctomycetes* and *Chlamydiales*, only few are also absent from Proteobectaria and represents the very small core PVC (or PC) genes set (Gimenez et al. 2011).

With the availability of new genomes, several cases of horizontal gene transfer were reported (Gimenez et al. 2011; Griffiths and Gupta 2002, 2006). The most interesting is certainly the ADP/ATP translocase that is present in multiple copies in all chlamydial genomes as well as in *Rickettsia,* green plant, and algae plastids. The current evolutionary scenario involves an ancestral gene duplication followed by an exchange between *Rickettsiae* and *Chlamydiae* and a transfer from *Chlamydiae* to plants (Greub and Raoult 2003). This latter transfer, dated to one billion years

Table 10.2 Characteristics of a few complete *Chlamydiales* genomes

	Chlamydia trachomatis D/UW-3/CX	*Chlamydia pneumoniae* CWL029	*Waddlia chondrophila* WSU 86-1044	*Protochlamydia amoebophila* UWE25	*Simkania negevensis* Z	*Parachlamydia acanthamoebae* UV-7
Genome size	1,042,519	1,230,230	2,116,324	2,414,465	2,496,337	3,072,383
GC content	41 %	40 %	44 %	34 %	38	39 %
% coding	89 %	88 %	92 %	82 %	91	90 %
Nb of protein coding genes	895	1122	1934	2028	2519	2788
Nb of tRNAs	37	38	37	35	35	40
Nb of rRNA operons	2	1	2	3	1	3
% repeats	0.04 %	0.7 %	4.9 %	1.5 %	nd	nd
Plasmid size	7,493	7,500	15,593	–	132,038	–

(Greub and Raoult 2003), evidences the long history of host parasitism by chlamydial ancestors. In addition, several studies highlighted the contribution of *Chlamydiales* to the establishment of plant and plant plastid functions by identifying several genes of probable chlamydial origin (Huang and Gogarten 2007; Suzuki and Miyagishima 2010; Collingro et al. 2011).

Chlamydia-related bacteria exhibit large variations in genome size, poor synteny, i.e., poor conservation of gene order, and numerous transposable elements (Horn et al. 2004; Bertelli et al. 2010). This suggests constraint to maintain dynamic genomes with higher genetic diversity and a larger adaptability to changing conditions. In contrast, the analysis of *Chlamydiaceae* genomes has also revealed streamlined genomes highly similar in gene content and gene order (Carlson et al. 2005; Read et al. 2000; Myers et al. 2009). Rearrangements, insertions, and deletion mainly arise in a region close to the terminus of replication termed the "plasticity zone" (Read et al. 2003). Evidence for gene decay in *C. pneumoniae* (Myers et al. 2009) and *C. trachomatis* (Thomson et al. 2008) substantiate the hypothesis of an ongoing process of genome reduction.

A recent study on ortholog proteins of members of the PVC superphylum evaluated that 12 % of branches carry indels under positive selection and a few branches carry indels evolving under purifying selection (Kamneva et al. 2012). Based on the rationale that small population size affects the extent of selection, they could confirm that free-living bacteria present a significantly higher percentage of branches under positive selection than *Chlamydiales* that comprise intracellular pathogens. Gene families under strong selection include ABC transporters, genes for general metabolism such as amino acid biosynthesis and bacterial secretion systems.

10.6 Chlamydial Replication

An intriguing but unstudied feature shared by *Planctomycetes* and *Chlamydiales* is the presence of two genes encoding for DnaA, the chromosomal replication initiator protein (Mackiewicz et al. 2004). In *E. coli,* this protein mediates the initiation of bacterial replication by interacting with repetitive non-palindromic sequences called DnaA boxes that are located close to *dnaA* gene at *oriC* region (Messer 2002). It is currently unknown if both copies are transcribed and retain their original function in members of the PVC superphylum.

Interestingly, the protein FtsZ that is a main organizer of the bacterial cell division in most Eubacteria and Archaea is absent in all genomes of *Planctomycetes* and *Chlamydiales* but is present in the genome of *Verrucomicrobiae* and in some genomes of *Lentispherae* (Bernander and Ettema 2010). The absence of FtsZ protein in *Chlamydiales* led some investigators to search for a protein that might act like FtsZ by accumulating at the cell midplane and forming a Z-ring to progressively complete binary fission of the cell. MreB, an analogue of tubulin, was shown to polymerize in vitro without requirement for ATP and to bind to key components of lipid II biosynthesis (Gaballah et al. 2011) and to FtsK (Ouellette et al. 2012).

Lately, Ouelette et al. (2012) demonstrated that MreB and the penicillin-binding proteins (PBP) are essential for chlamydial division. They proposed that MreB might act as a coordinator of bacterial division. Although van Niftrik et al. (2009) recently showed that another unrelated protein accumulates at the division ring of the anammox bacterium *Kuenenia stuttgartiensis*, no homolog of this protein was found among the *Chlamydiales* and other *Planctomycetes* proteins by sequence similarity search. Thus, the MreB homolog in *Planctomycetes* might play a similar role in cell division as for *Chlamydiales*.

10.7 Conclusions

Chlamydiaceae possess small genomes and are closely associated with higher eukaryotes. In contrast, *Chlamydia*-related bacteria, like other intracellular bacteria surviving within amoeba (Moliner et al. 2010), have two- to threefold larger genomes (Horn et al. 2004; Bertelli et al. 2010) and are able to infect a broader host range. Thus, the amoebae-resisting Chlamydiae might have been less constrained by reductive evolution, and their analysis might shed some light on the evolutionary history of *Chlamydiales* and *Planctomycetes*. Further comparisons between genomes of *Planctomycetes* and *Chlamydiales* will definitely provide interesting insights into the evolution and the biology of the PVC superphylum. Moreover, thanks to their rapid growth and the ease of amoeba culture, *Chlamydia*-related bacteria represent easier model organisms than classical *Chlamydia* such as *C. trachomatis* for the study of chlamydial replication and the determinants at play during the developmental cycle of *Chlamydiales.*

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Chapter 11
Planctomycetes: Their Evolutionary Implications for Models for Origins of Eukaryotes and the Eukaryote Nucleus and Endomembranes

John A. Fuerst and Evgeny Sagulenko

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Abbreviations

PVC	Planctomycete–Verrucomicrobium–Chlamydiae
LUCA	Last universal common ancestor
LACA	Last archaeal common ancestor
LBCA	Last bacterial common ancestor
LECA	Last eukaryote common ancestor
FECA	First eukaryote common ancestor
LBACA	Last bacterial and archaeal common ancestor
LPVCEAA	Last PVC eukaryote and archaeal common ancestor
MC	Membrane coating
ICM	Intracytoplasmic membrane
NPC	Nuclear pore complex

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11.1 Introduction

Understanding the evolution of cell organisation is fundamental for understanding evolution of complex cells such as those of the eukaryotes. Planctomycetes and some of their relatives such as the verrucomicrobia within the PVC superphylum (see Chap. 2) exhibit a compartmentalised cell plan not conforming to plans found in most Bacteria and Archaea, and analogous in some ways to that of eukaryotes. So the question immediately arises of whether there is any evolutionary link to eukaryotes which might explain such similarity, and whether regardless of any evolutionary homology, the evolution of compartmentalisation in PVC members can illumine our understanding relating to the general mechanisms underlying how cell complexity can evolve, e.g. how the eukaryotic nucleus originated. The complex planctomycete cell has implications for how such a complex process as endocytosis could have originated, how endomembrane systems evolve and how the DNA of the nucleoid could become enveloped by internal membranes which might form part of such wider endomembrane systems. We know that the last eukaryotic ancestor must have been quite a complex cell and that the endomembrane system and endocytosis and probably a multifunctional cytoskeleton must have been present (Dacks and Field 2007; Dacks et al. 2008; Elias et al. 2012; Field and Dacks 2009; Wickstead et al. 2010). Of course, the evolution of complex internal structure should also be correlated with evolution of new molecular cell biology features not known in other bacteria, e.g. transcription may be uncoupled from translation if there is an enveloped nuclear compartment as occurs in *Gemmata obscuriglobus,* and there may be a need for a nucleocytoplasmic transport system for transport of macromolecules across the nuclear envelope. In eukaryotes, the MC (membrane-coating) coatomer proteins such as clathrin are significant agents in eukaryote-characteristic cell biology processes, such as endocytosis and other membrane trafficking, and have been proposed in the ancestral form of protocoatomers as central to evolution of both endocytotic vesicles with their high membrane curvature and the nuclear pores, where nuclear envelope is associated closely with curved membrane (Devos et al. 2004) (see Chap. 3). Proteins homologous with eukaryote MC proteins appear to occur in PVC superphylum members and in the phyla *Planctomycetes*, *Verrucomicrobia* and *Lentisphaerae* (Santarella-Mellwig et al. 2010). Members of all three phyla possess cells compartmentalised by internal membranes (Lee et al. 2009) and at least *G. obscuriglobus,* and probably other planctomycetes, possess endocytosis abilities consistent with a mechanism involving clathrin-like MC proteins (Lonhienne et al. 2010). Multiple homologues occur in each PVC member with MC representatives. Phylogenetic and bioinformatics analysis suggests that the last common ancestor of PVC phyla already possessed MCs (Santarella-Mellwig et al. 2010) and thus perhaps endocytotic protein uptake ability. The phylogenetic significance of planctomycete compartmentalisation is already suggested by the striking distribution of the subtypes of compartmentalisation within the planctomycetes, all the phylogenetically closely related but diverse anammox planctomycetes have an anammoxosome, all

members of the Gemmata clade appear to possess a double-membrane nuclear envelope and a nucleoid is surrounded by that envelope within the pirellulosome in these species but not in other planctomycetes or PVC members. Once a compartmentalisation plan was invented, it seems to have been retained within particular clades, suggesting functional significance, one which in anammox planctomycetes has clear biochemical meaning. The molecular mechanisms evolved by PVC members may have evolved in an analogous way to those used by eukaryotes, and homology with eukaryote MC proteins is likely on the basis of protein structure as well as even primary sequence alignment.

There are several unique features of Planctomycetes or their members requiring evolutionary explanation, together indicating the unique status of the phylum within the Bacteria. Their status as Bacteria is consistent with not only 16S rRNA phylogenetics and oligonucleotide signature but also molecular markers such as Shine-Dalgarno sequences in mRNAs (Leary et al. 1998), bacterial promoters(Liesack and Stackebrandt 1989), secretion systems (Glockner et al. 2003), genes for lipid A of lipopolysaccharide(Sutcliffe 2010), at least some genes for peptidoglycan synthesis (Bernander and Ettema 2010; Pilhofer et al. 2008) and genes for bacterial-type flagella (Snyder et al. 2009). It is also consistent with their resistance to diphtheria toxin (Stackebrandt et al. 1984). However, they have a unique 'profile' of features not typically found in Bacteria. When these are taken individually, they are difficult enough to account for in evolutionary terms, but when viewed occurring together, they strengthen a suggestion of novel evolutionary mechanisms or affinities, e.g. sterols, protein cell walls, absence of peptidoglycan, absence of FtsZ and other than FtsK of cell division proteins of the Z ring divisome in genomes, presence of C1 transfer enzymes with unclear domain affinities, internal membranes and compartmentalised cell plan, condensed nucleoids and in anammox planctomycetes ATP synthase in internal compartment membranes (Fuerst and Sagulenko 2012). When we look more widely to the PVC superphylum, in the verrucomicrobia in addition to compartmentalised cells we find traces of an ancient eukaryote-like tubulin, and even some species that like the planctomycetes appear to have no peptidoglycan. The epistemological concept of consilience where independent pieces of evidence support an explanatory model seems applicable here, and an ideal evolutionary model will explain several of these unique features and predict new ones, some of which such as C1 transfer enzymes may be quite unexpected for a proto-eukaryote. They may nevertheless yield clues regarding relationships that could also explain such homologies (e.g. derivation from an ancient pre-domain LUCA or extensive gene transfer in a progenote or during eukaryogenesis). In this case, purely bioinformatic and phylogenomic evidence (especially based on only primary sequence data) may be of limited use without such an explanatory framework.

A complex cell plan in bacteria such as planctomycetes is related in a direct way to the importance of and controversy over the prokaryote–eukaryote dichotomy (Cavalier-Smith 2007; Dolan and Margulis 2007; Fuerst 2010; Martin and Koonin 2006b; Pace 2006, 2009; Whitman 2009). But the evolutionary meaning of that dichotomy has not been clear. Phylogenetically the types of different living organisms now appear as a trichotomy with acceptance of the three separate domains.

The original dichotomy of cell plans must now be seen in relation to the three domains, the degree to which they can be considered completely separate on a genomic and evolutionary basis and the relationship of the two domains the members of which display classical prokaryote organisation (Archaea and Bacteria)—i.e. without membrane-bounded nuclei and organelles—to the members of domain Eucarya and their complex organisation. Has the complex eukaryote structure been the result of cell fusion or other endosymbiotic events involving two or more domain members, e.g. from Archaea and Bacteria, or was there vertical reduction from a complex eukaryote-like LUCA ancestor? Or is the story even more complex, involving lateral gene transfers building up a eukaryote from many bricks of many origins? What are the implications for the concept of 'prokaryote' as a purely organisational term of planctomycetes and PVC bacteria with their more complex organisation than any other bacterium or archaeon and what are their implications for evolution of cell plans?

A planctomycete cell plan could indicate that a complex cell plan could evolve autogenously without the need for multiple partners or reduction from a more or equally complex ancestor (chlamydia is a possible example within the PVC superphylum of reductive cell plan, but these organisms have not been examined by cryofixation methods to check for the planctomycete plan). The existence of compartmentalisation in at least three phyla of PVC superphylum suggests that the PVC ancestor may have shared the cell plan of these phyla. It is also possible that planctomycete/PVC cell plans have evolved for specialised purposes connected with the particular water or soil habitats and nutritional opportunities of the ancestral species—e.g. that they are correlated with endomembrane development and vesicle forming proteins needed for macromolecule uptake (such a development may even have been very early in cells dependent on 'primordial soup' macromolecules). Development of endocytosis and special vesicles with lysosome-like digesting activity may have also implied the need for internal membrane protection of the DNA of the nucleoid and of the ribosomes.

11.2 Models for Origins of Eukaryotic Nucleus and Endomembranes

Theories for origins of eukaryote nuclei and endomembranes concentrate on various forms of a fusion or at least close mutualism between a member of the Archaea domain and a member of the domain Bacteria (Poole and Penny 2007). This sometimes involves a mutualism based on hydrogen transfer with various partners proposed (Martin and Muller 1998; Moreira and Lopez-Garcia 1998). More recently the origin of the nucleus has been proposed to be based on molecular consequences of an archaeon–bacterium fusion regarding intron invasion of protein-coding genes and its implications for development of gene splicing and the consequent separation of slow transcription and splicing of pre-mRNA from fast translation based on processed mRNA (Koonin 2006; Martin and Koonin 2006a). However, the inter-domain fusion

still remains central to these theories, together with a concept that the alphaproteobacterium as a partner in the fusion is needed somewhere in the process combined with the view that mitochondria were acquired before the nucleus. The archaeon–bacterium fusion has the advantage of attempting to explain the chimeric nature of the eukaryote genome. However, there are heretical and very reasonable views that interpret the supposedly chimeric nature of that genome quite differently with no necessity for acceptance of inevitable fusions as the only explanation for genome data and with a possibility of bioinformatics analysis of artefacts accounting for the appearance of an Archaea–Bacteria chimera (Kurland et al. 2003, 2006). And the archaeon–bacterium fusion has major disadvantages. These include the absence of any contemporary archaeon–bacterium fusion model and the improbability of a process where informational protein genes of the bacterial partner were lost and replaced by those of the archaeal partner. Such improbability follows from analyses indicating that modern eukaryote informational proteins are apparently more closely homologous to those of Archaea than to those of Bacteria (Forterre and Gribaldo 2010).

If symbiotic fusion between domains Archaea and Bacteria is not really necessary to explain eukaryote and nucleus origins, then we might look for the signs of other types of mechanism to explain such origins. One of the consequences of acceptance of PVC compartmentalisation and particularly of an organism such as *G. obscuriglobus* is that there is no longer a necessity to assume some form of cellular fusion as underlying the formation of the compartmentalised structural aspects of the eukaryote cell. Autogenous theories of the origin of the nucleus become more attractive once we have rejected that necessity to follow inter-domain fusion. This is so whether planctomycetes or their ancestors invented compartmentalisation or whether they inherited such a plan from a similarly complex ancestor and is also independent of any evolutionary homology between eukaryote-like characters in planctomycetes and eukaryote characters resembling them. Planctomycetes make an autogenous origin of organelles such as nuclei possible regardless of whether the mechanism of such origin is the result of convergent evolution or of vertical or horizontal evolutionary relationship.

If we require a model for autogenously evolving endomembranes and even nucleus-like structures in a planctomycete like the ancestor of *Gemmata* species, we might ask what selective pressures might be at play to make evolution of internal endomembrane systems by a bacterium or archaeon with simple cell plan desirable. We might also ask why such a gene- and energy-costly structure would be retained once acquired. Possible pressures might be associated with increased cell size and genome, need for increased surface area of internal membranes, development of anammox physiology or evolution of a new mode of cell nutrition via endocytosis.

11.2.1 Protective Chambers

Some of the most deeply branching planctomycetes appear to be anammox ammonium oxidisers (Chap. 4 and van Niftrik and Jetten 2012). These depend on the

anammoxosome compartment which plays a key role in their metabolism and possibly for protecting DNA from toxic intermediates like hydrazine or for preventing leakage of protons across the proton-motive force (pmf)-producing anammoxosome membrane in an organism with such a very slow growth rate (see Chap. 4). The anammox nutrition could provide a selective pressure for evolution of an internal membrane-bounded compartment, which then formed the basis for compartments in other later-evolving planctomycetes in which they acquired a different function (so that the function of compartments in anammox bacteria would be a preadaptation). In this early organism internal membranes such as the ICM, paryphoplasm, riboplasm and condensed nucleoids would already have been present, as well as the specialised anammoxosome. The condensed nucleoid may have been functional for UV resistance on the early Earth with its predicted minimal or no ozone layer and high UV levels (Cockell and Raven 2007).

11.2.2 Nutritional Selective Pressures for Endomembrane Adaptations

Autogenous origin of endomembranes via nutritional selective pressure might involve the type of transition proposed by Christian De Duve from an exoenzyme-secreting bacterium with ribosomes secreting proteins directly across the cytoplasmic membranes to an endocytosis-capable organism and eventually an organism with internal vesicles with bound ribosomes approaching rough endoplasmic reticulum (RER) (Fig. 11.1) (de Duve 2007).

The invention of some form of endocytosis capturing macromolecules from the external milieu must have been a key innovation in the origin or evolution of the eukaryotic cell. The last common ancestor of the eukaryotes (LECA) seems to have been quite complex and already possessed a complex endomembrane system and its major membrane-trafficking activities (Dacks et al. 2008; Field and Dacks 2009). There is no reason that a precursor first eukaryote common ancestor or proto-eukaryote also could not have had at least rudimentary elements of the essential endomembrane system.

There are even reasonable models for development of the endomembrane system from an initial bacterium secreting enzymes across the cytoplasmic membrane and via vesicle formation from that membrane (via MC proteins?) developing a new form of nutrition based on internalisation of macromolecules, as a precursor to development of phagocytosis (Fig. 11.1) (de Duve 2007). The occurrence of what appears to be a receptor-mediated endocytotic protein uptake in the planctomycete *G. obscuriglobus* together with eukaryote-homologous MC proteins is consistent with such a model. Of course an ancestor of this species rather than the contemporary representative of the lineage would have been the initiator of this nutritional mode, consistent with the occurrence of MC proteins in at least two other sister phyla of the PVC superphylum to which planctomycetes belong.

Giardia forms an interesting possible model of how such a mechanism may have worked in the First Eukaryote Common Ancestor (FECA) or a proto-eukaryote

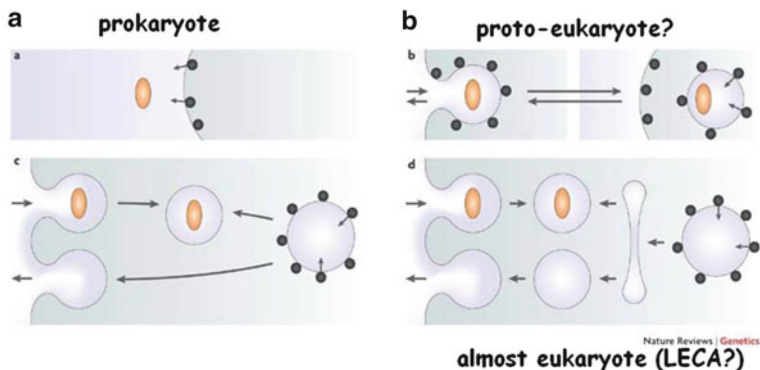

Fig. 11.1 Model for evolution of a proto-eukaryotic endomembrane system from invagination of plasma membrane associated with ribosomes secreting exoenzymes across the membrane. (**a**) Secretion of exoenzymes for food (*orange oval*) across cytoplasmic membrane (with bound co-translationally secreting ribosomes indicated via *black circles*) of a bacterium. (**b**) Invagination of cytoplasmic membrane to form endosome-like vesicle which can also perform protein digestion—ribosomes still bound to the vesicle mean that the vesicle contents are somewhat analogous to the RER lumen of eukaryotes. (**c**) Ribosome-bearing vesicles migrate into cell interior forming proto-ER, and some cargo digestion occurs in lysosome-like vesicles, but other digestion can still occur extracellularly via exocytosis of digestive enzymes. (**d**) ER and endocytic system differentiate, intracellularly active digestive enzymes are sorted by a proto-Golgi system to endosomes and lysosomes, and other secreted proteins are discharged outside the cell via exocytosis. We now have something approaching the endomembrane and membrane-trafficking system of the First Eukaryote Common Ancestor (FECA) or the Last Eukaryote Common Ancestor (LECA) (From de Duve 2007)

conforming to the De Duve mechanism for endomembrane evolution, regardless of whether the *Giardia* mechanism may represent reductive evolution from a more complex organisation (Lanfredi-Rangel et al. 1998). Unlike the situation in other eukaryotes, in *Giardia* the endosome vesicles are continuous topologically with the endoplasmic reticulum (ER) lumen rather than with the cytoplasm, and protein degradation occurs within the ER lumen (Abodeely et al. 2009). To some extent analogously, protein uptake vesicles in the planctomycete *G. obscuriglobus* are located in the paryphoplasm (like the ER lumen continuous with the space between nuclear envelope membranes) rather than the ribosome-containing pirellulosome cytoplasm. Protein degradation may also occur somewhere within the paryphoplasm/ER lumen equivalent. The analogy breaks down at a detailed level since *Gemmata* endocytic vesicles are contained within the paryphoplasm/ER lumen equivalent rather than being continuous with it.

It is not a large step or series of steps to evolve a nuclear envelope once we have an endomembrane system; the two are linked in modern eukaryotes, in the form of the continuity between outer nuclear envelope membrane and endoplasmic reticulum. The evolution of the nuclear pore complex (NPC) needed for transport across a nuclear envelope could have a common molecular origin with that of endocytosis, in the form of an ancestral MC protein as in the protocoatomer hypothesis, since membrane curvature in vesicle and in nuclear envelope membranes on either side of the nuclear pore complex is needed in either case, and homology exists between

some NPC and MC proteins. Nuclear pores have not yet been confirmed to exist in planctomycetes such as *G. obscuriglobus*, but they might be predicted to occur based on the double-membrane structure of the nuclear body envelope combined with occurrence of MC protein homologues in this organism. This would also be consistent with the De Duve model and highly significant in terms of evolutionary significance of planctomycetes and models for the origin of the eukaryotic nucleus, since it would further strengthen the concept of a true homology between planctomycete and eukaryote compartmentalisation and perhaps even between *G. obscuriglobus* nuclear bodies and the eukaryote nucleus.

The development of endocytosis may have been a necessary precursor to the evolution of phagocytosis and ability of unicellular ancestral proto-eukaryotes to engulf other cells as an extension of acquiring macromolecules as a mode of nutrition. In order to achieve this, however, a wall-less version of the organism originating endocytosis might have to evolve. This step has been proposed for a relative of the last archaeal common ancestor (LACA), an organism which may have been derived initially via loss of peptidoglycan synthesis from a bacterium (Cavalier-Smith 1981, 1987, 1988, 2010).

The archaeal relative in this model can be easily replaced by a wall-less PVC group ancestor or planctomycete ancestor. Such an organism could also have possessed not only endocytosis but also some form of phagocytosis (e.g. via ancestral *Prosthecobacter* tubulin microtubule cytoskeleton-based mechanics—see below). Planctomycetes would then appear to have replaced an ancestral peptidoglycan with a protein wall in an analogous manner to that proposed for Archaea in the wall-less archaeal 'phagocyte' model. This would be consistent with Scenario 3 (see below) in which an ancestral bacterium evolves compartmentalised cells and forms an ancestor to PVC, Eucarya and Archaea lineages.

Arguments against the potential connection between planctomycetes and eukaryotes based merely on cell and genome size (McInerney et al. 2011) are complicated and weakened by the enormous range of eukaryote cell sizes and genome sizes, effectively resulting in overlap with the bacteria. The cell of the eukaryotic alga *Ostreococcus,* for example, is 1 μm × 0.7 μm yet fits in a mitochondrion, chloroplast and membrane-bounded nucleus in that 'bacteria-size' boundary, while the admittedly parasitic eukaryote the microsporidian *Encephalitozoon cuniculi* has only a 2.9 Mb genome relative to the 9 Mb genome of *G. obscuriglobus*. Such overlaps make any assumptions about the nature of the cell size or genome size of ancient precursors of eukaryotes difficult to formulate or to make the basis for a rejection of homology.

11.3 Evolutionary Explanations for Planctomycete Compartmentalisation

The compartmentalised cells of planctomycetes and some species of related PVC superphylum members are unique among the Bacteria, as most dramatically demonstrated by the membrane bounded nucleus-like organelle of *G. obscuriglobus* and

the organelle of anammox planctomycetes linked to a unique biochemistry and lipid chemistry. These internal membrane-bounded compartments immediately call for comparison with the eukaryotic endomembrane system and membrane-bounded organelles such as the nucleus and even in the case of anammoxosomes with their membrane-bound ATP synthases, the mitochondrion (Chap. 4). Further than structure, there are now indications of functional analogies in the form of protein uptake by a mechanism resembling eukaryotic endocytosis. We cannot say yet that there is definite evolutionary homology with eukaryotes even in the case of the nucleated *G. obscuriglobus* but the case has been made that homology is more likely and more parsimonious an explanation for the eukaryote-like features of an organism like *G. obscuriglobus* than analogy or convergence(Fuerst and Sagulenko 2012). In the case of the MC proteins (Chap. 3) homologous with clathrin-like proteins of eukaryotes associated with mechanisms of endocytosis and formation of vesicular endomembranes, we even have a molecular level homology with eukaryotes shared between planctomycetes and other PVC superphylum members (though this may be complicated by cross-occurrence in the *Bacteroidetes* phylum (Devos 2012). Other potential clues may be found in analysis of such elements as signal peptides, which in anammox planctomycetes at least seem to be more similar to those of eukaryotes than to those of Bacteria (Medema et al. 2010), consistent with the finding in the non-anammox planctomycete *Rhodopirellula baltica* of N-terminal export signal peptides with eukaryote-homologous domains including cadherin and calx-β (Studholme et al. 2004). Distinctive but sporadic eukaryote-homologous molecular features known so far include discoidin domains in some proteins (Studholme et al. 2004) and an integrin homologue (Jenkins et al. 2002a). So what is the evolutionary meaning of this probable homology—how did it come about? This is closely related but not identical to the question 'how did planctomycete compartmentalisation evolve?', since it may be that it evolved elsewhere but was retained in planctomycetes and relatives from a common ancestor where it actually evolved first or was the product of large scale gene transfers from a lineage where it had originated. Possible explanations for PVC compartmentalisation can be summarised in at least three major models (Figs. 11.2, 11.3 and 11.4). Some of these hypotheses can be used to make predictions and perhaps these can stimulate focused experimental programmes and phylogenetic and bioinformatic analysis and search projects. *Scenario 1* (Fig. 11.2) assumes an ancestral eukaryote-like LUCA with compartmentalisation with internal membrane-bounded nuclear organelle and retention of compartmentalisation in the PVC superphylum and possibly in *Ignicoccus* of Archaea. *Ignicoccus* has an 'outermost membrane' which is energised and contains ATP synthase, H(2):sulphur oxidoreductase and AMP-forming acetyl-coenzyme A synthetase, elements of the ATP-synthesising, electron transport and CO_2-fixation pathways in this archaeon (Kuper et al. 2010) (Mayer et al. 2012) and possesses an inner membrane surrounding the ribosome-containing cytoplasm; vesicles are found between the two membranes (suggesting *Ignicoccus* should be tested for macromolecule uptake)—it is the most likely analogue of planctomycete cell plan in the Archaea. Such compartments are assumed lost in most other Bacteria and Archaea. A LUCA with some complex cell features of a proto-eukaryote has been proposed for

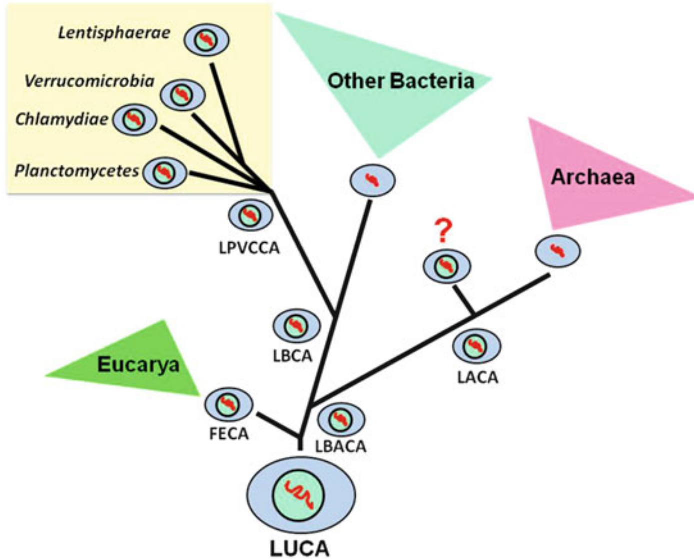

Fig. 11.2 Scenario 1 model for explanation of compartmentalisation of PVC superphylum species (*yellow box*). LUCA (Last Universal Common Ancestor) is a complex cell with internal membranes and at least one membrane enclosing the nucleoid (*red*). This compartmentalisation is retained in PVC members and Eucarya and perhaps some deep Archaea (*question mark*) but independently lost in most Bacteria and Archaea. The LBACA (Last Bacterial and Archaeal Common Ancestor), the LBCA (Last Bacterial Common Ancestor) and LACA (Last Archaeal Common Ancestor) as well as FECA (First Eukaryote Common Ancestor) and the LPVCCA (Last PVC Common Ancestor) retain compartmentalisation from LUCA, while such compartmentalisation is lost in most Bacteria and Archaea. Note that only PVC phyla with cultured members have been included here

separate reasons, for example, uncertainty of three-domain phylogenies, resemblance of eukaryotes with proposed RNA World molecular biology and the distribution of phylogenetically related protein fold superfamilies (Forterre and Philippe 1999; Glansdorff 2000; Glansdorff et al. 2008; Kurland et al. 2007; Penny and Poole 1999; Wang et al. 2007). Scenario 1 is very similar to one earlier proposed 'retention scenario' for the origin of modern compartmentalised cells(Forterre and Gribaldo 2010). Scenario 1 is also similar to that proposed in another discussion of PVC evolutionary significance (Reynaud and Devos 2011), but in that scenario rather than necessarily assuming a complex LUCA, the complex plan is assumed to be a shallower feature of the last common ancestor of both the PVC superphylum and the last common ancestor of Eucarya and Archaea. The other two scenarios in Figs. 11.3 and 11.4 do not assume a eukaryote-like LUCA, and the topology of the three-domain tree is conventional with a Bacterial root. In *Scenario 2* (Fig. 11.3) there is convergent evolution of compartmentalisation in the ancestor of the PVC superphylum and in the ancestor of Eucarya, with HGT from PVC to Eucarya-supplying genes evolved first in the PVC superphylum such as the tubulins of *Prosthecobacter* and the MC clathrin-like proteins correlated with

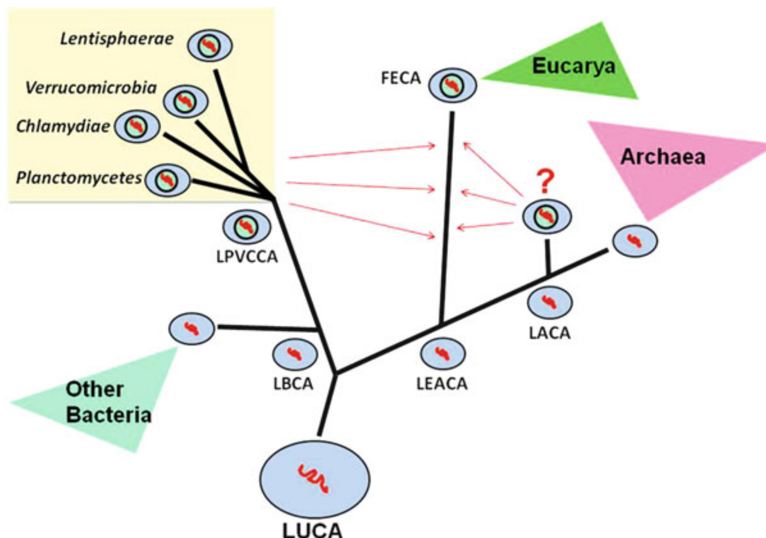

Fig. 11.3 Scenario 2 model for explanation of compartmentalisation of PVC superphylum species (*yellow box*). LUCA is not compartmentalised and LBCA and LACA are also simple non-compartmented cells. The last common ancestor of all Eucarya and Archaea (LEACA) is also not compartmented. Independently compartmentalisation is invented in the ancestors of LPVCCA, Eucarya and some Archaea within Crenarchaeota (e.g. ancestral to modern *Ignicoccus*). HGT (Horizontal Gene Transfer) from ancestral PVC members and ancestral Crenarchaeota and/or Thaumarchaeota (*question mark*) donates features of eukaryality such as MC proteins and tubulins (from PVC ancestors) and actins and ESCRT systems (from ancestral Crenarchaeota and Thaumarchaeota) to the lineage leading to the FECA within Eucarya. FECA's compartmentalisation and endomembrane systems may be dependent on the ensemble of these transfers

receptor-mediated endocystosis in *G. obscuriglobus*. In this view, PVC members are a 'toolkit' for eukaryality rather than being directly descendant to or from eukaryotes. Archaea may also have been involved in such gene transfer, making up a complement of proto-eukaryote genes including actins, histones and the ESCRT system, and virus genomes could supply missing components (Forterre 2011). However, fusion and engulfment was not necessarily essential for such transfers—there is evidence for inter-kingdom gene transfer between bacteria and eukaryotes on a smaller scale (Figge and Cerff 2001; Lawrence et al. 2011). An alternative to this scenario is that the PVC superphylum members have evolved their cell plan via convergent evolution but that no gene exchange has occurred between PVC members and Eucarya and that any analogous molecular and structural features are coincidental or reflect shared selective pressures towards acquisition of compartmented cells by the two groups (McInerney et al. 2011). In *Scenario 3* (Fig. 11.4), evolution of compartmentalisation occurs in a common ancestor of PVC, Eucarya and Archaea, with the PVC ancestor assumed to have diverged very early during the origin and radiation of domain Bacteria. The root LUCA organism is assumed as Bacteria-like and not compartmentalised. So this scenario involves invention of

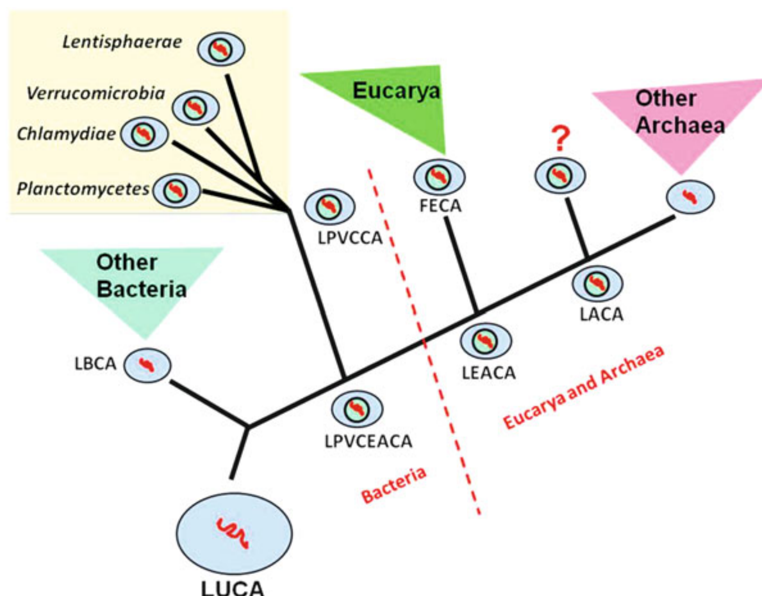

Fig. 11.4 Scenario 3 model for explanation of compartmentalisation of PVC superphylum species (*yellow box*). LUCA is not compartmentalised and LBCA is not compartmentalised, but the last common ancestor of the PVC superphylum, Eucarya and Archaea (LPVCEACA) is compartmentalised, an invention which occurs along the lineage leading from the bacterial root of the domain tree in this case. The arbitrary demarcation of lineages in the Bacterial domain and those no longer distinguishable as Bacteria forming the root lineage of Eucarya and Archaea sister domains is indicated by the *red dotted line*. LPVCCA, LEACA and FECA are compartmentalised, but this feature is lost on the lineage leading to most of the Archaea, except perhaps for some Crenarchaeota such as *Ignicoccus* or deep-branching Archaea yet to be isolated (*question mark*)

compartmentalised cells, endomembrane systems and nucleus in an ancestor of PVC superphylum and the other two non-Bacteria domains. A recent hypothesis regarding origin of eukaryotes is somewhat similar in that it assumes a compartmentalised ancestor of Eucarya and Archaea but rejects the PVC superphylum as related to that pre-eukaryote (Vesteg and Krajcovic 2011).

Conceivably a planctomycete such as an anammox planctomycete with both ether-linked and ester-linked lipids could form the origin of these distinctive lipid classes in membranes of Archaea on the one hand and Eucarya in the other, but we do not know enough about the stereoisomers of these planctomycete lipid classes (known to be distinct in Archaea and Eucarya) to know how plausible this might be. LUCA may well have had membranes with quite a complex lipid composition (Lombard et al. 2012). In addition, the anammox lipids are unique among the three domains of life concerning the structure of their hydrocarbon chains, consisting as they do of a ladder of concatenated cyclobutane rings to form a characteristic ‘ladderane’ lipid (cf. isoprenoid long-chain alcohol-derived chains in Archaea and fatty acid-derived hydrocarbon chains in Bacteria) (see Chap. 4).

Scenarios such as 1 and 2 not involving some type of conversion of a Bacteria member to a member of the Eucarya lineage have the advantage of not requiring invention of a distinctive domain-specific 16S ribosomal RNA as defines the domain Eucarya. Need for such invention is also a disadvantage of various fusion or endosymbiotic hypotheses of eukaryote origins. Relation of planctomycetes to ancient precursor cells as in Scenarios 1 and 3 may be consistent with claims that planctomycetes at least may be deep branching based on phylogenetics of conserved rRNA positions (Brochier and Philippe 2002) or proteome phylogenetics (Jun et al. 2010). If homology with eukaryote structure or function is very ancient, we might expect as has been found that a bioinformatics signal for molecular sequence homology of genes with cell biology relevance is quite weak and will pose challenges which can be met only by considering higher structural levels for proteins.

These evolutionary scenarios might prove valuable for our progress if only to form the basis for future experimental or bioinformatic tests of these hypotheses and for prediction of results of future experimental tests. For example, Scenario 1 predicts that the archaeon *Ignicoccus* or another archaeon retaining features of a LUCA of proto-eukaryote complexity might prove to possess endocytotic ability similar to planctomycetes. It also predicts that compartmentalisation might also be retained from the complex LUCA in phyla other than PVC superphylum members, or features such as internal membranes with bound ribosomes but without clear plasma membrane origins may be found in other phyla, e.g. cyanobacterial internal complexity may reflect such origins. Scenario 2 predicts that a number of homologous genes will be found to be shared by PVC members and eukaryotes in addition to those found, but that due to the ancient HGT proposed, secondary and tertiary structure of proteins will have to be employed to find them. Such measures will also have to be applied to detect any ancient homology with a eukaryote-like LUCA or proto-eukaryote FECA in Scenarios 1 and 3. Planctomycetes are known to possess very high proportions of hypothetical proteins with unknown function in their proteomes, meaning that such 'dark genomic matter' may hide relevant homologues and be expected if the relationship is an ancient one. If Scenario 3 is correct then we should find more homologues between PVC members and Archaea than have been presently determined, e.g. regarding components or structure of protein cell walls, a type of wall occurring in both planctomycetes and some Archaea members. Regarding even simple genome annotation-based analyses, *R. baltica* has been claimed to have ca. 8 % of its proteome as homologous to domain Eucarya proteins (Glockner et al. 2003). Out of 3,380 ORFs identified for *R. baltica* in that study using an expect cut-off of $<1\times10^{-3}$, 8 % were assigned as homologous to proteins from the domain Eucarya, while other bacteria such as *Thermotoga maritima* and *E. coli,* for example, display only 2 and 0.4 % hits, respectively, to Eucarya proteins (Glockner et al. 2003). Some other whole genome-based analyses also using BLAST approaches only have argued for absence of any significant level of homology of planctomycetes (*R. baltica*, *Blastopirellula marina*, *G. obscuriglobus* and *Kuenenia stuttgartiensis*) to eukaryotes, noting possibility of database-based distortions (Fuchsman and Rocap 2006). These and other conclusions may need revision after analysis of secondary and tertiary protein structure comparisons possible with

emerging methods. Rejection of the hypothesis of at least genes significant for compartmentalisation and endomembranes may be premature based only on primary sequence homology searches.

In Scenarios 1–3 we have considered autogenous or ancestral development of compartmentalised cells, and not various possible fusion hypotheses. However, even in a symbiotic fusion scenario, planctomycetes have been proposed as relevant, e.g. in the scenario contemplated where a compartmentalised PVC ancestor was the bacterial partner in a archaeon–bacterium fusion involving a mesophilic member of the Thaumarchaeota, a modern group with a wide range of eukaryotic features such as type IB topoisomerase and histones. This archaeon would also contribute proto-eukaryotic features such as the ESCRT system and actins, so that tubulins and actins, the key cytoskeletal proteins for eukaryality, would be present in the fusion and with distinct similarities already with the homologous eukaryotic proteins. The critical context of this analysis simultaneously constructing and assessing this proposal indicated the need for its rejection along with other even less likely fusion hypotheses (Forterre 2011; Forterre and Gribaldo 2010). In that scenario or series of alternative scenarios (Fig. 11.5), the planctomycete-like cell nuclear envelope or the ICM would form the basis for the nuclear envelope, depending on whether the fusion partner was a *Gemmata*-like ‘synkaryote’ with nuclear body or a simpler *Pirellula*-like ‘akaryote’ without a double-membrane-enveloped nuclear compartment but with an ICM. MC-like vesicle and nuclear pore proteins, ester-based membrane phospholipids, the ICM membrane, MC proteins, tubulins and sterol-synthesising enzymes would also be donated to the proto-eukaryote by this PVC member, but much of the PVC genome might be lost with the archaeon taking on the major genomic role. Such a PVC-like partner clearly would have a set of ancestral properties combining known properties of several contemporary PVC members, many of which may have by now lost some of these characters. To complete the eukaryote-characteristic complexity, this scenario also involves invasions of the early proto-eukaryote by viruses such as ancestors of NCLDV (nucleo-cytoplasmic large DNA viruses) to account for other eukaryote-specific protein families not found in PVC bacteria or Thaumarchaeota. Some major problems of eukaryote origins are not solved by this or any other fusion scenario. These problems include the unique features of eukaryote viruses and the occurrence of three domain-specific versions of rRNA and thus ribosomes rather than only two versions as expected from fusion hypotheses, as well as the apparent uniqueness of the initial fusion event. These problems and especially the hypothesis of inter-domain cell fusions are not easily explicable in terms of a uniformitarian philosophy of causation dependent on contemporary mechanisms, e.g. where Archaea–Bacteria fusions have not yet been discovered (Forterre 2011; Poole and Penny 2007).

We can also consider scenarios not so much from a phylogenetic perspective as from a cell biology perspective, in terms of the descent and fate of membranes and membrane compartments. In a cell biology model compatible with Scenario 1 (Fig. 11.6a), a proto-eukaryotic ancestor might have at least some of the features of endomembranes and their topological relations to the cell, but the features are modified during subsequent evolution such that the paryphoplasm evolves as a separate

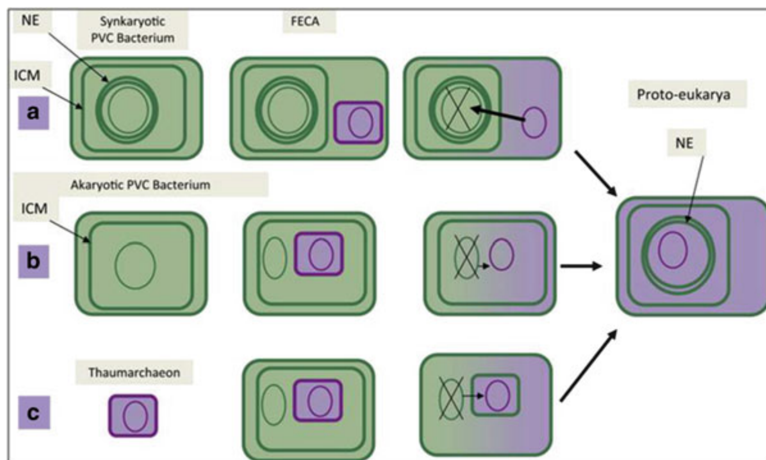

Fig. 11.5 Scenarios according to the PTV hypothesis for origin of the eukaryotic nuclear envelope involving fusion between (**a**) a PVC bacterium such as an ancestor of *Gemmata obscuriglobus* with intracytoplasmic membrane (ICM) and double-membrane bounded nucleoid (a 'synkaryotic' PVC cell) or (**b**) and (**c**) a PVC bacterium such as an ancestor of *Pirellula staleyi* or *Blastopirellula marina* with the simplest planctomycete cell plan of only an ICM (an 'akaryotic' PVC cell), in each case with an archaeon in the Thaumarchaeota. The scenarios eventually result in evolution of a proto-eukaryote with nuclear envelope and endomembrane derived from the PVC partner. In (**a**) the eukaryotic nuclear envelope and endomembrane is derived directly from the PVC bacterium nuclear envelope and ICM, in (**b**) the eukaryotic nuclear envelope is derived from the ICM of the PVC bacterium and in (**c**) the eukaryotic nuclear envelope is derived directly from the cytoplasmic membrane of the thaumarchaeon, but this has been replaced with one derived from the PVC partner ICM. Bacterial chromosomes, membranes with bacterial-type phospholipids and the bacteria-derived cytoplasm including ribosomes are in *green*, archaeal chromosomes, membranes with Archaea-type phospholipids and Archaea-derived cytoplasm including ribosomes are in *purple*. The First Eukaryotic Common Ancestor (FECA) resulting from the fusions is transformed into the proto-eukaryote organism before any mitochondrial endosymbiosis has occurred, and extensive viral invasion has not yet remodelled any of these organisms. In (**a**) and (**b**) the thaumarchaeon cell disappears after formation of FECA, but the archaeal chromosome becomes the major part of the genome of the proto-eukaryote, surrounded by nuclear envelope membranes derived from the PVC bacterium, which may also have donated some genes needed for this. In (**c**), there is an archaeal chromosome in the proto-eukaryote, but the archaeal membrane is somehow transformed (in an unlikely transition) into one with bacterial (i.e. PVC ICM)-type lipids which comprise all membranes of the proto-eukaryote. In all scenarios the cytoplasmic ribosomes are derived from the archaeal partner (From Forterre 2011)

specialised compartment for endocytotic vesicle trafficking and quasi-lysosomal protein digestion in the final *Gemmata*-like planctomycete, perhaps as an adaptation to low osmolarity and high turgor pressure habitats such as freshwater. In a second cell biology scenario compatible with Scenario 2 (Fig. 11.6b), there is donation not only of individual genes but of the gene sets governing cell compartmentalisation from a *Gemmata*-like ancestor, such that the paryphoplasm and ICM are remodelled and the paryphoplasm broken up to form the more dispersed ER and endomembrane system of the first eukaryote common ancestor. In both cases, the basis for the

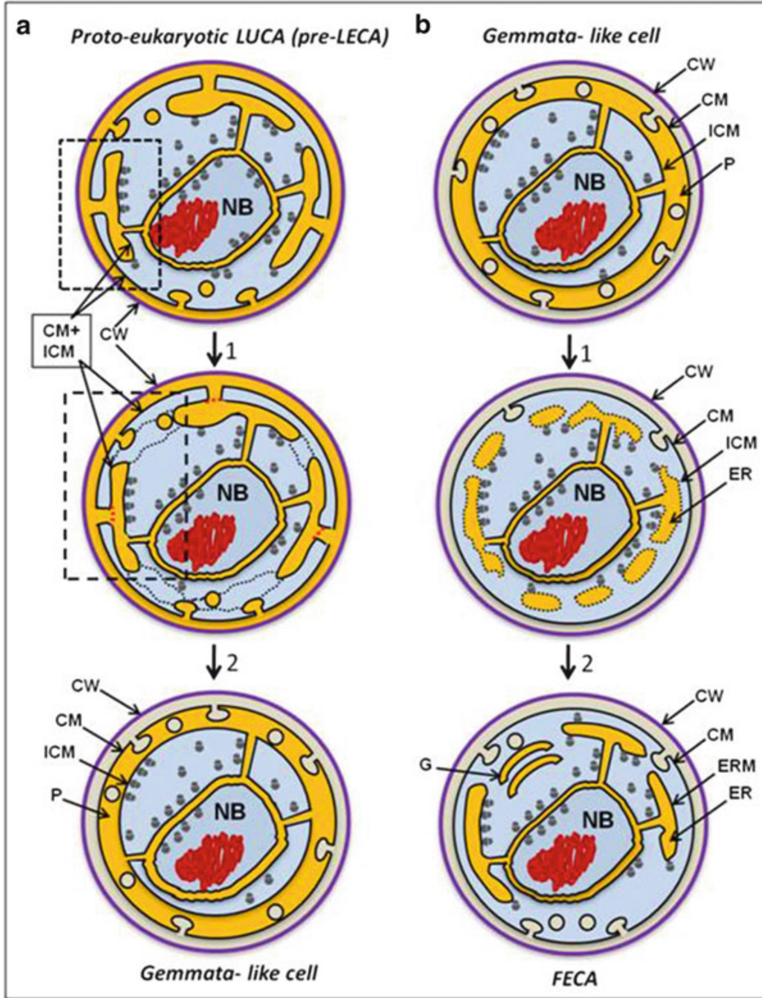

Fig. 11.6 Two possible models for internal membrane evolution explaining (**a**) origin of *Gemmata obscuriglobus*-like compartmentalisation from a eukaryote-like ancestor, (**b**) origin of proto-eukaryote organisation in the First Eukaryote Common Ancestor (FECA) from a *G. obscuriglobus-like* ancestor. In (**a**), reductive evolution from a proto-eukaryotic LUCA with a double-membrane-bounded nucleus connected to other endomembranes results in a *Gemmata-type* planctomycete cell plan with three major compartments including a double-membrane-bounded nuclear body (NB) and paryphoplasm. The proto-eukaryote has endocytosis ability and endocytosis vesicles for protein uptake pass directly from cytoplasmic membrane to cytoplasm and ribosomes are bound to endomembranes including both sides of the nuclear envelope (unlike modern eukaryotes). ER membranes directly communicate (*dotted box*) with plasma membrane, and translation is not compartmented (again in contrast to modern eukaryotes). In stage 1 of the evolution, ER membrane loses continuity with the plasma membrane, ER vesicles fuse (*dotted lines*) and endocytic vesicle formation now occurs in a specialised paryphoplasm compartment (P) equivalent to the former ER lumen. Such a transformation might be selected due to the increased efficiency of endocytosis in a confined compartment, with the advantage of efficient lysosome-like

model is the similarity between the paryphoplasm and pericisternal space continuous with it in the nuclear envelope on the one hand and the lumen or cisterna of the ER of eukaryotes on the other. As indicated above, there is at least one unicellular eukaryote with simpler cell organisation, *Giardia*, where the ER and the endocytic systems are not separated so that protein degradation occurs in the ER (Lanfredi-Rangel et al. 1998). Even admitting that the *Giardia* case may be secondarily developed in this parasite, this is quite compatible with the possibility of evolution of an ER from a paryphoplasm of *Gemmata* where protein degradation following endocytic protein uptake occurs (Lonhienne et al. 2010).

In terms of functional aspects of compartmentalisation evolution compatible with, for example, Scenario 3, the PVC cell plan could be explained by evolution from an ancestral anammox planctomycete which had developed an anammoxosome to contain the anammox metabolic apparatus and protect the cell from its toxic intermediates. Other non-planctomycetes and eukaryotes could then have developed other varieties of compartmentalisation building on the basic mechanisms. However, we do not yet know that any anammox planctomycetes have MC protein homologues or other molecules explaining internal membrane development (none

Fig. 11.6 (continued) degradation of macromolecular nutrients from other community cells. In stage 2, a *Gemmata*-like cell evolves capable of endocytosis by cytoplasmic membrane infolding into the paryphoplasm compartment. This compartment has become specialised for endocytosis and expansion of lysosomal nutrient degradation in low osmolarity, high turgor pressure habitats. Note that the periplasm of the proto-eukaryotic LUCA cell has now become the paryphoplasm and the paryphoplasm of the *Gemmata*-like cell is now topologically distinct from the periplasm between cytoplasmic membrane and cell wall (but the contents of the endocytic vesicles within the paryphoplasm are topologically equivalent to periplasm contents and the external milieu). The nucleus and pirellulosome within the ICM are preserved as accompanying consequences and correlates of the endomembrane system, as a mechanism of chromosome segregation without contact of DNA with cytoplasmic membrane and as compartments specialised for DNA replication and mRNA transcription and translation. Paryphoplasm is continuous with the pericisternal space between the nuclear envelope membranes, as the ER lumen of the proto-eukaryote is continuous with the analogous space in the proto-eukaryote. In (**b**), a proto-eukaryotic cell plan is evolved from a *Gemmata-like* planctomycete ancestor of modern *G. obscuriglobus,* already capable of competitively useful endocytotic nutrient acquisition, and perhaps also with some cytoskeletal elements such as those tubulins present in some modern verrucomicrobia. In stage 1 of its evolution, separate compartments of paryphoplasm are walled off (forming some isolated vesicles that disperse—indicated by *dotted lines*), making possible greater membrane surface with bound ribosomes for protein synthesis and complex processing of proteins through different compartments. Exocytosis is needed for transport of proteins to the cell wall and plasma membrane. In stage 2 resulting in FECA, endomembrane is differentiated such that some form of simple Golgi apparatus allows protein processing via trafficking through the Golgi—this is similar to the simple Golgi equivalent in *Giardia*. Endocytotic vesicle formation occurs now directly into the cytoplasm as in modern eukaryotes. In the next stage (not illustrated), the cell wall would be lost and phagocytosis of other cells in a microbial community would become possible on the basis of the mechanisms supplied by membrane trafficking and cytoskeletal protein evolution. *CW* cell wall (*purple*), *CM* cytoplasmic membrane, *ICM* intracytoplasmic membrane, *NB* nuclear body, *P* paryphoplasm, *ER* endoplasmic reticulum, *ERM* endoplasmic reticulum membrane, *G* Golgi apparatus. Topologically equivalent compartments appear in the same colour (ER and paryphoplasm (*yellow*), ribosome-containing cytoplasm (*blue*)). Periplasm is *grey*, and chromosomal DNA is *red* (From Fuerst and Sagulenko 2012)

could be found in Kuenenia using the same methods revealing those in planctomycetes and verrucomicrobia—see Santarella-Mellwig et al. 2010). However, the Scenario 3 model modified with anammox involvement does have the advantage of explaining the lipid linkage-type distribution in Eucarya and Archaea (though it would require two complete changes of lipid hydrocarbon chain type from the unique ladderanes of anammox). To develop this further, we would need more data on how anammox ICM and anammoxosome membranes are generated within the anammox cell and on stereoisomer types of lipid as well as ladderane synthesis.

11.4 PVC Superphylum and Evolution of Compartmentalisation

There is increasing gene sequence and genomics-based support for the relationships between phyla summarised in the concept of the PVC superphylum, at least for planctomycetes, verrucomicrobia and chlamydiae (Griffiths and Gupta 2007) (Gupta et al. 2012; Hou et al. 2008). The compartmentalised *Poribacteria* may well prove to have different affinities (Gupta et al. 2012). Assuming *Poribacteria* are now excluded on recent phylogenetic grounds as a *Bacteroidetes* member from the strict PVC superphylum (Gupta et al. 2012), species with cells compartmentalised via internal membranes occur in at least established three phyla of this superphylum—*Planctomycetes*, *Verrucomicrobia* and *Lentisphaerae* (Lee et al. 2009). Though not explicitly interpreted as such by the original authors, published micrographs of chemically fixed cells of some members of the *Chlamydiae* strongly suggest the occurrence of internal membranes and compartments in at least the deep-branching *Parachlamydia acanthamoebae* and perhaps also *Criblamydia sequenensis* ((Greub and Raoult 2002; Thomas et al. 2006)—see Chap. 10). Re-examination of such species by cryofixation methods is needed to confirm such significant possible structures consistent with PVC membership, but cell plan loss in such a specialised group of intracellular parasites is also likely. The occurrence of compartmentalisation in several phylogenetically related phyla suggests not only that an ancient common ancestor may have shared the major features of the shared cell plan but that since this ancestor was likely to have been closer to the root organism of the domain Bacteria than individual phylum members; the last bacterial common ancestor may have also shared this cell plan, one perhaps lost in all other bacterial phyla (other than possibly *Poribacteria,* if this is a *Bacteroidetes* phylum member as now suggested (Gupta et al. 2012)) and the phylum *Proteobacteria* represented only by one marine species *Sulfitobacter pontiacus*, where a simple pirellulosome-type compartment may occur (Sorokin et al. 2005). Another possibility is that among the Bacteria, cell compartments evolved in the ancestor of PVC superphylum phyla only. A third interesting suggestion is that the last common ancestor of PVC superphylum members (LPVCA) was a sister clade to the last common ancestor of Archaea and Eucarya, with features retained from the LUCA which were not necessarily kept by the Archaea or Eucarya, but with some eukaryote- and Archaea-like features already developed (Reynaud and Devos 2011). Such

a scheme avoids the need for extensive loss of eukaryote-like features from a last universal common ancestor from all bacteria and Archaea except the PVC superphylum members. It may have advantages concerning lipid composition distribution since anammox planctomycetes at least are known to have both ether- and ester-linked lipids (Sinninghe Damste et al. 2004) (of an admittedly very unusual ladderane structure not found elsewhere in nature (Sinninghe Damste et al. 2002)).

We noted above that planctomycetes have a number of unique features, some of which are consistent with an evolutionary homology of such features with those of eukaryotes. When we look at the wider PVC superphylum, we see more support for such consistency. Firstly, at least several members of the phylum *Verrucomicrobia* (representing at least four subdivisions) and one species of phylum *Lentisphaerae* possess cells compartmentalised via a major ICM membrane dividing the cell into ribosome-free paryphoplasm and ribosome-containing pirellulosome, as in the simplest planctomycetes (Lee et al. 2009). Secondly, species of the verrucomicrobial genus *Prosthecobacter* possess a homologue of tubulin more similar to eukaryotic tubulin than other bacterial tubulin-family proteins such as FtsZ (Jenkins et al. 2002b). This protein not only can polymerise to form protofilaments but it can also form a microtubule assembly of simpler protofilament assembly than eukaryotic microtubules (Pilhofer et al. 2011). Such bacterial microtubules share with eukaryotic microtubules important features such as straight protofilaments and similar protofilament interactions (Pilhofer et al. 2011). Although horizontal gene transfer from eukaryotes has been proposed as the origin of verrucomicrobial tubulins, from structural and polymerisation condition considerations, it has been suggested that the verrucomicrobial tubulins BtubA and BtubB represent transfer of a proto-tubulin gene from an ancient rather than modern eukaryote, at a stage where both alpha and beta tubulins had evolved via gene duplication (Martin-Galiano et al. 2011). The last common ancestor of modern tubulins appears to have evolved to form heterodimers with properties permitting tube formation but without need for chaperones. The 5-protofilament microtubule-forming tubulins of *Prosthecobacter* have therefore been proposed as a form primordial to the chaperone-dependent modern tubulins forming 13-filament microtubules (Pilhofer et al. 2011). If this is so, there seems no reason to be certain of the polarity of any gene transfer, and the evolved primordial verrucomicrobial tubulin could have been transferred to a proto-eukaryote consistent with the ‘toolkit for eukaryality’ scenario for PVC evolutionary roles. A wall-less PVC ancestor with such tubulin could even form the basis for development of a phagocytic proto-eukaryotic ancestor of PVC, Eucarya and Archaea consistent with Scenario 3 as mentioned above. A proto-eukaryotic LUCA possessing such a tubulin with retention in *Prosthecobacter* and loss in all other Bacteria would also be consistent with the data, but of course considerably less parsimonious unless the PVC group is assumed an anciently divergent one. Loss of the verrucomicrobial tubulins appears to have occurred even within the genus as well as among most other verrucomicrobia, since *Prosthecobacter fluviatilis* lacks the genes for these tubulins (as judged only from PCR evidence so far) (Takeda et al. 2008). However, the observation of reactivity with anti-eukaryote tubulin antibodies of tubules in the cells of the verrucomicrobial epixenosome ectosymbionts of marine ciliate protists (Petroni et al. 2000) suggests that the distribution pattern and evolutionary relationships of

verrucomicrobial tubulins may turn out to be more complex, not excluding presence of genes for these tubulins in a common ancestor of verrucomicrobia. While still members of phylum *Verrucomicrobia*, these ectosymbionts of protists are phylogenetically distinct from members of the genus *Prosthecobacter* (Petroni et al. 2000). Genome sequences for epixenosomes could be quite valuable for confirming any presence of BtubA/BtubB genes homologous to those of *Prosthecobacter* and tracing the origins of these cytoskeletal evolution markers. Another possible occurrence of interesting tubule structures in PVC members is within the anammoxosomes of anammox planctomycetes, but the protein composition of these structures is not clear and may even be related to the enzymes used in ammonium oxidation by these organisms (see Chap. 4). Thirdly, the eukaryote-homologous MC (membrane-coat) proteins so significantly correlated with endocytosis in the planctomycete *G. obscuriglobus*, and confirmed as associated with membrane vesicles in that species (Lonhienne et al. 2010), are also found distributed through several species of the phylum *Verrucomicrobia* and in one species of phylum *Lentisphaerae* (Santarella-Mellwig et al. 2010). They have not been found in phylum *Chlamydiae* so far (Santarella-Mellwig et al. 2010). The MC protein homologues of the PVC superphylum phyla appear to share a common ancestor (Santarella-Mellwig et al. 2010). Although we have no evidence for any form of macromolecule uptake in verrucomicrobia or lentisphaerae, representative strains with MC proteins (e.g. *Verrucomicrobium spinosum*, *Pedosphaera parvula* and *Chthoniobacter flavus* and *Lentisphaera araneosa*) should be tested experimentally for this ability.

Two of the major features above, compartmentalisation and MC proteins, are shared by the planctomycetes, verrucomicrobia and lentisphaerae, supporting their occurrence in a common ancestor of the PVC phyla. So far, there is no evidence for close homologues of eukaryotic tubulin or even of any bacterial FtsZ tubulin homologue in planctomycetes. The occurrence of tubulins in verrucomicrobia may be an instance of retention of an ancestral feature lost in other phyla or may indicate that we have not searched the proteomes of such phyla with suitable methods for detecting deep structural homologies. A pattern of loss may well have occurred, tubulins are found in the four described *Prosthecobacter* species, at least two of which also have an FtsZ (Pilhofer et al. 2007), but only a possibly deep-branching FtsZ is found in *V. spinosum* (Pilhofer et al. 2007; Yee et al. 2007). Verrucomicrobial tubulins seem to be related to a very ancient type of tubulin, perhaps corresponding to deep gene duplication events inventing the tubulin gene family.

11.5 Timing of Planctomycete and PVC Evolution Relative to Appearance of Eukaryotes: Insights from the Geological Record?

Our understanding of the relevance of planctomycete and PVC compartmentalisation to that of eukaryotes might be helped by a reasonable model for appearance of the planctomycetes and other PVC members within the history of life on Earth and

for at least the approximate time of their evolution. And it would help also to have a good idea of when the eukaryotes first appeared as a distinctive group. As is expected in geomicrobiology, however, as with the date for the explosive radiation of the Bacterial phyla (David and Alm 2011), there is little reliable data on when the planctomycetes or PVC superphylum members may have first appeared as distinct groups relative to other phyla. One study based on ‘slow’ conserved positions of 16S rRNA claims that the planctomycetes may be one of the most deep-branching phyla within the domain Bacteria, deeper than hyperthermophiles such as Aquifex (Brochier and Philippe 2002), but this has been criticised in another study (Barion et al. 2007). A recent phylogenetic analysis based on proteomes distinguishes planctomycetes as the deepest phylum of Bacteria but at the same time separates it from other PVC members (Jun et al. 2010). And controversy surrounds the dating of the first eukaryote in the geological or especially microfossil record (Buick 2010; Javaux et al. 2010; Parfrey et al. 2011; Rasmussen et al. 2008). A recent comparison of multi-gene data and the most confident eukaryote microfossil data suggest that the eukaryote LECA may have emerged between 1.67 and 1.87 Ga (billion years) ago (Parfrey et al. 2011). But there is also evidence from biogeochemical markers in bitumens containing bacterial hopanes and probable eukaryotic steranes that eukaryotes may have existed even as early as 2.46–2.67 Ga ago. Such early coexistence of Bacteria and Eucarya suggests that the opportunity for the HGT in Scenario 2 from planctomycetes and Archaea to a pre-eukaryote above could have been quite ancient. A controversial claim has suggested microfossils consistent with eukaryote cell identity in estuarine sediments 3.2 Ga old (Javaux et al. 2010) (Buick 2010), which might be consistent with the concept of a eukaryote-like LUCA and an ancient Eucarya rRNA lineage and thus Scenario 1 above. The only estimate for planctomycete divergence within the context of a phylogenetic analysis employing thousands of gene families but based on inclusion of *R. baltica* as the only planctomycete in the analysis suggests planctomycetes may have diverged from other Bacteria 3.05–2.67 Ga ago (David and Alm 2011). Yet the most relevant group of planctomycetes within a geochemical context would be the anammox planctomycetes that could have lived on an early Archaean anaerobic Earth and possibly been the very first biological producers of nitrate via a consequence of their pathway of CO_2 fixation using anaerobic nitrite oxidation as a source of reducing electrons (Vlaeminck et al. 2011). And phylogenetics of the selected C1 transfer enzymes that do occur in planctomycetes (though possibly not in anammox planctomycetes) indicating their extreme divergence from those of other organisms suggests in some perspectives an ancient involvement of planctomycetes in evolution of methane generation or oxidative transformations (Chistoserdova et al. 2004) (see Chap. 8), processes which must have been quite early in Earth history (possibly > 3.5 Ga ago) on geological and isotopic analysis grounds (Arndt and Nisbet 2012). But in other perspectives phylogenetics of the planctomycete C1 transfer enzymes suggests an instance of HGT from Archaea (Bauer et al. 2004). Concerning wider connections to PVC members, the thermophilic methane-oxidising verrucomicrobium *Methylacidiphilum infernorum* and possible related verrucomicrobia found in ecology studies of methane-rich habitats are also of interest geomicrobiologically and

phylogenetically, but their C1 transfer enzymes seem to differ from those in planctomycetes (Hamdan et al. 2008; Hou et al. 2008) (see Chap. 9). The ability to synthesise simple sterols in *G. obscuriglobus* (Pearson et al. 2003) and presence of some eukaryote-type sterol synthesis enzymes (Summons et al. 2006) in this species indicates that the simplest known eukaryote-homologous sterol pathway may have evolved in planctomycetes and suggests that at least some of the most relevant planctomycetes to eukaryote relationships may have evolved after there was sufficient oxygen from cyanobacterial photosynthesis to support sterol synthesis, a process known to require molecular oxygen (Galea and Brown 2009). There may be a close connection between ability to synthesise sterols and membrane compartmentalisation and endomembrane vesicles (Summons et al. 2006). Sterol synthesis may have enabled greater membrane deformation ability (Bacia et al. 2005) and paved the way for evolution of endocytosis and exocytosis as seen initially in *G. obscuriglobus* among planctomycetes. This would be consistent with a possible role for such organisms in evolution of proto-eukaryotes by contribution of sterol synthesis genes. The oxygen content of transmembrane proteins in eukaryotes and selected bacteria has suggested that oxygen content of protein amino acids may correlate with appearance of cellular compartmentalisation and that this may relate to atmospheric oxygen concentration and aerobic metabolism (Acquisti et al. 2007; Baudouin-Cornu and Thomas 2007). Though this is controversial (Sasidharan et al. 2008), at least one of other study has confirmed some aspects of these results concerning the oxygen content of eukaryotic transmembrane proteins, and strengthens suggestions concerning compartmentalisation (Vieira-Silva and Rocha 2008). Very interestingly, *R. baltica*, the only planctomycete included in the original study, displayed transmembrane proteins conforming to the oxygen atomic composition of eukaryotes rather than (non-compartmentalised) prokaryotes. This may be related to the size and number of communication-related transmembrane proteins and the extent of their extracellular domains (Acquisti et al. 2007), which suggests for us a testable hypothesis postulating large extracellular domain size in non-anammox planctomycete transmembrane proteins as well as a high oxygen content of their proteins, and also that if any further compartmentalised planctomycete proteomes are examined in species other than *R. baltica*, a eukaryote-type oxygen composition of transmembrane proteins should be found.

Among the PVC superphylum, the phylum *Chlamydiae* without free-living representatives is likely to be a more recent offshoot of the PVC superphylum. However, there are interesting connections of chlamydial with plant genes going back to the primary photosynthetic plastid acquisition by eukaryotes (Huang and Gogarten 2007). These suggest some potential ancient and possibly endosymbiotic associations of PVC members related to the amoeba inhabitant *Protochlamydia* with eukaryotic algae, and they could be relevant to possible HGT events between PVC members and proto-eukaryotes suggested in Scenario 2 (Huang and Gogarten 2007).

For future progress on the timing of planctomycete evolution in relation both to other Bacteria and to the Eucarya, there is a need for an integrated approach. This could include possible contributions of geomicrobiology to plumb the depths of anammox history (using potential stable biomarkers such as the anammox-specific

ladderanes (Hopmans et al. 2006)) and phylogenetics of several evolutionarily interesting systems such as sterol synthesis and C1 transfer enzymes in a phylogenomic context, as well as analysis of fold signature families of relevant proteins with eukaryotic homology such as MC proteins and tubulins.

11.6 Conclusions

Planctomycetes and related organisms within the PVC superphylum are important for understanding evolution of the diversity of cellular organisation plans since they are important for understanding how cell compartmentalisation may have originated. The types of cell structural plans they display include those with greater or lesser similarities to the eukaryote cell plan, but these plans could be analogous or homologous to that plan. In favour of homology is the coincidence of structural, functional and some molecular features with similarity to eukaryote features, exemplified most dramatically by the occurrence of both nucleus-like structures and receptor-mediated endocytotic protein uptake in the planctomycete *G. obscuriglobus*, with the latter correlated with structural homologues of clathrin-like MC proteins of eukaryotes. The occurrence of microtubule-forming cytoskeletal proteins homologous to eukaryote tubulins and occurring as filaments in the verrucomicrobia member *Prosthecobacter* is also a significant eukaryote homology within the related phylum *Verrucomicrobia*, one suggesting a very ancient relationship or gene transfer. We have proposed some evolutionary scenarios here to explain any possible homologies between planctomycetes and eukaryotes. A wide range of scenarios is important to consider since for testing such homology, we may not know the sensitivity needed by our methods of analysis in the search. For example, if Scenario 1 is true, then our tests may not be able to plumb these depths of time if we do not appreciate fully the antiquity of that connection (we may be compelled to examine protein structure more than sequence to reveal any connection). We also need to apply direct experimental approaches testing possible cell biology phenomena with potential eukaryote homology, such as endocytosis, since the 'bottom-up' approaches depending on genomics alone may confront such limitations. However, even if a PVC ancestor invented compartmentalisation completely independently of any phylogenetic link with eukaryote compartmentalisation, such a remarkable example of convergent or parallel evolution would comprise a significant model for understanding the mechanisms by which cell compartments can evolve. Such analogous mechanisms would be expected to be novel ones at the molecular level since they would have no homologues in eukaryotes, though they might have within the Bacteria. So the future of such a 'win–win' situation for future researchers of the evolutionary implications of planctomycetes and relatives, whether analogy or homology with eukaryote systems is revealed, is bound to be bright. Planctomycetes may be descendants of evolutionary container ships on a course to a eukaryote port or ships with only a planctomycete destination, but the cargo we can now unload from their study will be scientific treasure indeed.

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Chapter 12
A Final Word: The Future of Planctomycetology and Related Studies

John A. Fuerst

The chapters of this book have ranged over a wide world of the phylum *Planctomycetes* and significant regions of related bacteria in the PVC superphylum. They have provided the basis for the reader to enter this world and hopefully participate in adding to our knowledge of these fascinating organisms. Here we might attempt to sketch the sort of questions which these studies stimulate, perhaps in many cases forming the basis for future productive research programs:

1. Can many of the fascinating morphotypes such as *Planctomyces bekefii* found during the origins of planctomycete science be cultured? If not, can their relationships to other planctomycetes be determined by culture-independent methods?
2. Do the phyla *Planctomycetes, Verrucomicrobia,* and *Chlamydiae* share a common ancestor, and if so, what features of the genome and phenotype might be shared as a signal of such ancestry? As indicated in Chaps. 3, 4, and 10, we already have solid evidence of the relationship between these phyla and features shared by them in the case of some of the member phyla, but future work is needed to consolidate and confirm these analyses and to investigate the common characteristics which might link these relatively distinct phyla within a PVC superphylum.
3. What is the detailed composition of the paryphoplasm of planctomycetes like *Gemmata obscuriglobus* and verrucomicrobia like *Verrucomicrobium spinosum*, and can its clear distinction from periplasm of Gram-negative proteobacteria be confirmed by future approaches? What is unique about the composition of the protein-rich cell wall in planctomycetes, and how is it related to the evolution of other major types of bacterial cell wall?

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4. Will new examples of the type of cell compartmentalization found in many PVC members also be revealed in other bacteria and even Archaea? Is the structure of cells of *Ignicoccus* the same fundamental compartmentalization type as that of simple planctomycetes and verrucomicrobia, and if so, did it evolve via the same mechanism?
5. In what sense are the PVC superphylum and its members relevant to our understanding of the evolution of eukaryote cell organization? The question of whether cell organization and cell biology features of some PVC members and related genes such as the MC protein homologs are homologs of eukaryote features on the one hand or analogs on the other, the product of some type of convergent evolution, is still one requiring detailed further investigation for resolution.
6. What is the true extent and diversity of the PVC superphylum? How many phyla belong to it? Members of the phylum *Lentisphaerae* may repay much more detailed investigation of their cell structure and function, and other potential members not yet investigated or cultured may confirm a wider extent to this superphylum.
7. Is the eukaryote-like feature of endocytosis-like protein uptake in *Gemmata obscuriglobus* displayed by any other member of the PVC superphylum, especially those with homologs of MC proteins of eukaryotes, and to what extent is it relevant to understanding the evolution of endomembrane systems in the last eukaryotic common ancestor? What is the detailed molecular mechanism of endocytosis-like protein uptake in *Gemmata obscuriglobus*?
8. What is the exact deep relationship between the MC proteins of eukaryotes such as clathrin and some nucleoporins and the homologs of MC proteins found in many PVC superphylum members? Can such predicted homology be confirmed? Are there features of such proteins relating to necessary interaction with other ancillary proteins needed for vesicle formation during endocytosis? How are they related if at all to endomembrane system functions in planctomycetes other than endocytosis-like protein uptake?
9. Can experiments testing models proposed for anammox biochemistry in anammox planctomycetes confirm generation of a proton motive force across the anammoxosome membrane? To what extent is the anammoxosome analogous to the energy-generating internal mitochondrion organelle of eukaryotes?
10. How can cryoelectron tomography and other state-of-art electron and light microscopy methods be applied to PVC member cells, and how can approaches using them be designed to answer significant questions about cell function as well as structure?
11. The discovery of several new genera of acidophilic planctomycetes has had the benefit of confirming yet again the wide distribution and thus evolutionary and probable ancestral significance of the shared cell plan characteristic of planctomycetes, conserved as it is in several completely new genera. What is the full diversity of a relatively specialized group of planctomycetes such as those acidophilic species found in peat bogs of the arctic tundra, and what is their ecological role within the acidic wetland microbial community? Does the

diversity of this group suggest that many more planctomycetes with specialized nutritional or cultural requirements and physiology are awaiting discovery in tropical and marine as well as arctic and freshwater habitats?

12. Can genetic systems for significant model planctomycetes be developed to the degree that the green fluorescent protein-based techniques so dramatically useful in eukaryotic and bacterial cell biology can be applied to questions about cell compartments and their protein composition?
13. Can comparative genomics be applied to solving the problem of what gene sets may be specific to groups sharing cell compartment features such as the double-membrane-bounded nucleoid of *Gemmata* clade strains, and can these insights be confirmed by other non-bioinformatic approaches?
14. What deep relationships exist between the C1 transfer genes of planctomycetes and those of other bacteria and to Archaea? Do they stand alone in the evolutionary spectrum, and if so, how could they have evolved and what is their relevance to the early evolution of methanogenesis and methanotrophy? What function do they perform for organisms apparently not capable of environmental C1 compound utilization?
15. To what extent are genes specific to the methane oxidation and other physiological features of the acidophile verrucomicrobium *Methyloacidiphilum* related to lateral gene transfer and from where were they derived and in what ecological circumstances? Are cells of *Methyloacidiphilum* compartmentalized in any way analogous or homologous to the compartmentalization found in several other genera of verrucomicrobia and in planctomycetes? Could such compartmentalization be relevant to the quite specialized physiology of these organisms?
16. Is the signal for PVC superphylum membership linking phyla such as *Planctomycetes* with phyla such as *Chlamydiae* strong enough that more experimental evidence for this link will be possible to find? Perhaps the details of the life cycle of chlamydiae and their infectivity and pathogenicity for amoebal and animal cells are linked to shared features of cell organization in PVC members going back to their common ancestor. Such links might have profound implications for understanding chlamydial cell biology and disease processes, as well as suggesting simple experimental models for study of chlamydial genes in more amenable culturable model bacteria.

Finally, as in all of science, but perhaps no more dramatically than in the case of "planctomycetology" and the microbiology and molecular biology of related bacteria, questions about planctomycetes and related PVC members give rise to more questions as well as more data and knowledge. The chapters of this book have clearly demonstrated the unique features of planctomycetes and related bacteria and their potential to act as new models for structure, origins, and biology of the living cell. Through their use and the understanding that may be produced, we might even achieve a unification of cell biology cutting across the artificial boundaries of the prokaryote–eukaryote dichotomy.

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