NOTE

Molecular and ultrastructural confirmation of classification of ATCC 35122 as a strain of *Pirellula staleyi*

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A freshwater isolate from Campus Lake, Baton Rouge, LA, USA, strain ATCC 35122 (= ICPB 4362 = Schmidt CLPM White = Tekniepe BT2 white), which had been proposed as a putative reference strain for '*Planctomyces staleyi*' (later reclassified as *Pirellula staleyi*), has been re-examined to establish its relationship to the type strain of *Pirellula staleyi*, ATCC 27377^T. 16S rRNA sequencing confirms its very close relationship to ATCC 27377^T and its membership of the order *Planctomycetales*. Ultrastructural characteristics are also consistent with its membership of the planctomycetes and of the genus *Pirellula*. These characteristics include polar crateriform structures and the occurrence of the unique internal, single-membrane-bounded compartment enclosing the nucleoid and ribosome-like particles, the pirellulosome, and a polar cap region. Cells of strain ATCC 35122 often displayed pointed, hump-like protrusions opposite each other on the cell, constituting prosthecae, and these were also found to be present on cells of strain ATCC 27377^T. The original identification of ATCC 35122 as a strain of *Pirellula staleyi* is confirmed on both molecular and phenotypic grounds.

Keywords: planctomycetes, *Planctomycetales*, *Pirellula staleyi*, ultrastructure, phylogenetics

Strain ATCC 35122 (= ICPB 4362 = Schmidt CLPM White = Tekniepe BT2 white) was originally isolated by Jean Schmidt and Mortimer Starr (Schmidt & Starr, 1982) as a 'white' subclone of ICPB 4232, isolated from the freshwater Campus Lake, Baton Rouge, LA, USA (Tekniepe *et al*., 1981), and was proposed as a putative reference strain for '*Planctomyces staleyi*' by Starr *et al*. (1983). This decision is consistent with ultrastructure, resistance to peptidoglycan synthesis-targeting antibiotics and DNA G+C content and with comparison with the proposed type strain of this species, ATCC 27377^T, originally isolated from the freshwater Lake Lansing, MI, USA, and classified as *Pasteuria ramosa* (Staley, 1973). Metchnikoff (1888) first described *Pasteuria ramosa*. However, the isolate described by Metchnikoff was a parasite of species of the crustacean *Daphnia* with clear morphological differences from ATCC 27377^T and so, in 1983, the latter strain was reassigned to the genus *Planctomyces* as the proposed type strain of '*Planctomyces staleyi*' (Starr *et al*., 1983). The proposed type strain of '*Planctomyces staleyi*' was later reassigned by Schlesner & Hirsch (1984) to the genus *Pirella*, on the basis of 16S RNA oligonucleotide catalogue data, DNA base composition and re-examination of phenotypic data. This genus name was later corrected nomenclaturally to *Pirellula* by the same authors, because of homonymy with a fungal genus (Schlesner & Hirsch, 1987). However, no 16S rRNA sequence or other molecular data existed to establish the true relationship of ATCC 35122 either to planctomycetes or to *Pirellula staleyi*. We have therefore re-examined this strain to establish its phylogenetic relationships.

For electron microscopy, ATCC 35122 was grown on M1 medium agar (Schlesner, 1994) at 28 °C for 14 days before harvesting for cryosubstitution. Cells were rapidly frozen by plunging into liquid propane or in a high-pressure freezer (Leica EMPACT) and then cryosubstituted in 2·0% (w/v) osmium tetroxide in acetone at −70 °C for 2 days. Cells were then warmed gradually to room temperature before infiltration with

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The GenBank accession number of the 16S rRNA sequence of strain ATCC 35122 is AF399914.

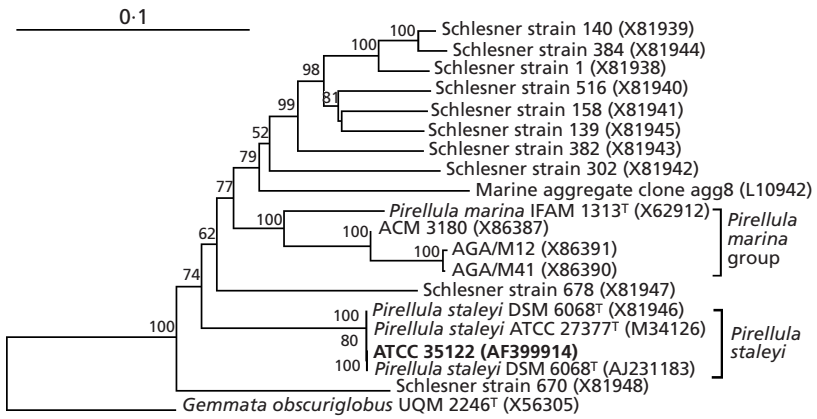

Fig. 1. Phylogenetic tree derived from neighbour-joining distance-based analysis of aligned 16S rRNA sequences, showing the relationship of ATCC 35122 to existing *Pirellula* species and related strains in culture (accession numbers are shown in parentheses). Bar, 0·1 nucleotide substitutions per site.

Epon resin and polymerization. Ultrathin sections were stained with uranyl acetate/lead citrate and examined via a JEOL 1010 transmission electron microscope. For negative staining, the strain was grown on M1 medium at 28 °C for 9 days and a suspension was transferred to a pioloform-coated specimen support grid and negatively stained with 1·0 % (w/v) uranyl acetate plus 0·4 % sucrose. For phase-contrast microscopy, a 5 µl suspension of ATCC 35122, grown on M1 medium agar at 28 °C for 8 days, was placed on the surface of a 3·0 % (w/v) agarose slab on a slide and allowed to soak in. A cover slip was placed over the agarose and cells were viewed using a Zeiss Axioplan2 fluorescence microscope. Photographs were taken with a Nikon 34 mm camera, controlled by Zeiss Axiophot2 software and using Kodak Max 400 35 mm colour film.

Attachment of cells to glass during growth was examined after 20 days growth in a broth composed of 0·5 % peptone and 0·05 % yeast extract in a base of Hutner's basal salts and vitamin solution, as used by Staley (1973).

A nearly complete 16S rRNA sequence was derived via cycle sequencing of the 16S rRNA gene (amplified by PCR using primers 27f and 1525r) employing ABI fluorescent dideoxynucleotide chain terminators and sequencing primers 342r, 687r, 945f, 1101r and 1114f, followed by electrophoresis on an ABI automated sequencer (Australian Genome Research Facility). Resulting sequences were aligned against reference sequences in the ARB software database (Strunk & Ludwig, 1995) and neighbour-joining distance-based phylogenetic analysis was performed with TREECON for Windows 1.3b (Van de Peer & De Wachter, 1994), Kimura's two-parameter distance measure and using 100 bootstrap resamplings, with *Gemmata obscuriglobus* as the outgroup. Nucleotides at *Escherichia coli* positions 36–1485 were used in the alignment for phylogenetic analysis.

16S rRNA sequencing confirms the membership of strain ATCC 35122 in the order *Planctomycetales* and its very close relationship to strain ATCC 27377^T. The close relationship to the three existing 16S rRNA sequences for ATCC 27377^T is illustrated in the phylogenetic distance-based tree (Fig. 1). The sequence entries for the species used are those deposited for strain DSM 6068^T by E. Stackebrandt as GenBank accession no. X81946 and by F. A. Rainey as accession no. AJ231183, and for ATCC 27377^T (as '*Planctomyces staleyi*') by C. R. Woese and H. Oyalzu as accession no. M34126. Strain DSM 6068^T is equivalent to ATCC 27377^T and is merely an accession of the type strain within a different culture collection. Strain ATCC 35122 forms a monophyletic group with the most recent existing database sequence for *Pirellula staleyi* ATCC 27377^T, accession no. AJ231183, with a bootstrap confidence level of 100 %, and all extant sequences for *Pirellula staleyi* cluster with high confidence, as expected. In the alignment used for the tree, a 19-base indel region between *E. coli* positions 1280 and 1300 occurs as a deletion in sequence X81946 relative to all other available database sequences for ATCC 27377^T. When undetermined nucleotide positions and this indel region are not taken into account, the 16S rRNA gene sequence for ATCC 35122 displays 100 % similarity to all existing database entries for the sequence of ATCC 27377^T and its equivalent in the DSMZ. In particular, alignment of the sequence for ATCC 35122 with the most recent and complete available sequence for the equivalent of ATCC 27377^T held in the DSMZ, accession no. AJ231183, displays 100 % identity over positions 32–1524 (*E. coli* numbering). According to criteria proposed for species distinction using 16S rRNA sequences (Stackebrandt & Goebel, 1994), two isolates could have 99·8 % similarity in 16S rRNA and still comprise distinct species as judged by DNA–DNA hybridization; distinct species status for ATCC 35122 therefore cannot be excluded. However, it appears to be at least a member of the same rRNA species complex or rRNA superspecies, categories that have been proposed for organisms with virtually identical 16S rRNA sequences (Stackebrandt & Goebel, 1994; Fox *et al.*, 1992). A detailed study of DNA–DNA hybridization with all cultured planctomycete species will be necessary to answer this question.

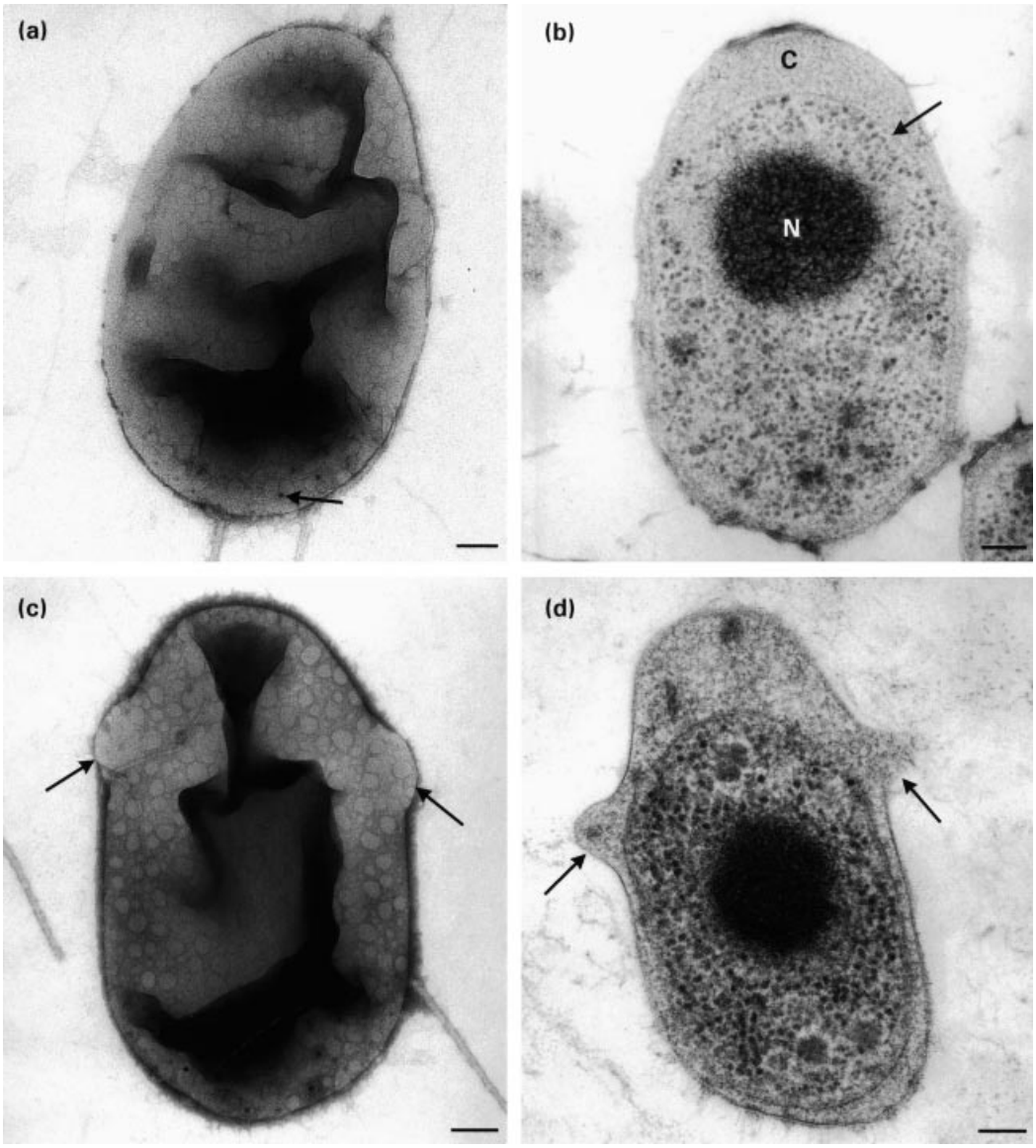

Fig. 2. Transmission electron micrographs of cells of strain ATCC 35122. (a) Negatively stained cell showing polar distribution of crateriform structures (arrow) and teardrop shape of the cell. (b) Thin section of cryosubstituted cell showing the internal pirellulosome compartment (arrow) containing the condensed fibrillar nucleoid (N) and ribosome-like particles and the surrounding polar cap region (C). (c) Negatively stained cell showing hump-like protrusions (arrows) on either side of the cell towards one pole and crateriform structures at the opposite pole. (d) Thin section of cryosubstituted cell showing hump-like protrusions (arrows) on either side of the cell as well as a pirellulosome containing a condensed fibrillar nucleoid. Bars, 100 nm.

Ultrastructural characteristics are also consistent with membership of ATCC 35122 within the planctomycetes and within the genus *Pirellula*. Crateriform structures of identical polar distribution and diameter (12 nm) to those on cells of ATCC 27377^T (as found by Starr *et al*., 1983) were displayed on negatively stained cells via transmission electron microscopy (TEM) (Fig. 2a) and are characteristic of *Pirellula staleyi* in their confinement to less than half the cell surface, relative to their larger extent on *Pirellula marina* (Schlesner, 1986). Cells are tapered at one end, consistent with the teardrop cell shape of *Pirellula staleyi* (Schlesner & Hirsch, 1984). Strain ATCC 35122 also possesses a unique internal, single-membrane-bounded compartment, the pirellulosome, enclosing the nucleoid and ribosome-like particles, and a polar cap region, which we have previously shown also to occur in *Pirellula staleyi* and *Pirellula marina* (Lindsay *et al*., 1997, 2001) and which is visualized optimally after preparation of cells via cryosubstitution (Fig. 2b). Earlier studies using chemical fixation would have had more difficulty visualizing the compartmentalized structure of

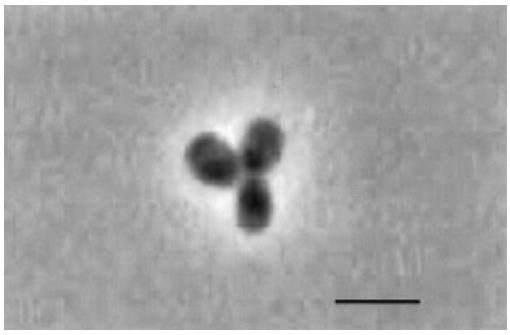

Fig. 3. Phase-contrast light micrograph of a rosette of strain ATCC 35122 showing polar differentiation of each cell into phase-light and phase-dark regions. Bar, 5 μm.

Pirellula staleyi cells, but a micrograph published by Staley (1973) does in fact show a band of darker-staining cytoplasm around the cell rim consistent with the 'polar cap region' in cryosubstituted material of ATCC 27377^T (Lindsay *et al*., 1997) and ATCC 35122 in the present study. The study of Staley (1973) also shows phase-contrast micrographs with phase-dark and phase-light regions within cells of ATCC 27377^T, consistent with compartments seen in cryosubstituted cells of this type strain and in the present study of ATCC 35122 (Fig. 3).

A unique feature seen in both negatively stained cells and in thin-sectioned cells was the occurrence of 'hump' protrusions including both cell wall and cytoplasm (Fig. 2c, d). These protruded 50–111 nm from the cell and were 200–260 nm in diameter measured at the base of the structure (from thin sections and negatively stained cells). There were one or two visible per cell, and never more than two, and when two were visible these were distributed in a characteristic manner opposite each other on the cell near the narrow pole. They appear to conform to the definition of prosthecae as cellular appendages or extensions of the cell containing cytoplasm (Staley, 1968). The confirmation of the prosthecate nature of the protrusions on cells of strain ATCC 35122 via thin sections fully establishes the occurrence of structures fulfilling the definition of true prosthecae in a division of the *Bacteria* other than the proteobacteria, verrucomicrobia or green sulfur bacteria (Hedlund *et al*., 1997; Trüper & Pfennig, 1992). Their shape and dimensions are more comparable to the conical, wart-like prosthecae on the surface of *Verrucomicrobium spinosum* than to the longer structures in other prosthecate bacteria (Schlesner, 1987). However, the prosthecae of cells of strain ATCC 35122 were smaller than prosthecae of *V. spinosum* and there were very many fewer per cell than the numerous prosthecae of *V. spinosum* cells. Since this species is a member of a division of the domain *Bacteria* separate from the planctomycetes, the verrucomicrobia (Hedlund *et al*., 1997), it would appear that prosthecae have now been found in four separate divisions of the *Bacteria*; planctomycetes, verrucomicrobia, green sulfur bacteria and proteobacteria. Prosthecae have previously been reported on the basis of negative staining to occur in some isolates of planctomycetes (Schlesner, 1994). The single 'unicorn' structures found on cells of a prawn-derived, *Planctomyces brasiliensis*-like isolate (Fuerst *et al*., 1997) are similar to the hump-like protrusions found on cells of strain ATCC 35122. The hump-like protrusions are distinct from the 'cornicula' cell-surface protrusions observed in negatively stained preparations of morphotype II of the planctomycetes from freshwater enrichments, since 'cornicula' are different in shape, much smaller in dimensions and appear to consist of subunits (Schmidt & Starr, 1979). The parent strain of the ATCC 35122 clone, ICPB 4232, was not noted to possess any unusual surface projections by Tekniepe *et al*. (1981), but examination of their Figs 3A and 3F reveals projections on the surface of negatively stained cells that are comparable to those we observed on ATCC 35122, as might be expected considering the derivation of the latter as a single clone from ICPB 4232 that does not change pigmentation during ageing of colony growth. We re-examined the type strain of *Pirellula staleyi*, strain ATCC 27377^T, by negative staining and TEM and found that it also possesses one or two 'hump'-like prosthecae, although these are distributed further from the narrow cell pole than in ATCC 35122. Occurrence of prosthecae on both *Pirellula staleyi* strains ATCC 27377^T and ATCC 35122 is consistent with their close relationship as determined from sequence analysis. The 'hump'-like prosthecae could well perform a useful surface area-increasing function for nutrient uptake in these aquatic bacteria. However, there are only one or two per cell, so some function other than increasing surface area seems more likely, such as attachment or decrease in sedimentation rate (Hedlund *et al*., 1997). Functions that are proposed for prosthecae include increasing surface area (e.g. *Ancalomicrobium*), reproduction (daughter cells borne on hyphal tips, e.g. *Hyphomicrobium*) and stalk function (e.g. *Caulobacter*) (Schmidt, 1971; Semenov & Staley, 1992).

Consistent with the descriptions of Schlesner & Hirsch (1984) and Starr *et al*. (1983), fibrillar, rigid, well-defined stalks are not present in either *Pirellula staleyi* strain and we found no evidence that fascicles or irregular holdfasts are clearly visible enough to be useful in identification. A feature of strain ATCC 27377^T connected with cultural characteristics noted by Staley (1973) and possibly connected with holdfast properties was the tendency of its cells to attach to glass surfaces during growth, and this characteristic was also displayed by strain ATCC 35122.

In summary, strain ATCC 35122 is confirmed as a member of the planctomycetes on the basis of 16S rRNA sequence. The original identification of strain ATCC 35122 based only on phenotype and its close relationship to the type strain of *Pirellula staleyi* ATCC 27377^T is now confirmed on molecular grounds by 16S

rRNA sequence analysis. The ultrastructural properties of strain ATCC 35122 are consistent with its membership of the genus *Pirellula* and the species *Pirellula staleyi*.

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References

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

Fuerst, J. A., Gwilliam, H. G., Lindsay, M., Lichanska, A., Belcher, C., Vickers, J. E. & Hugenholtz, P. (1997). Isolation and molecular identification of planctomycete bacteria from postlarvae of the giant tiger prawn, *Penaeus monodon*. *Appl Environ Microbiol* **63**, 254–262.

Hedlund, B. P., Gosink, J. J. & Staley, J. T. (1997). Verrucomicrobia div. nov., a new division of the Bacteria containing three new species of *Prosthecobacter*. *Antonie van Leeuwenhoek* **72**, 29–38.

Lindsay, M. R., Webb, R. I. & Fuerst, J. A. (1997). Pirellulosomes: a new type of membrane-bounded cell compartment in planctomycete bacteria of the genus *Pirellula*. *Microbiology* **143**, 739–748.

Lindsay, M. R., Webb, R. I., Strous, M., Jetten, M. S. M., Butler, M. K., Forde, R. J. & Fuerst, J. A. (2001). Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. *Arch Microbiol* **175**, 413–429.

Metchnikoff, E. (1888). *Pasteuria ramosa*, un représentant des bactéries a division longitudinale. *Ann Inst Pasteur* **2**, 165–170.

Schlesner, H. (1986). *Pirella marina* sp. nov., a budding, peptidoglycan-less bacterium from brackish water. *Syst Appl Microbiol* **8**, 177–180.

Schlesner, H. (1987). *Verrucomicrobium spinosum* gen. nov., sp. nov.: a fimbriated prosthecate bacterium. *Syst Appl Microbiol* **10**, 54–56.

Schlesner, H. (1994). The development of media suitable for the microorganisms morphologically resembling *Planctomyces* spp., *Pirellula* spp., and other *Planctomycetales* from various aquatic habitats using dilute media. *Syst Appl Microbiol* **17**, 135–145.

Schlesner, H. & Hirsch, P. (1984). Assignment of ATCC 27377 to *Pirella* gen. nov. as *Pirella staleyi* comb. nov. *Int J Syst Bacteriol* **34**, 492–495.

Schlesner, H. & Hirsch, P. (1987). Rejection of the genus name *Pirella* for pear-shaped budding bacteria and proposal to create the genus *Pirellula* gen. nov. *Int J Syst Bacteriol* **37**, 441.

Schmidt, J. M. (1971). Prosthecate bacteria. *Annu Rev Microbiol* **25**, 93–110.

Schmidt, J. M. & Starr, M. P. (1979). Corniculate cell surface protrusions in morphotype II of the *Blastocaulis-Planctomyces* group of budding and appendaged bacteria. *Curr Microbiol* **3**, 187–190.

Schmidt, J. M. & Starr, M. P. (1982). Ultrastructural features of budding cells in a prokaryote belonging to morphotype IV of the *Blastocaulis-Planctomyces* group. *Curr Microbiol* **7**, 7–11.

Semenov, A. & Staley, J. T. (1992). Ecology of polyprosthecate bacteria. *Adv Microb Ecol* **12**, 339–382.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Staley, J. T. (1968). *Prosthecomicrobium* and *Ancalomicrobium*: new prosthecate freshwater bacteria. *J Bacteriol* **95**, 1921–1942.

Staley, J. T. (1973). Budding bacteria of the *Pasteuria-Blastobacter* group. *Can J Microbiol* **19**, 609–614.

Starr, M. P., Sayre, R. M. & Schmidt, J. M. (1983). Assignment of ATCC 27377 to *Planctomyces staleyi* sp. nov. and conservation of *Pasteuria ramosa* Metchnikoff 1888 on the basis of type descriptive material. Request for an Opinion. *Int J Syst Bacteriol* **33**, 666–671.

Strunk, O. & Ludwig, W. (1995). ARB – a software environment for sequence data. Department of Microbiology, Technical University of Munich, Munich, Germany.

Tekniepe, B. L., Schmidt, J. M. & Starr, M. P. (1981). Life cycle of a budding and appendaged bacterium belonging to morphotype IV of the *Blastocaulis-Planctomyces* group. *Curr Microbiol* **5**, 1–6.

Trüper, H. & Pfennig, N. (1992). The family Chlorobiaceae. In *The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, vol. IV, pp. 3583–3592. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. Schleifer. New York: Springer.

Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570.