The planctomycetes: emerging models for microbial ecology, evolution and cell biology

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Planctomycetes

The planctomycetes are an unusual but widely distributed group of budding bacteria which are proving to be of increasing relevance to at least three major areas of research in microbiology: microbial ecology, molecular evolution and cell biology. They constitute one of the phylogenetically distinct major 'phyla' of the domain Bacteria defined using 16S rRNA sequence analysis (Woese, 1987), and form a group so deeply branching it has been proposed as a new bacterial order, Planctomycetales, and family, Planctomycetaceae (Schlesner & Stackebrandt, 1986). Cultured planctomycetes which have been analysed with respect to their cell wall chemistry display the unusual feature of not synthesizing the otherwise universal Bacterial cell wall peptidoglycan; this feature is shared only with the chlamydiae and mycoplasmas among the Bacteria (Konig et al., 1984; Liesack et al., 1986). Planctomycetes also exhibit some unusual molecular features, including short 5S rRNA and, in at least two species, unlinked rrn operon organization (Bomar et al., 1988; Liesack & Stackebrandt, 1989). Gemmata obscuriglobus, a budding bacterium isolated from a freshwater dam in Queensland, Australia (Franzmann & Skerman, 1984), is a member of the order Planctomycetales and is thus within the domain Bacteria, yet possesses a membrane-bounded nuclear body, an organelle hitherto known only in the Eucarya. The unique ultrastructure of this bacterium is of great potential significance to understanding the ways in which eukaryote cell organization may have evolved.

The planctomycetes were initially discovered as aquatic freshwater and planktonic micro-organisms with a distinctive morphology due to their occurrence in rosettes connected by a non-cellular stalk. The type species of the genus Planctomyces, Planctomyces bekefii, was first observed in a pond in Budapest, Hungary, although interpreted at that time as a planktonic fungus (Gimesi, 1924). 'Planctomyces eossus', a similar morphotype, was much later also interpreted in this way (Hortobagyi, 1965). Pl. bekefii characteristically occurs as rosettes of spherical cells (Fig. 1), joined together by non-cellular stalks, originally interpreted as fungal conidia and fungal conidiophores, respectively (Schmidt et al., 1981). The cell pole opposite to the stalk possesses tapering multifibrillar 'spire' appendages while pili occur over the entire cell. Pl. bekefii from the original type locality was eventually shown to possess a prokaryotic ultrastructure (Schmidt & Starr, 1980a), as was this species from North American enrich-
and similar morphotypes seen in natural enrichments. Their assignment as members of the Planctomycetales depends at present solely on morphological and ultrastructural features such as yeast-like budding, ‘crateriform structures’, absence of peptidoglycan cell wall layer and production of non-cellular multifibrillar stalk-like structures, features known to occur in cultured planctomycetes which have been characterized via molecular phylogenetics. The order Planctomycetales and the family Planctomycetaceae were defined not only on the basis of 16S rRNA oligonucleotide catalogues and sequence data, but also on the basis of such distinctive phenotypic characters (Schlesner & Stackebrandt, 1986).

The representatives of the planctomycetes isolated in pure culture include four genera and seven species: Planctomyces maris, Pl. limnophilus and Pl. brasiliensis, Pirellula staleyi and Pl. marina, Gemmata obscuriglobus and Isosphaera pallida (Bauld & Staley, 1976; Hirsch & Müller, 1985; Schlesner, 1986, 1989; Staley, 1973; Franzmann & Skerman, 1984; Giovannoni et al., 1987b). Of these, P. staleyi, Pl. limnophilus and G. obscuriglobus are from freshwater habitats, Pl. maris is marine, Pl. marina is from the brackish Baltic Sea, Pl. brasiliensis is from a hypersaline pond and I. pallida is from a hot springs habitat, although only moderately thermophilic (optimum growth temperature 41 °C). Additional strains in pure culture include the freshwater Pl. staleyi strain ICPB 4232 (Schmidt CLPM white, Tekniepe BT2 white, ATCC 35122; Tekniepe et al., 1981; Schmidt & Staley, 1982; Starr et al., 1983), bacteria resembling Pirellula species from the giant tiger prawn Penaeus monodon (Fuerst et al., 1991) and the 257 Planctomyces-like, Pirellula-like, spherical and some possibly prosthecate planctomycetes from fresh, brackish, marine and hypersaline waters isolated by Schlesner (1994). Isolation methods vary from use of micromanipulation (Franzmann & Skerman, 1984) and relatively non-selective enrichments (Hirsch & Müller, 1986) to the very selective medium based on N-acetylglucosamine as carbon source and ampicillin and cycloheximide as selective agents employed with great success by Schlesner (1994). Isolation and identification methods have been usefully reviewed previously (Starr & Schmidt, 1989; Staley et al., 1992).

Electron microscopy has been essential for confirming the yeast-like budding division mode and the presence of planctomycete-characteristic crateriform structures and multifibrillar appendages in putative planctomycete isolates. Future preliminary identification of planctomycetes will apply 16S rRNA sequence-based oligonucleotide probes such as those which can be based on Escherichia coli positions 47–53 of the planctomycete 16S rRNA (Liesack & Stackebrandt, 1992; Liesack et al., 1992) as well as electron microscopy. Crateriform structures are cell surface structures characteristic of all known planctomycetes, appearing as electron-dense circular regions after negative staining of whole cells (Figs 2 and 3), with diameters of 12 nm or 5–7 nm; in I. pallida such structures are as large as 27 nm in diameter, and in other planctomycetes, enlargement of the structures up to 30 nm can occur with cell maturation (Tekniepe et al., 1981;
which may normally fill such holes after SDS treatment but also with loss of protein, allowing stain accumulation (Schmidt et al., 1987a). Planctomycete crateriform structures may not be holes in the wall but solid structures more similar to circular protein arrays with a geometry allowing stain accumulation (Schmidt & Starr, 1979b). Neither those protrusions nor crateriform structures bear any simple relation to the distribution of fimbriae, which are common appendages on planctomycete cells. Crateriform structures may not be holes in the wall but solid structures more similar to circular protein arrays with a geometry allowing stain accumulation (Schmidt & Starr, 1979b). However, isolated planctomycete walls do show perforations consistent with holes, but also with loss of protein, which may normally fill such holes after SDS treatment

used in isolation (see later). Presence or absence of crateriform structures has been used along with other structural features to include or exclude planctomycete-like morphotypes in natural enrichments as true members of the planctomycetes (Schmidt & Starr, 1979b; Starr et al., 1984). Their distribution is useful taxonomically to distinguish Planctomyces and Gemmata from Pirellula strains, the latter displaying distribution of the larger form of these structures on the budding, ‘reproductive’ pole of the cell only (Fig. 3). Although they are claimed to be unique to planctomycetes, the array of 8.5-nm-diameter rings on the surface of Helicobacter mustelae (O'Toole et al., 1994), the circular perforations of peptidoglycan layers in cyanobacteria (Gügliemi & Cohen-Bazire, 1982) and the crateriform-like structures of the aerobic bacteriochlorophyll-synthesizer Porphyrobacter (Fuerst et al., 1993) should be noted as possible analogous structures. However, they remain a very useful diagnostic feature for members of the planctomycetes.

The other unusual major surface structure of Pi. bekefi, the non-cellular non-prosthecate multifibrillar stalk, which in that species radiates from the centre of rosettes, is not of universal occurrence among the planctomycete genera. It is however a feature characteristic of the genus Planctomyces, occurring in Pi. limnophilus, Pi. maris and Pi. brasiliensis of the strains in pure culture. Although the stalks of Pi. bekefi and similar morphotypes are thick enough (at least 0.5 μm wide) to be visible by light microscopy, those of Pi. maris are too thin at 0.1 μm to be above the usual resolution limits (Bauld & Staley, 1976). Stalks may be tubular, ribbon-like or rope-like, comprised of twisted fibrils. Another more irregular form of multifibrillar appendage is found in at least Pi. staleyi among other planctomycetes; the significance of such appendages was one of the bases for the distinction of the genus Pirellula (Schlesner & Hirsch, 1984) and molecular evidence has confirmed this distinction on much firmer grounds (Liesack et al., 1992). Multifibrillar stalk-like appendages have also been found outside the planctomycetes, in the proteobacterium Porphyrobacter (Fuerst et al., 1993). Although the non-cellular stalks of Gallionella ferruginea contain fibres, these are claimed to be completely inorganic, consisting of ferric hydroxide only (Hanert, 1989).

At times in the history of planctomycete studies, nomenclature has been confusing, and those entering the field should be aware of the fact that the organism now known as Pi. staleyi was once thought to be a rediscovery of Pasteuria ramosa described in the 19th century by Elie Metchnikoff (Staley, 1973), and that this species has also been classified as a member of the genus Planctomyces (Starr et al., 1983). The latter view is now obsolete as a result of a reassignment (Schlesner & Hirsch, 1984) and in the light of later 16S rRNA sequence data. However, it is still confusingly preserved in many phylogenetic trees and small subunit rRNA databases because of a GenBank 16S rRNA sequence entry (M34126) under Planctomyces staleyi for the important type strain of this species, ATCC 27377. An additional source of confusion was the need to reject the genus name Pirella and replace it with Pirellula, due to

![Fig. 3. Electron micrograph of a negatively stained Pirellula-like planctomycete from the giant tiger prawn, showing a teardrop-shaped cell possessing crateriform structures (arrows) with polar distribution and a complex sheathed flagellum. Bar, 0.5 μm.](image-url)
prior use of *Pirella* for a fungal genus (Schlesner & Hirsch, 1987). The planctomycetes were once commonly referred to as the *Blastocaulis-Planctomyces* group, reflecting a genus name used for uncultured freshwater lake planctomycetes (Henrici & Johnson, 1935; Hirsch, 1972; Schmidt & Starr, 1981).

**Ecology**

**Classical ecology**

Originally studied as freshwater organisms, the planctomycetes are proving to be quite widely distributed in marine, hypersaline and even terrestrial soil habitats. Even in freshwater and brackish water habitats, planctomycetes can be isolated from habitats of diverse trophic status, including oligotrophic, eutrophic, very eutrophic and frankly polluted habitats (Schlesner, 1994). By use of selective media based on ampicillin resistance of the peptidoglycan-rich planctomycetes and utilization of N-acetylgalactosamine as sole carbon and nitrogen source, isolates have been derived from such diverse sources as sewage treatment sludge, cattle manure, garbage dump leakage water, the meromictic saline Ekho Lake in Antarctica, alkaline chalk mine, acid bog water and the pitcher of the insectivorous plant *Nepenthes* sp., as well as brackish water of the Baltic Sea (Schlesner, 1994). *In situ* morphological evidence suggests that groundwater may also be a planctomycete habitat (Hirsch & Rades-Rohkohl, 1983; Ekendahl *et al.*, 1994).

Little is known concerning physiology and metabolism of planctomycetes, but strains in pure culture are all chemoheterotrophs capable of growth in air, with some such as *P. maris*, *G. obscuriglobus* and *I. pallida* being obligate aerobes (Bauld & Staley, 1976; Franzmann & Skerman, 1984; Giovannoni *et al.*, 1987b). Others, such as *P. marina* and *P. limnophilus*, are facultative in the sense of being capable of carbohydrate fermentation (Schlesner, 1986; Hirsch & Müller, 1985). Presence of cytochrome *c* oxidase and nitrate reduction ability in at least some strains, such as *P. marina* and *P. brasiliensis*, suggest possession of electron transport chains similar to other facultative aerobic Bacteria (Schlesner, 1986, 1989), but quinone composition is unusual for aerobes (see later). It may also be relevant to planctomycete electron transport chain composition that oxygen uptake in *I. pallida* is cyanide sensitive (Giovannoni *et al.*, 1987b). Considering the physiological diversity of members of an equivalent distinct Bacterial phylum such as the Proteobacteria, anaerobic, chemooautotrophic and photosynthetic representatives of planctomycetes may well be discovered. Carbohydrates have been identified as the major carbon sources, yet gelatin hydrolysis can be performed by *P. limnophilus*, *P. brasiliensis* and *P. marina* (Hirsch & Müller, 1985; Schlesner, 1986, 1989). Starch is hydrolysed by *P. brasiliensis*, *P. marina* and *G. obscuriglobus*. Planctomycetes typically exhibit slow growth, with the shortest generation time recorded for *P. maris* at 13 h (Bauld & Staley, 1976), identical to the generation time recorded for *G. obscuriglobus* (Franzmann & Skerman, 1984), but a generation time in excess of 100 h has been observed for *P. maris* in a glucose-seawater salts medium. Many planctomycete strains may require relatively dilute media, as nutrient-rich media (above 0.75 g peptone, yeast extract and glucose 1% ) have been found to be inhibitory to *P. marina* (Schlesner, 1994), and the obligate oligotrophy of *I. pallida* has already been noted.

*I. pallida* possesses several especially interesting ecological features; moderate thermophily (growth up to 55 °C), obligate oligophily (growth inhibited with glucose above 0.025%), gliding motility (unique among budding Bacteria), phototactic ability (unique among heterotrophic Bacteria), and ability to form gas vesicles (Giovannoni *et al.*, 1987b; Giovannoni & Castenholz, 1989). Since these organisms occur in hot springs as plankton or as components of cyanobacteria-dominated microbial mats, the latter two abilities may be related to vertical axis positioning. Phototaxis in a non-phototroph without bacteriochlorophylls (but with carotenoids) may be useful for study of phototaxis independently of photosynthesis (Giovannoni *et al.*, 1987b). *I. pallida* may be widely distributed, since in addition to its type locality in Warm Springs, Oregon, isolations have been achieved from other North American hot springs (Giovannoni & Castenholz, 1989), and cloned 16S rDNA sequences identified as from *I. pallida* reported from the Octopus Spring cyanobacterial mat, Yellowstone National Park, USA, confirm evidence from microscopy that this species is abundant in the top mat layers at this location (Ward *et al.*, 1987, 1990). *I. pallida* has been reported to grow in co-culture with a hot-springs phototroph, *Heliothrix oregonensis*, a co-culture maintained successfully only under aerobic conditions in the light, and apparently obligate for growth of *H. oregonensis* (Pierson *et al.*, 1985). In the mats of the alkaline hot springs at Warm Springs, Oregon, *H. oregonensis* was consistently found associated with *I. pallida* and a few specific cyanobacteria in a bright orange layer in O₂-saturated water at 45–56 °C and with high solar radiation exposure.

Many valuable but isolated limnological observations concerning planctomycetes exist, and we know that *P. bekefii* morphotypes, at least, occur in freshwater at widely dispersed geographical locations, including Hungary, Denmark and many other European sites, Vietnam, Venezuela, Israel, Australia and the USA (Schmidt & Starr, 1980a,b; Kristiansen, 1971; Hortobágyi, 1968; Lewis *et al.*, 1986; Schmaljohann *et al.*, 1987; Staley *et al.*, 1992; Pedroso-Alío & Brock, 1982). Association with eutrophic or polluted water has been reported, along with high pH values (6.8–9.4), high conductivity and temperate summer temperatures, and it has been suggested that the decomposition of phytoplankton maxima resulting in H₂S production and rising iron and manganese concentrations may favour *P. bekefii* blooms (Kristiansen, 1971). *P. bekefii* and *P. condensus* have been correlated with a seasonal increase in heterotrophic phytoplankton in a municipal water reservoir (Granberg, 1969). Culturable freshwater planctomycetes have also been reported as being most numerous in eutrophic habitats (Staley *et al.*, 1980). Continued ecological research on *P. bekefii* will be hampered by the absence of culture techniques for this...
organism, and by the present lack of any molecular technique for its identification as a planctomycete and determination of phylogenetic homogeneity. This should be resolved easily with methods such as cloning of 16S rDNA amplified from micromanipulated cells and in situ hybridization using planctomycete-specific fluorescent oligonucleotide probes. The question of whether *Pl. bekefii* may serve as a useful environmental indicator organism for eutrophic conditions can then be addressed. *Planctomyces* rosettes can occur in densities of as high as 3.5 x 10^3 ml^-1 in British lakes, but in a thorough multiple regression and seasonal study of Esthwaite Water, English Lake District, no clear relationship with any physico-chemical variable was found, though numbers increased at the onset of overturn in September (Jones, 1978). *Pl. bekefii*, *Pl. guttaformis* and *Pl. stranskae* have been noted to often occur in freshwater bodies following or accompanying algal or cyanobacterial blooms, laboratory enrichments of planctomycetes may be favoured by ambient light and consequent phototroph development (Schmidt & Starr, 1980b; Starr & Schmidt, 1989) and freshwater *Pirellula* species can occur as epiphytes of ensheathed cyanobacteria, which may relate their occurrence to cyanobacterial bloom formation (Staley, 1981).

An interesting biogeochemical aspect of rosette- and stalk-forming *Planctomyces* morphotypes observed in native freshwater habitats is their accumulation of iron and/or manganese on the non-cellular stalk (Schmidt et al., 1981, 1982). Energy-dispersive X-ray spectroscopy has shown encrustation with such elements, with a possibility that at least in some specific locations, manganese may be exclusively accumulated by one morphotype (morphotype 1a; *Pl. bekefii*) and both iron and manganese by another (morphotype 1b; *Pl. crassus*) (Schmidt et al., 1981, 1982). Passive accumulation of oxidized iron is a likely explanation for iron encrustation, but biologically catalysed oxidation of reduced manganese is a property to be considered in investigating the eco-physiology of such bacteria, if and when they are isolated in pure culture.

Possible associations of planctomycetes with crustacea have been an issue ever since Metchnikoff's report of associations of pear-shaped parasitic micro-organisms with cladoceran 'water fleas' of *Daphnia* spp. (Metchnikoff, 1888). It now seems more likely that this parasite was the non-planctomycete endospore-forming *Pa. ramosa*, phenotypically similar to *Pa. penetans* and *Pa. thornei*, pathogens of plant-parasitic nematodes (Sayre et al., 1983; Starr & Sayre, 1988). True *Pirellula*-like bacteria were later found occurring attached to the carapace of *Daphnia pulex* (Staley, 1973). More recently, the isolation of bacteria resembling *Pirellula* species has been recorded from the hepatopancreas of the giant tiger prawn *Penaeus monodon* (Fuerst et al., 1991); these bacteria and further isolates from post-larvae of that crustacean have been shown to be phylogenetically closely related to *Pirellula* or *Planctomyces* species on the basis of 16S rRNA sequences (J. A. Fuerst and others, unpublished). Such bacteria may inhabit the prawn haemolymph as well as the gut, and may possibly be selected for especially within baculovirus-infected prawns, though they occur in healthy prawns in at least low numbers. The exact nature of their association with this crustacean remains to be determined.

**Molecular ecology**

The application of the methods of molecular ecology were first applied to the problem of planctomycete distribution and diversity in a study of acid soil (pH 4.2) from a subtropical Australian environment (Liesack & Stackebrandt, 1992; Stackebrandt et al., 1993). The clone library resulting from amplification of 16S rRNA genes derived from direct lysis of the bacterial community within the soil matrix revealed seven clones hybridizing with planctomycete-specific oligonucleotides under stringent conditions. Sequencing revealed that planctomycete-specific signature sequences (a valuable list of which is presented in Table 1 of Liesack & Stackebrandt, 1992) were shared also by these clones, and that the phylogenetic relationships of the sequences spanned the diversity of at least three of the described genera of the family *Planctomyces*, a surprising finding since members of these genera were all originally described and isolated from aquatic habitats and *Isospheera* only from hot springs. While one clone was related to *I. pallida*, and another to *Pl. limnophilus*, the remaining clones branched off adjacent to *G. obscuriglobus*, and shared with that species a unique bulge loop of 13 nucleotides around position 1005 (*E. coli* 16S rRNA numbering), a slightly enlarged bulge loop between positions 1024-1036, and a higher G+C base ratio for 16S rDNA than for *Planctomyces* and *Pirellula*, a feature also shared with *I. pallida*. However, except for the case of the close relationship of clone MC100 to *Pl. limnophilus*, these authors stressed the speculative nature of the allocation of the clones clustered with *Isospheera* and *Gemmata* to those genera. In addition to these clear-cut planctomycete clones, a further group of clones (soil cluster III) appeared to be related to planctomycetes more distantly, forming a novel main line of descent within the Bacteria, sharing a common ancestry with planctomycetes and with chlamydia, and also sharing synapomorphic 16S rDNA signature nucleotides with both the latter groups.

That planctomycetes may prove to be of global significance for nutrient cycling processes in marine habitats is suggested by the recognition of their possible significance as components of macroscopic marine phyto-detrital aggregates (> 0.5 mm) also known as 'marine snow'. Macroscopic detrital aggregates have been suggested to be important agents in the flux of biogenic carbon from the sea surface to the deep ocean, with biological and chemical transformations on sinking aggregates having a profound influence on the quantity and quality of organic matter reaching the deep sea. Such aggregates can serve as localized microhabitats and may harbour transient micro-aerophilic or anaerobic microhabitats in otherwise aerobic environments. Therefore, processes not occurring in planktonic non-particulate seawater habitats but important to marine chemistry, such as methanogenesis and denitrification, as well as aerobic processes utilizing the products of anaerobic decomposition, may be catalysed by macroaggregate or particulate-associated microflora.
and sequence, include a negative diphtheria toxin reaction (protein synthesis resistant to this ADP-ribosylating toxin), lipids with glycerol esters of fatty acids and DNA-dependent RNA polymerase with subunit pattern characteristic of eubacteria (Schlesner & Stackebrandt, 1986). 16S rRNA oligonucleotide cataloguing using Planctomyces or Planctomyces-like organisms and Pirellula (then Pirella), including Pl. limnobius, Pi. staleyi and Pi. marina, also indicated their membership of a single line of descent equivalent in depth to other major lines of the eubacterial kingdom (Stackebrandt et al., 1986a). The similarity in phenotype (e.g. reproduction, morphology and aerobic physiology) of extant genera is, however, unusual relative to other aerobic prokaryotes of comparable phylogenetic depth. A family Planctomycetaceae and order Planctomycetales for Planctomyces and Pirella was proposed on the basis of genotypic and phenotypic coherency and a phylogenetic depth comparable to that of other eubacterial divisions (Schlesner & Stackebrandt, 1986). ‘Planctomyces and relatives’ was recognized as one of the major (e)ubacterial divisions based on 16S rRNA oligonucleotide catalogues, of status equivalent to such extensive divisions as what is now the class Proteobacteria and the Gram-positive Bacteria (Woese, 1987).

There is direct molecular evidence for the distinctiveness of the planctomycetes relative to other bacteria. Certain oligonucleotides present in 16S rRNA of iso-chronically evolving eubacteria, that is, CACAAG, CUUAAG, AAUAAG, UUCCCG, AUACCG and AUUCUACG, were found to be missing from the planctomycetes originally examined by oligonucleotide cataloguing, including the brackish water strains from Kiel Fjord, Baltic Sea, IFAM 1310 and IFAM 1317, later assigned to the genus Pirellula and to Pl. maris, respectively (Schlesner & Hirsch, 1984), and strain IFAM 1319, a strain of uncertain position later assigned to Pirellula (Liesack et al., 1986). Of these, CUUAAG, AUACCG and AUUCUACG can be confirmed as absent from all known planctomycete 16S rRNA sequences on the basis of more recent sequence data. The absence of these otherwise universal oligonucleotides can be interpreted as implying that these universal oligonucleotides evolved later in the evolution of the eubacteria, after the ancestor of Planctomyces and relatives had branched off, and the lack of peptidoglycan in planctomycetes from this perspective may likewise be explained by evolution of peptidoglycan synthesis at a stage later than that at which planctomycetes diverged (Stackebrandt et al., 1984). These interpretations apply only if a relatively ancient lineage for the planctomycetes is assumed. Examination of planctomycete 16S rRNA sequences for signature positions characteristic of the domains shows that all planctomycetes possess a G:C pair at position 52:359, like Archaea and Eucarya but unlike most Bacteria (except the chlamydiads and the deep-branching radioresistant micrococci and relatives phylum), which have a pyrimidine:purine pair, an unusual G:U pair at position 53:358 shared only with chlamydiads and the deep-branching green non-sulphur phylum, and at position 933:1384 an A:U in common with Archaea and Eucarya.
but unknown in any other Bacteria except the green sulphur phylum. The latter signature is also absent from the acknowledged deepest phyla of the Bacteria such as the thermotogas and Aquifex. The S5 rRNA of planctomycetes exhibits an unusual and very short primary structure of only 109–111 nucleotides relative to the 118 base minimal length for other Bacteria and Archaea, the absence of an insertion at position 66 unique among prokaryote S5 rRNA molecules, and the presence of numerous base-pair transversions relative to the minimal S5 rRNA secondary structure for Bacteria (Bomar et al., 1988).

Are they related to chlamydiae?
An important question for future resolution is the nature of the relationship of planctomycetes to the chlamydiae. On the basis of 16S rRNA sequences, a remote relationship of Pi. staleyi to Chlamydia psittaci supported by shared higher order structures has been postulated (Weisburg et al., 1986). Cloned 16S rRNA gene sequences of C. psittaci and Pi. staleyi were used in comparisons with representatives of other Bacteria and a remote but specific relationship was found between the chlamydiae and that single planctomycete representative when signature positions with a composition unique to and characteristic of particular groupings were compared; evolutionary distances showed no specific relationship of the chlamydiae to any other of 400 partial 16S rRNA sequences of eu-bacteria (Weisburg et al., 1986). Twenty-five such related signature positions were found, higher than the chlamydiae shared with any other eubacterial sequence. Secondary structure of the 16S rRNA also indicated some shared features with chlamydiae distinct from other Bacteria in the region covering positions 40 to 400. There is however only one known shared phenotypic character, the absence of peptidoglycan in their cell walls (otherwise a universal feature of walled Bacteria, i.e. excluding mycoplasmas from consideration). With respect to the possible connection with Chlamydia and the possible ancient status of planctomycetes, it is of interest that phylogenetic trees based on nucleotide and amino acid sequences for elongation factor Tu have indicated possible early divergence of Chlamydia (as well as Thermotoga) from the domain Bacteria root (Cousineau et al., 1992). The question of possible relationships of planctomycetes to chlamydiae has been closely connected with the problems of shifting branch position of the planctomycetes in phylogenetic analysis and the problem of whether planctomycetes represent an ancient lineage or whether a rapid evolutionary rate has distorted their position within phylogenetic tree topologies.

Ancient or rapidly evolving?
There have been two interpretations of phylogenetic analyses of the planctomycetes based on 16S rRNA sequences, one in which the planctomycetes appear to be an ancient lineage, and the other in which the planctomycetes are seen as a rapidly evolving lineage. These interpretations are complicated by the variable results of phylogenetic analyses including the group. From the first perspective, they might represent descendants of an ancient group of Bacteria branching immediately after divergence of the ‘ur-eubacteria’ from the progenote (universal common ancestor) and before the main radiation of the Bacterial lines of descent (Stackebrandt et al., 1984). From the second perspective, planctomycetes may appear to be an older group than actually is the case due to a higher evolutionary rate causing artificially deep branching within phylogenetic trees (Schlesner & Stackebrandt, 1986). A third position could be imagined consistent with both of these views, where planctomycetes branched early from other phyla and also diverged via a rapid evolutionary rate.

The well-known tree based on 16S rRNA oligonucleotide catalogues (Fig. 11 in Woese, 1987) shows ‘planctomycetes and relatives’ as a relatively shallow branching division very distant from the deepest branching Thermotoga. In a recent maximum likelihood analysis based on 16S rRNA sequences using 253 species of Bacteria and Archaea, clustering of planctomycetes with chlamydia is also obtained (Olsen et al., 1994). Such conclusions differ from an earlier 16S rRNA oligonucleotide catalogue analysis yielding a deep-branched position of planctomycetes and the fimbriate prosthecate non-budding bacterium Verrucomicrobium spinosum, with both separated from chlamydia (Albrecht et al., 1987).

The phylogenetic difficulties were illustrated clearly in one study in which the planctomycete position varied according to which sequences were included in the analysis (Liesack et al., 1992). When either Planctomyces or Pirellula sequences only were included as planctomycete representatives, planctomycetes clustered with C. psittaci, and dendrogram topology resembled that emerging without inclusion of planctomycetes. If, however, G. obsciruglobus was the only representative planctomycete sequence, the planctomycete line branched off deeply, between lines defined by Bacteroides/Chlorobacteri and Thermus/Chloroflexus. Inclusion of I. pallida resulted in its position as the deepest branch in the Bacteria, and influenced the relationships between Thermus thermophilus and Thermotoga maritima by formation of a common root, a situation also found when both G. obsciruglobus and I. pallida were included. If all six planctomycete sequences available were included, a new position of planctomycetes between the Thermotoga and the Chloroflexus/Thermus lines of descent resulted. Such inconsistencies were considered unique for analyses of Bacteria except perhaps for mycoplasmas. A ‘fast clock’ interpretation invoking elevated mutation rate in the planctomycetes, and within that group, an even more rapid rate for G. obsciruglobus and I. pallida, was used to explain these results.

Recent publications from three separate research groups contain trees derived from 16S rRNA gene sequences which exhibit relatively deep-branch positions for the planctomycetes (Neefs et al., 1993; Van de Peer et al., 1994; Embley et al., 1994). Van de Peer et al. (1994) employed a novel statistical method for determining the confidence of the tree topology involving repeated
random sampling of selected sequence sets from a dataset of different representatives of 18 taxa corresponding to the divisions or phyla of the Bacteria but including also all the subdivisions of one of these, the Proteobacteria. By examining the frequency of different clusters among 1000 trees resulting from this protocol, a consensus tree resulted in which planctomycetes branch as the sister group of all remaining taxa except the thermotogales, and separately from the chlamydiae (Fig. 4). A higher probability was attributed to the early divergence of planctomycetes than to their forming a binary cluster with the chlamydiae, a topology which did occur but only at a lower frequency.

Analysis of the translated amino acid sequence of partial sequence (79%) for a protein-coding gene in planctomycetes, atpD, coding for the β-subunit of ATPase in *Pi. marina*, indicated a deeper branch position for this species than other Bacteria for which homologous sequence was available (Rönner et al., 1991). Sequencing of further examples of ancient conserved proteins, such as elongation factors, topoisomerases, DNA-dependent RNA polymerase, and heat-shock proteins, for planctomycetes will contribute valuable data for evolutionary analysis of the group. Although a 23S rRNA sequence exists for *Pi. marina* (Liesack et al., 1988), one phylogenetic analysis using this sequence disagrees with 16S rRNA and atpD analyses in that *Pi. marina* groups with the Proteobacteria (Schleifer & Ludwig, 1989), while another distinguishes

One SS rRNA-based phylogenetic analysis recognizes the planctomycetes as a cluster including Planctomyces, Pirellula, Gemmata and Isosphaera, but they occur in a loose grouping with the low mol% G+C Gram-positive Bacteria and quite distinct from the Proteobacteria (Van Den Eynede et al., 1990), yet some common structural features shared between the SS rRNA molecules of planctomycetes and proteobacteria have suggested phylogenetic affinities (Bomar et al., 1988).

What then are the arguments for and against the case of planctomycetes being an ancient lineage or a rapidly evolving (tachytelic) but relatively recent lineage? In favour of an ancient lineage is the internal phylogenetic depth of the group, comparable with that of other Bacterial phyla or divisions, and the absence of certain universal Bacterial oligonucleotides in planctomycete 16S rRNA, as well as the absence of peptidoglycan, both the latter suggesting evolution of the group before acquisition of either the particular signature oligonucleotides or ability to synthesize peptidoglycan. Deep branching in itself may not indicate ancient lineage if the group has exhibited a rapid evolutionary rate giving rise to extreme sequence divergence. On the side of rapid evolutionary rate may be the lower similarity values of planctomycetes with members of other Bacterial phyla as compared with values found between those phyla themselves (Liesack et al., 1992). In a similar manner to the tachytelic mycoplasmas, but also to the deep branching green non-sulphur (Chlororflexus) division, planctomycetes display uniqueness in variants of highly conserved oligonucleotide families (Woese et al., 1985). Also, the occurrence of many unusual phenotypic and genotypic idiosyncrasies, including short SS rRNA and split rRNA operons, may be an indication of highly derived unique features which result from a fast clock (Liesack et al., 1992). At present the question must be considered unresolved, at least until complete relative rate tests are performed. Relative rate tests using comparison of sequence changes between planctomycetes and other Bacteria against a third outgroup reference sequence might be able to resolve the question of how much planctomycetes differ in their evolutionary rate from other Bacteria, but such analysis has not appeared, and might be difficult to interpret due to dependence on assumed phylogeny (Sarich & Wilson, 1967; Fitch, 1976). More complex approaches involving determination of decay of internal symmetry of a gene may be needed to read the planctomycete molecular clock (Gibbs & Dugaiczyk, 1994). Establishment beyond reasonable doubt whether planctomycetes and chlamydiae are sister groups may also be needed to resolve this phylogenetic problem (Woese, 1991).
evolution of Bacteria via molecular phylogenetics. Changes in branch positions and phylogenetic depth with the molecule sequenced and organisms included challenge presently available analysis techniques. The interesting and difficult features of this model, which strain the limits of contemporary phylogenetic methods, may generate approaches to analysis which may even be useful with less divergent and problematic groups of micro-organisms.

Molecular cell biology

Cell wall and membranes

The first unusual feature of planctomycete cell biology to be found was the absence of peptidoglycan in their cell walls. This had been indicated first from ultrastructural evidence, and was confirmed by determination of wall chemical composition with respect to absence of muramic acid and diaminopimelic acid in \( \text{Pi. maris, Pi. staleyi, Pi. marina} \) and related strains, and in \( G. \text{obscureiglobus and I. pallida} \) (König et al., 1984; Liesack et al., 1986; Stackebrandt et al., 1986b; Giovannoni et al., 1987a). A strain of \( \text{Pi. marina} \) (IFAM 1313) was found to have an SDS-resistant wall composed mainly of a protein rich in glutamate (König et al., 1984). An additional five strains of \( \text{Pirellula} \), including \( \text{Pi. staleyi} \), and three \( \text{Planctomyces} \) strains, including \( \text{Pi. maris} \), displayed a major protein component rich in proline and stabilized to a significant degree by disulphide bonds as indicated by a high cystine content, explaining resistance of envelopes to 10% SDS (Liesack et al., 1986). \( G. \text{obscureiglobus} \) has a wall relatively low in cystine (Stackebrandt et al., 1986b). Perforations consistent with planctomycete-specific crateriform structures appear also in isolated planctomycete walls (Liesack et al., 1986; Stackebrandt et al., 1986b; Giovannoni et al., 1987a). Lack of peptidoglycan was consistent with the known resistance of these bacteria to peptidoglycan-synthesis-targeting antibiotics such as penicillin-G, ampicillin, vancomycin, n-cycloserine and cephalotin (König et al., 1984). The resistance to \( \beta \)-lactam antibiotics observed for the uncultured species \( \text{Pi. bekefi} \) in enrichments (Schmidt & Starr, 1989) is thus consistent with its probable true relationship to the planctomycetes, as is its cell wall structure. It has been suggested that the absence of peptidoglycan from planctomycetes may be consistent with an ancient origin for this division of the Bacteria, perhaps representing retention of the characteristics of a bacterium evolving prior to invention of peptidoglycan (Stackebrandt et al., 1984). This view may be a useful source of hypotheses with respect to cell wall synthesis pathways.

Cellular fatty acid and chemotaxonomic analysis has revealed some unusual features in the planctomycetes. Lipids of planctomycetes examined possess fatty acids in the ester linkage typical of the Bacteria and Eucarya rather than of Archaea (Kerger et al., 1988; Giovannoni et al., 1987a). In \( \text{Pirellula sp.} \) and \( \text{Planctomyces sp.} \), including \( \text{Pi. staleyi, Pi. marina, Pi. limnophilus and Pi. maris} \), the major ester-linked fatty acids of phospholipids are palmitic, palmitoleic and oleic acids, a pattern more typical of microeukaryotes than of eubacteria (Kerger et al., 1988).

Presence of the 18-carbon monounsaturated 18:1o9c, not normally a major component of bacteria, is characteristic of the order \( \text{Planctomycetales} \) (Kerger et al., 1988). Evidence for presence of lipopolysaccharide lipid A, presumably from the planctomycete wall, was also obtained (though not direct evidence of LPS as such), as indicated by the presence of hydroxy fatty acids of unique 3-hydroxy pattern sufficiently unusual to perhaps form the basis of signature biomarkers for planctomycete presence in a habitat (Kerger et al., 1988). Evidence for 3-hydroxy acids has also been obtained for \( I. \) pallida (Giovannoni et al., 1987a). Presence of even-numbered 3-hydroxy fatty acids was confirmed in a more recent study where distribution of different types of those compounds was found to correlate well with the distinction between \( \text{Planctomycetes} \) and \( \text{Pirellula} \), but such compounds also occurred in non-planctomycete prosthete bacteria such as \( \text{Prosthecococcus} \) (Sittig & Schlesner, 1993). A unique cyclopropane fatty acid 13,14cy21:0 is found in some strains resembling \( \text{Planctomyces} \). In contrast to prosthete proteobacteria, menaquiones rather than ubiquinones are found in planctomycetes even though grown under aerobic conditions (Sittig & Schlesner, 1993).

The above features emphasize the phenotypically distinct nature of the planctomycetes, consistent with the molecular sequence evidence for their novelty.

Genetic organization

Unusual features of planctomycete molecular cell biology include the unusually short 5S rRNA with special structural characters and the unusual \( \text{rrn operon} \) organization involving separation of the 16S rRNA gene from the 23S rRNA gene. Evidence for such a separation has been found in at least two genera so far, in \( \text{Pi. marina} \) (Liesack & Stackebrandt, 1989) and \( \text{Pi. limnophilus} \) (Menke et al., 1991). In \( \text{Pi. marina} \), each of the two sets of 16S rRNA genes are separated by at least 85 and 44 kb, respectively, from the closely linked 23S–5S rRNA genes. \( \text{Pi. limnophilus} \) possesses two unlinked 16S and 23S rRNA genes separated from each other by at least 43 kb, while the organization of the five \( \text{rrn operons} \) found in \( G. \text{obscureiglobus} \) could not be unambiguously determined (Menke et al., 1991). Although linkage of 16S and 23S rRNA genes into an \( \text{rrn operon} \) with order 5′-16S-23S-5S-3′ is typical for Bacteria, there are enough exceptions, such as some proteobacteria in addition to the planctomycetes and the deep-branching \( \text{Thermus} \), to indicate an absence of phylogenetic significance for this trait. A by-product of this research was the discovery of a –10 Pribnow box-like promoter region (TATCTAT) in the 5′-flanking regions of the 23S–5S operons from \( \text{Pi. marina} \) similar to P2 of the 5S G operon in \( \text{E. coli} \), although homologies to a typical –35 region were not found (Liesack & Stackebrandt, 1989). The putative Pribnow box was followed by a GC-rich sequence similar to the discriminator region under stringent control in stable RNA and ribosomal protein genes of \( \text{E. coli} \). Conserved elements similar to sequences involved in antitermination control in rRNA operons of \( \text{E. coli} \) and \( \text{Thermus thermophilus} \) were also identified in these flanking regions. A
putative processing signal and rho-independent terminator with GC-rich hairpin were found in the 3'-terminal region of the 23S–5S rDNA operons. Homologies may exist between organization of the antitermination boxes in the 23S–5S operons of *Pi. marina* and those of *Thermus thermophilus*. This remains our only knowledge of the possible organization of genes with respect to transcriptional control in the planctomycetes.

**The membrane-bounded nuclear body: new plans for cell architecture and new models for cell biology?**

The budding mode of reproduction in the planctomycetes is one which could well form a model for cell differentiation in a similar way to the successful use of *Caulobacter* for this purpose. The life-cycle of at least one *Pirellula*-like strain suggests division of this cycle into at least three distinct phases: swarmer cell maturation, budding and mother cell 'resting' between buddings, and pilus synthesis and flagellum loss are correlated with the maturation phase (Tekniepe et al., 1981). Multiple successive daughter cells can appear from one locus of the mother cell.

The character which most clearly positions the planctomycetes as a significant group for cell biology, however, is the membrane-bounded nuclear body of *G. obscuriglobus* (Fuerst & Webb, 1991). This body is visible after cryo-fixation techniques such as cryo-substitution (Fig. 5) known to prevent artifactual membrane organelles such...
as mesosomes, as well as after conventional chemical fixation without freezing. Recent results have demonstrated that the shape and structure of G. obscuriglobus can be markedly influenced by osmotic effects of buffer concentration during conventional chemical fixation for electron microscopy (Lindsay et al., 1995). The structure of chemically fixed cells had originally suggested that the DNA of this species might be ‘packaged’ in a phase-dark inclusion (Franzmann & Skerman, 1984). I. pallida has been reported to possess nuclear material located in a distinct region of the cell when 4',6-diamidino-2-phenylindole (DAPI) staining of DNA is applied, and may also prove to have a compartmentalized cell organization (Staley et al., 1992). Possible occurrence of membrane-bounded nuclear regions in this and other planctomycete species is presently under investigation. In G. obscuriglobus, as in eukaryote nuclei, there are two nuclear membranes, separated by a relatively electron transparent space. The nuclear body is visible in DAPI-stained cells via fluorescence microscopy, DNA can be demonstrated inside the body by anti-dsDNA monoclonal antibody, and all the cell DNA appears to be contained within the body.

Several evolutionary scenarios may account for the occurrence of such an organelle in what is phylogenetically a member of the Bacteria. These structures might be evolutionarily homologous with eucaryal nuclei, and represent retention of a character shared with a common nucleated proto-eukaryote ancestor. Alternatively, convergent evolution may have resulted in a structure analogous to the eukaryotic nucleus, perhaps even similar in function as well as structure. A third alternative is that the planctomycete nucleus merely reflects a fortuitous internal membrane hypertrophy with a different function to membranes of eucaryal nuclei. The first two hypotheses imply that molecular cell biological features unique within the Bacteria may be discovered. For example, nuclear-targeted proteins bearing nuclear localization signals, perhaps with homology to those used by eucarya, may be discovered if the first hypothesis is true. Alternatively, proteins with unique planctomycete-specific signals would be expected if the second applies.

The occurrence of nuclei in planctomycetes implies that it may not be necessary to invoke either a central role for phagocytosis (Cavalier-Smith, 1975) or an endosymbiotic mechanism involving phagocytic engulfment (Lake & Rivera, 1994; Sogin, 1991) to explain the origin of the eukaryotic nucleus. The planctomycete case demonstrates that an analogous structure can occur within a rigid-walled non-phagocytic cell, presumably one with a non-phagocytic ancestor in which the original genome engulfment by a purely internal membrane had occurred. If evolutionary homology of planctomycete nuclei with those of eucarya is assumed, hypothetical planctomycete phyllogenies consistent with possible modes for eucaryal origins include considering planctomycetes (1) as ‘ancient living fossils’ retaining an ancestral feature shared with the last common ancestor of the three domains (this implies a proto-eucaryal common ancestor) or (2) as advanced ‘missing links’ between Bacteria and Eucarya or more precisely, descendants of such links retaining their significant structural innovations (which implies a late eucaeryl origin and may be inconsistent with trees clustering Archaea closer to Eucarya than Bacteria). If we assume these nuclei are the result of convergent evolution, phylogenetic problems are avoided.

Regardless of the true phylogeny of the planctomycete nucleus, the occurrence of a membrane-bounded genome in such organisms has many intriguing implications for the study of cell biology. Some predictions which can be made are that there should be some type of nuclear localization signal in nuclear-directed proteins such as DNA-binding topoisomerases and polymerases (e.g. gyrase and DNA-dependent RNA polymerase), and some form of nuclear pore analogue. Weaker predictions are that introns may be found in some protein-coding genes of planctomycetes (which would have major implications for the ‘introns-early’ vs ‘introns-late’ controversy) together with correlated RNA-splicing mechanisms involving spliceosomes and small nuclear ribonucleoprotein particles, and that cytoskeletal proteins necessary for distribution of membrane-bounded nuclei during division might occur. Sequences with homology to nuclear localization signals have already been claimed to occur in the proteobacteria Agrobacterium tumefaciens and Neisseria gonorrhoeae, bacteria not known to possess membrane-bounded nuclei, but with intimate eukaryotic host relationships (Citovsky et al., 1992; Pohlnet et al., 1990).

In summary, the planctomycetes are a novel group of organisms with great potential for increasing our knowledge of microbial ecology, bacterial evolution and cell biology. Their wide distribution in nature implies important and hitherto unsuspected roles for them in ecosystem processes, especially concerning the marine ecosystem and contributions of particulate fractions of seawater to global biogeochemistry. The problems posed by their evolutionary relationships challenge contemporary phylogenetic analysis. Finally, and perhaps most significantly, they have challenged our understanding of possible fundamental plans for structural organization of the cell. The molecular implications of their ultrastructure may have wide implications for cell biology and an emerging perspective on the expanding common ground shared by prokaryote and eukaryote biology.

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