

Planctomycetes – a phylum of emerging interest for microbial evolution and ecology

John A. Fuerst

*Department of Microbiology and Parasitology, University of Queensland,
Brisbane, Queensland 4072, Australia
j.fuerst@mailbox.uq.edu.au*

Planctomycetes are a group of budding, peptidoglycan-less bacteria of increasing significance for microbial evolution, ecology, cell biology and genomics. Studies of both cultured isolates and clone library sequences from natural communities have enriched this significance. Their display of unusual distinctive features such as compartmentalized cell organization, ability of some species to grow anaerobically and autotrophically via oxidation of ammonium, and the possession of large genomes combined with their wide distribution in a variety of habitats reinforces an increasing interest in them.

1) Introduction to the Planctomycetes

Planctomycetes are an example of one of several groups of prokaryotes the true significance of which for microbiology, and for ecology and biology as a whole, is becoming recognized due to insights from the application of molecular sequencing and phylogenetics in combination with microbial ecology, modern electron microscopy preparative methods and chemotaxonomy. Such groups also include the verrucomicrobia (Hedlund *et al.*, 1997); (Janssen *et al.*, 2002), the acidobacteria (Hugenholtz *et al.*, 1998) (Liles *et al.*, 2003) and TM7 phyla (Hugenholtz *et al.*, 2001) among the Bacteria, and the Korarcheota and mesophilic crenarcheotes among the Archaea (DeLong, 1998a; DeLong, 1998b). However, the riches of knowledge awaiting the deep study of bacterial diversity are being exemplified nowhere better than by our increasing understanding of the potential importance of the planctomycetes. This is an unusual yet deceptively non-‘extreme’ group of bacteria, like actinomycetes initially

mistaken for fungi (Starr & Schmidt, 1989), and resembling Archaea in their possessing protein cell walls, but brought back into the Bacterial fold with the application of electron microscopy, 16S rRNA phylogenetics and the determination of a bacteria-like reaction to diphtheria toxin (Stackebrandt *et al.*, 1984; Starr & Schmidt, 1989). Many of the early observations and species designations were based on natural microbial communities or enrichments (Starr & Schmidt, 1989), and even now some of those species such as the rosette-forming *Planctomyces bekefi* (type species of the genus) remain uncultured, and enrichments such as bioreactor cultures remain an important contributor to our knowledge of new planctomycetes. The planctomycetes, organisms within the order *Planctomycetales*, are members of the distinct phylum *Planctomycetes* (also known as a ‘division’) of Domain Bacteria (Garrity *et al.*, 2003), a phylum which represents a deep-branching group within the Bacteria on the basis of 16S rRNA sequence phylogenetics (Schlesner & Stackebrandt, 1986); (Fuerst, 1995; Van De Peer *et al.*, 1994). A recent important phylogenetic study applying an alignment of only slowly evolving positions to tree generation suggests that this division may be the deepest branching among the Bacteria, rather than hyperthermophiles like the Aquificales (Brochier & Philippe, 2002) though there is controversy about this conclusion (Di Giulio, 2003).

Planctomycetes are distinctive for their peptidoglycan-less cell walls and budding reproduction and other cell organization features of great evolutionary significance discussed below. These bacteria have been identified in diverse freshwater, marine and soil habitats and even invertebrate animals (Fuerst, 1995; Fuerst *et al.*,

1997; Neef *et al.*, 1998; Schlesner, 1994; Staley *et al.*, 1992; Wang *et al.*, 2002). They have been isolated as chemoheterotrophs from an equally diverse range of habitats, using for example selective media based on their inherent resistance to antibiotics targeting peptidoglycan synthesis and their predilection for N-acetylglucosamine as a carbon substrate (Schlesner, 1994), but only representatives of 4 genera exist in pure culture, *Pirellula*, *Planctomyces*, *Gemmata* and *Isosphaera*. We know already that many more remain to be isolated in pure culture, and that their physiological range is wider than axenically cultured strains suggest. A unique group of planctomycetes, the autotrophic ‘anammox’ planctomycetes, comprising at least distinct 3 ‘Candidatus’ genera (“Brocadia”, “Kuenenia” and “Scalindua”) (Kuenen & Jetten, 2001; Schmid *et al.*, 2000; Schmid *et al.*, 2003), perform a novel type of autotrophic metabolism based on anaerobic oxidation of ammonium, the ‘anammox’ process; these exist in culture so far only in bioreactor mixed cultures, though these can be quite enriched in anammox planctomycetes as the major component of the microbial community (Schmid *et al.*, 2003; Strous *et al.*, 1999). A filamentous morphotype known to wastewater microbiologists as “Nostocoida limicola” III occurring in activated sludge appears to be closely related to the gliding moderate thermophile *Isosphaera pallida* (Liu *et al.*, 2001).

Planctomycetes are sometimes grouped for the purpose of organizing sequence databases with the verrucomicrobia, organisms forming another distinct phylum of the domain Bacteria, differing from planctomycetes in possession of peptidoglycan but like the phylum *Chlamydiae* sometimes linked to planctomycetes in phylogenetic trees based on 16S rRNA (for an example with significant bootstrap confidence support (see (Janssen *et al.*, 1997) but for rejection of such relationships see (Ward *et al.*, 2000)). Planctomycetes and verrucomicrobia share certain 16S rRNA signature nucleotides (Derakshani *et al.*, 2001), and they also share the phenomenon of possessing some significant homologs with eukaryote genes of importance in cell biology. These homologs already include one with integrin alpha-V in the case of *Gemmata obscuriglobus* (Jenkins *et al.*, 2002a), and with tubulin in the case of *Prostheco bacter dejongeii* (Jenkins *et al.*, 2002b). The question of evolutionary interest which arises immediately from such observations is- do such homologs reflect their

inheritance from a common ancestor of the 2 divisions of the Bacteria, or were they acquired by separate lateral transfer from eukaryotes at a later time in evolution? If the former, might not planctomycetes and verrucomicrobia share specific eukaryote homologs? Related questions are whether the Bacterial homologs perform similar functions to those known in eukaryotes e.g. to form cytoskeletal microtubules functioning in chromosome segregation in the case of tubulin, or in communication between cell external environment and cytoskeleton in the case of integrin. Both planctomycetes and verrucomicrobia have implications for cell biology and the evolution of eukaryote cell organization, the former due to their possession of membrane-bounded cell compartments, the latter due to their possession of proteins which represent the closest homologs to eukaryote tubulins within the Bacteria. Some analogous considerations may apply to the proposed similarly tentative relationship between planctomycetes and the chlamydia. Such a relationship is consistent with some 16S rRNA signatures and sometimes interpreted as loosely linking all these 3 phyla together, but so far often without statistical confidence in phylogenetic analysis.

2) Planctomycetes and implications of membrane-bounded nucleoids in bacteria of cell biology

Planctomycetes have been shown to possess a number of characteristic structural features, including distinctive types of membrane-bounded compartments within cells of at least 4 genera (Lindsay *et al.*, 2001). These features have only been clearly revealed by the application of cryosubstitution and other freezing cryotechniques to preparation of cells for electron microscopy, but are also being revealed by fluorescence microscopy of living cells. Thus, *Gemmata obscuriglobus* displays a double-membrane-bounded organelle, the nuclear body, enveloping the nucleoid and all the cell’s DNA as well as ribosome-like material (Fuerst & Webb, 1991; Lindsay *et al.*, 2001). Members of the genus *Pirellula* possess a unique organelle, the pirellosome, a single-membrane-bounded organelle also containing the nucleoid DNA as well as other ribosome-like material, and ammonium-oxidizing anammox species have an additional internal anammoxosome compartment surrounded by a single

membrane and with unique structural and functional properties. (Lindsay *et al.*, 1997; Lindsay *et al.*, 2001). Cell compartments have in fact been found to be common to all planctomycete species examined, and are elements of a new type of cell plan hitherto unknown among prokaryotes yet shared by all planctomyetes so far examined by application of cryosubstitution techniques (Lindsay *et al.*, 2001). These structures pose a challenge to the existing classification of known living cells on the basis of organization (prokaryote vs. eukaryote). They raise significant questions about whether they represent analogs or homologs of eukaryote cell structure, occurring in organisms that appear to be valid members of the Domain Bacteria on the basis of rRNA sequence, and about function concerning location of transcription and translation, transport between compartments, and how nucleoids are distributed during division. Cytoskeletal proteins other than the FtsZ common in many Bacteria may be involved in cell division and perhaps nuclear body distribution. Some planctomyetes such as those performing the 'anammox' process display unique types of autotrophic physiology unknown in other Bacteria or Archaea, and these may also be dependent on the compartmented cell structure the planctomyetes provide, with specialized enzymes concentrated exclusively in special membrane-bounded organelles, the anammoxosomes (Lindsay *et al.*, 2001). Of special interest to planctomycete and evolutionary cell biology is that anammoxosomes contain tubule structures visible via cryopreparative techniques for electron microscopy, and that the nucleoid is attached to the anammoxosome membrane. Such tubule structures are interesting as possible analogs or precursors of cytoskeletal structures of eukaryotes such as microtubules, especially in the context of the discovery of tubulin homologs in *Prostheco bacter dejongei* (Jenkins *et al.*, 2002b) and actual tubules in the uncultured symbionts of protozoans called 'epixenosomes' (Petroni *et al.*, 2000; Rosati *et al.*, 1993). *Prostheco bacter* and epixenosome symbionts are members of the verrucomicrobia phylum, which some phylogenetic analyses suggest are related at a deep level to the planctomyetes, though the validity of this possible relationship is still unclear. The planctomyetes thus form significant models for the understanding of fundamental questions in evolutionary cell biology, about the origins of eukaryote nucleus and cytoskeleton.

Cell compartmentalization in the planctomyetes has implications for formulating models of evolution of eukaryotes and eukaryote-specific proteins. The compartmentalization phenomena found in planctomyetes suggests that an endogenous rather than endosymbiotic origin for the eukaryote nucleus, e.g. (Lake & Rivera, 1994) is at least a possibility. Ultrastructure of eukaryote nuclear envelope and pore complexes also does not favour endosymbiotic origins for nuclei (Poole & Penny, 2001). The various stages needed for endogenous membrane enfolding of the genome may be represented 'frozen' in different planctomycete genera. Planctomyetes form a model for how a eukaryote-like nucleus might have formed within one cell lineage without the need for symbiotic events, and if so molecular correlates of such evolution may also be found in this group. Some of these correlates and proteins may be analogous or even homologous to those specific to eukaryotes e.g. those concerned with nucleocytoplasmic transport of protein and RNA through the nuclear envelope.

Planctomycete cell compartmentalization also has other implications for cell biology, concerning the relationship of functional cell biology to structural compartmentalization. A major finding of investigations of planctomycete ultrastructure is that in representatives of all cultivated genera, all the DNA of planctomyetes like *Gemmata obscuriglobus* and *Pirellula marina* is contained within a membrane envelope, and no DNA is found outside this envelope or in contact with any cytoplasmic membrane apposed to cell wall (Lindsay *et al.*, 1997; Lindsay *et al.*, 2001). In particular, in *G. obscuriglobus*, all cell DNA is confined to the double-membrane-bounded nuclear body. This implies firstly that chromosome segregation must require a special mechanism in these organisms different from those in non-compartmented bacteria, since the chromosomal DNA cannot be attached to the cytoplasmic membrane as in the classical 'replicon' model for segregation in bacteria.

Since DNA is confined to a membrane-bounded nuclear region, it would appear that transcription must also be confined to this region, and this implies that some of the translation in these planctomyetes may be uncoupled from transcription and therefore resemble the molecular cell biology of protein synthesis in eukaryote cells.

3) Buds from the tree of life- Planctomycetes and the Last Common Ancestor of the Domains of life?

Phylogenetic relationships between the cultured planctomycetes themselves are relatively clear e.g.(Fuerst *et al.*, 1997) (Gripenburg *et al.*, 1999; Ward *et al.*, 1995). The phylogenetic position of the planctomycetes relative to other Bacteria however has been the subject of controversy, with some analyses deducing a fast evolutionary rate and artefactually deep position within Bacteria (Liesack *et al.*, 1992), and others a deep branching position implying an ancient lineage within the Bacteria. Such problems have sometimes been attributed to phylogenetic analysis problems such as long branch attraction (Jenkins & Fuerst, 2001). One of the most recent analyses (Brochier & Philippe, 2002) using an advanced phylogenetic analysis method employing slowly evolving positions of 16S rRNA has deduced that the planctomycetes may in fact be the deepest branching phylum within the Bacteria, instead of hyperthermophiles like *Aquifex*. This may then imply that cell compartmentalization may be quite an ancient feature retained in the planctomycetes but lost in all other members of the Bacteria. This is consistent with some views of the history of the 3 domains of life which would see the Last Common Ancestor as a complex eukaryote-like cell (Forterre & Philippe, 1999; Forterre *et al.*, 1992; Glansdorff, 2000). However alternative phylogenetic analyses attempting to contradict this view have already been advanced, although even in one of those analyses planctomycetes are relatively deep-branching (Di Giulio, 2003); the analysis based on multiple genes derived from genomic data supports a distinct planctomycete phylum but does not support deep-branching (Glöckner *et al.*, 2003). Some genomic evidence from *Pirellula* sp. strain 1 suggests an absence of any relation to low%G+C or high%G+C Gram-positive bacteria and a possible relationship to Gram-negative bacteria based on genes needed for lipid A and flagellum basal body protein synthesis (Glöckner *et al.*, 2003).

4) Ammonium-oxidizing chemoautotrophic planctomycetes performing the Anammox process

Planctomycetes isolated in pure culture have all been cultivated as heterotrophs, but with the discovery of the anaerobic ammonium oxidizing

anammox planctomycetes in wastewater-processing bioreactors it is clear that the diversity of the planctomycetes now must encompass chemoautotrophs. A new deep-branching member of the planctomycetes, “*Candidatus* Brocadia anammoxidans”, was discovered to be the dominant member of an anaerobic wastewater treatment bioreactor microbial community performing a novel anaerobic ammonium oxidation (the Anammox process) (Strous *et al.*, 1999). Cells of this organism possess a unique membrane-bounded compartment, the anammoxosome, specific to anammox planctomycetes (Strous *et al.*, 1999);(Lindsay *et al.*, 2001), and a unique membrane lipid, a concatenated cyclobutane chain lipid termed a ‘ladderane’ appears to be present in the anammoxosome envelope, in some cases also ether-linked (Damste *et al.*, 2002). A second species, “*Candidatus* Kuenenia stuttgartiensis”, and a further distinct species of this genus have been discovered by European groups in the last 2 years (Egli *et al.*, 2001; Schmid *et al.*, 2000). These morphotypes have not yet been cultured, and appear to be bacteria with some of the slowest generation times in the laboratory (up to 3 weeks in the laboratory). Several recent reviews describe the present knowledge of their diversity, physiology and applied significance in waste remediation (Jetten *et al.*, 2003; Jetten *et al.*, 2001; Schmidt *et al.*, 2003; Strous *et al.*, 2002). The anammox planctomycetes may form only part of an even larger group of organisms branching deeply within the phylum (Chouari *et al.*, 2003).

Several genome projects on anammox planctomycetes are being planned and one of those is in progress at this time, such is the intense interest they have generated. The genome project initiated by the group at University of Nijmegen is described at <http://www-microbiol.sci.kun.nl/tech/genomics.html>. One of the major enzymes specific to the anaerobic ammonium-oxidizing ability of these organisms-hydroxylamine oxidoreductase-HAO- is localized entirely within the anammoxosome compartment (Lindsay *et al.*, 2001); this data has been central to the development of a model for the mechanism of anaerobic ammonium oxidation in these bacteria involving a pivotal role for knowledge of location of the HAO enzyme (Jetten *et al.*, 2001). A remarkable recent finding is that the anammoxosome membrane possesses unique ‘ladderane’ lipids with cyclobutane rings with ether linkages to the glycerol backbone in

some cases, and which confer much greater density to the anammoxosome membrane than ordinary membranes (Sinninghe Damsté *et al.*, 2002). Another unusual feature of anammoxosomes is that cytoskeleton-like tubules occur inside anammoxosomes and are sometimes arranged in organized patterns. The nature of the tubule structures in anammoxosomes is unknown, but the discovery of bacterial tubulin in verrucomicrobial *Prostheco bacter* makes it conceivable that such tubules are also composed of a cytoskeletal protein homologous with tubulin.

5) Planctomycetes planctomycetes everywhere-recent results from molecular ecology

The application of cultural approaches had already revealed and is still revealing a wide distribution of planctomycetes especially in aquatic habitats of a wide variety geographically and ecologically from freshwater pond to ocean, and from Antarctica to Australia e.g. (Fuerst *et al.*, 1997; Schlesner, 1994; Tan & Ruger, 1999; Wang *et al.*, 2002).

However, techniques of direct molecular ecology employing clone library approaches to estimating microbial community diversity in environmental habitats as well as FISH (fluorescent in situ hybridization) approaches have broadened and deepened our knowledge, revealing the ubiquitous distribution of planctomycetes not only in habitats where they have been commonly cultured but also many where culture has not yet been attempted. As a distinct phylum of Bacteria, planctomycetes have lent themselves to design and application of specific oligonucleotide FISH probes such as PLA46 and PLA886, though interestingly PLA886 can cross-react with some eukaryotes (Neef *et al.*, 1998). Planctomycetes appear to be quantitatively as well as qualitatively significant in some direct molecular ecology studies. A recent finding that planctomycetes are one of the dominant microbial components of a coarse-grained marine shelf sediment of the Middle Atlantic Bight with relatively low organic carbon content and probably contributing to the high biocatalytic filtration occurring in such sediments is a dramatic example of their unsuspected significance ecologically (Rusch *et al.*, 2003). Planctomycetes can occur as quite significant proportions of the total microbial community in other habitats also- for example in a study of Yellowstone National Park

soil recently heated by a geothermal event, 18% of clones in a bacterial 16S rDNA library of the community belonged to the planctomycete phylum (Norris *et al.*, 2002). Studies of quantitative distribution of members of the domain Bacteria in any molecular ecology study can be influenced by insufficiency of classical Bacteria-specific probe sequences such as EUB338 to 'see' planctomycetes (Neef *et al.*, 1998), and because of this most studies will now employ a mix of probes including the planctomycete-sensitive EUB338II for this purpose (Daims *et al.*, 1999).

Also in the marine habitat is the important discovery of quantitatively significant ammonium-oxidizing anammox planctomycetes in the Black Sea, the world's largest anoxic basin (Kuypers *et al.*, 2003), and anammox activity in an anoxic bay off the coast of Costa Rica (Dalsgaard *et al.*, 2003), indicating a significant role for anammox planctomycetes in the global nitrogen cycle, as biological agents for molecular nitrogen regeneration in anoxic conditions. Genus *Pirellula* planctomycetes appear to be significant members of the microbial community of rice roots in flooded rice field microcosms (Derakshani *et al.*, 2001). Evidence from using RT-PCR to estimate actively metabolizing community members in a freshwater sediment indicates that *Pirellula*-like planctomycetes can be active in anoxic conditions (Miskin *et al.*, 1999).

Although planctomycetes had originally been observed and cultured from aquatic habitats, one of the results from molecular ecology approaches has been appreciation of their wide occurrence in soil. This started with the pivotal demonstration of uncultured planctomycetes in the notable MC clones from a natural forest soil at Mt. Coot-tha in subtropical Brisbane Australia (Liesack & Stackebrandt, 1992), and has continued with many other studies, including the geothermal heated soil at Yellowstone with high fractions of planctomycetes noted above (Norris *et al.*, 2002), soil from the Amazon rainforests (Borneman & Triplett, 1997), cultivated soil where planctomycetes can contribute up to 7.2% ± 4.2% of total soil rRNA measured by filter hybridization (Buckley & Schmidt, 2003), and a quantitative FISH study with a planctomycete-specific Pla5a probe showing that even in a pristine forest soil from Switzerland, 7 ± 3 % of DAPI-

stained cells were planctomycetes (Zarda *et al.*, 1997). Of applied significance is the occurrence of planctomycetes in the communities of wastewater treatment plants and activated sludge digestors (Chouari *et al.*, 2003; Liu *et al.*, 2001), in communities active in bioremediation, e.g. a sulfidogenic 2-bromophenol-dehalogenating consortium where planctomycetes were one of the 4 phylotypes found (Knight *et al.*, 1999), the occurrence of a clone with a nearest match an *Isosphaera* Schlesner 657 strain in a library from a biomass production chamber using soybean plants for purification of human hygiene waste water at Kennedy Space Center (Kerkhof *et al.*, 2000), and another *Isosphaera* match in a community from a bioregenerative life support system in the Lunar-Mars Life Support Test Project at the Johnson Space Center (Sakano *et al.*, 2002).

The fact that planctomycetes seem to have been and are now nearly everywhere is illustrated dramatically by the finding that 4.8% of the clones from a community library from the Paleolithic Altamira cave paintings in Spain consist of planctomycetes (Schabereiter-Gurtner *et al.*, 2002). Perhaps we have been looking at them for quite a long time.

6) The Whole Story - Genome Sequencing Projects for Planctomycetes

The contributions of whole genome sequencing to our understanding of the full potential and the evolution of members of domains Bacteria and Archaea have been dramatic in recent years. Possessing some of the largest genomes known in the Bacteria (e.g. 9 Mb for *Gemmata obscuriglobus*), planctomycetes were always going to wait a while for their genomic secrets to be revealed, but recently several planctomycete genome projects have commenced and in some cases are well underway with multiple coverage already achieved. The whole genome sequence from a marine planctomycete affiliated with the genus *Pirellula*, though apparently distinct enough to be placed in a new yet-to-be-published genus "*Rhodopirellula*" as "*Rhodopirellula baltica*", has now been completed and published {Glockner, 2003 #130} by the REGX project research group at Max Planck Institute of Marine Biology, Bremen, Germany (<http://www.regx.de/>); it is available at the REGX web site as well as for search at NCBI. Among

notable results are the occurrence of 8% best BLAST homology hits of the ORFs detected with eukaryote sequences, a remarkable number of sulfatases of so far unknown function, a large number of genes with signal peptide sequences which may be correlated with protein transport across the intracellular pirellulosome compartment, and the occurrence of some genes suggesting that some elements of a peptidoglycan synthesis apparatus may be present. Some genomic data also exists for *Pirellula marina* including some eukaryote-like genes (Jenkins *et al.*, 2002a). Data from the TIGR (The Institute for Genomic Research) project on *Gemmata obscuriglobus* is now available for bioinformatics searching on web sites at both TIGR and NCBI (National Center for Biotechnology Information). 1,250 contigs have now been assembled with mean contig size 7,388 bases, and total assembled sequence 9,235,199 bases- giving a coverage of ca. 8.1x. (see <http://www.tigr.org/tigr-scripts/ufmg/ReleaseDate.pl> for present status). A previous study of the same strain had revealed interesting genes such as integrin alpha-V (Jenkins *et al.*, 2002a). In addition a commercial project on a soil isolate Wa-1 of *Gemmata* led by James T. Staley of University of Washington and implemented at Integrated Genomics of Chicago has been in progress, which will complement the *G. obscuriglobus* results very well and provide data for intragenomic comparisons (<http://wit.integratedgenomics.com/GOLD/index.cgi?want=Prokaryotic+Ongoing+Genomes>).

The biotechnologically and environmentally important anammox planctomycetes are now also the subject of genome sequencing efforts, with a project on "*Candidatus Kuenenia stuttgartiensis*" underway in an effort coordinated by the University of Nijmegen (see <http://www-microbiol.sci.kun.nl/tech/genomics.html>).

There is even some metagenomic data concerning occurrence of planctomycetes in the ocean which has been valuable in determining some potential problems with use of the 27f primer to detect planctomycetes in molecular ecology PCR (Vergin *et al.*, 1998).

A completed annotation of even just the available full (but not yet closed) genome sequence of *Gemmata obscuriglobus* will mean that identification of proteins relevant to its unique cell plan such as those involved in cell division, chromosome

segregation, cytoskeleton and nucleoid compartmentalization will be facilitated, and this will help solve the problem of this cell plan's evolutionary meaning. The availability of several planctomycete genomes will help not only this phylogenetic effort but also assist understanding of the genomic profile correlating with the unique physiology of the ammonium-oxidizing anammox organisms, though genomes for several different genera of these are desirable for comparative confirmation of any interesting results. Particularly interesting should be the comparison of genes identified in planctomycetes with members of other divisions of Bacteria of unclear but suggested relationship to planctomycetes such as verrucomicrobia and chlamydia, and the comparison with other organisms with protein cell walls including members of the Archaea such as crenarcheotes.

7) Conclusions

The planctomycetes are a good example of how a once neglected and relatively obscure phylum of Bacteria can find increasing appreciation within the microbiological and broader scientific community when just a small amount of research attention is paid to them, and of the value of increasing our knowledge of microbial diversity to our potential understanding of wider problems in biology as a whole. The most significant of these, the question of the nature of the Last Universal Common Ancestor and the origin of eukaryotes, may yet place planctomycetes in centre stage in the grand drama of our search for evolutionary answers in deep time.

Acknowledgements

I thank the Australian Research Council for funding research on planctomycetes in my laboratory.

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