International Journal of Systematic and Evolutionary Microbiology (2001), **51**, 195–202 Printed in Great Britain

NOTE

Phylogeny of the filamentous bacterium '*Nostocoida limicola*' III from activated sludge

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Five strains of the filamentous bacterium '*Nostocoida limicola*' III were successfully isolated into pure culture from samples of activated sludge biomass from five plants in Australia. 16S rRNA gene sequence analyses showed that all isolates were members of the *Planctomycetales*, most closely related to *Isosphaera pallida*, but they differed phenotypically from this species in that they did not glide and were not thermotolerant. The ultrastructure of these '*N. limicola*' III isolates was also consistent with them being *Planctomycetales*, in that they possessed complex intracellular membrane systems compartmentalizing the cells. However, the arrangements of these intracellular membranes differed between isolates. These data confirm that '*N. limicola*' III is phylogenetically unrelated to both '*N. limicola*' I and '*N. limicola*' II, activated sludge filamentous bacteria which share morphological features in common with '*N. limicola*' III and which have been presumed historically to be the same or very similar bacteria.

Keywords: '*Nostocoida limicola*' III, *Planctomycetales*, *Isosphaera*, activated sludge bulking

Although morphological descriptions of many filamentous bacteria causing the operational problems of bulking and foaming in activated sludge plants were published several years ago (Eikelboom, 1975), it is only recently that progress in resolving their systematics has been made (Kämpfer, 1997; Seviour & Blackall, 1999). Some bacteria still await taxonomic clarification, mainly because they have not yet been isolated and characterized. Until this information is available and they are better understood, it is unlikely that the problems they cause in activated sludge plants will be controlled reliably. These bacteria are generally difficult to culture on artificial media (Kämpfer, 1997; Seviour & Blackall, 1999) and, with no information on their nutritional requirements, attempts at media design must be empirical (Seviour & Blackall, 1999). Once in pure culture, it is then possible to use their 16S rRNA gene sequences to elucidate their phylogeny and design rRNA targeted probes for their *in situ* identification (Amann *et al*., 1995; Kämpfer, 1997; Seviour & Blackall, 1999), providing tools for studying their population dynamics.

'*Nostocoida limicola*' is one of these filamentous bacterial morphotypes. Originally described by van Veen (1973) as a Gram-positive bacterium with coccoid cells in chains, it was considered subsequently to represent three morphotypes '*N. limicola*' I, II and III, distinguished from each other almost exclusively on their individual cell dimensions (Eikelboom & van Buijsen, 1983). The important question of whether these three are morphological variants of a single bacterium or quite different organisms which happen to resemble each other when viewed under the microscope, has now been partially answered. Thus, several isolates of '*N. limicola*' I have been grown (J.-R. Liu, P. Burrell, E. M. Seviour, J. A. Soddell, L. L. Blackall & R. J. Seviour, unpublished). Based on their 16S rRNA gene sequence data, some are closely related to *Lactosphaera pasteurii* (Janssen *et al*., 1996), now known to be almost identical to the bulking filament *Trichococcus flocculiformis* (Stackebrandt *et al*., 1999) described previously by Scheff *et al*. (1984). Others emerge as *Streptococcus* species, closely related to

Abbreviations: FISH, fluorescence *in situ* hybridization; ICM, intracytoplasmic membrane.

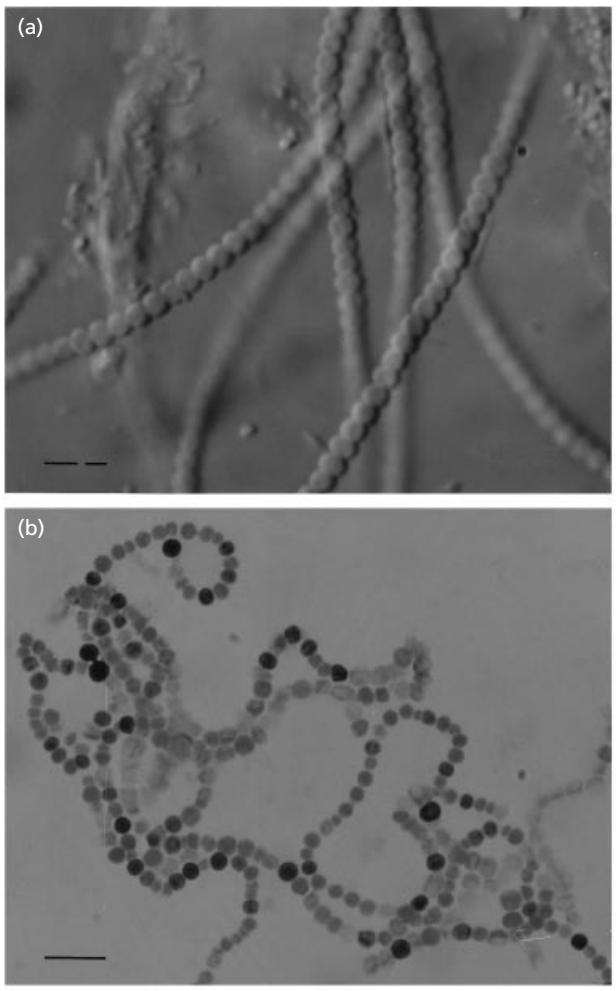

Fig. 1. Filaments of large regular cocci in chains from the Tongala activated sludge biomass sample which are characteristic of '*N. limicola*' III (a) and a pure culture of strain Ben 220 from Tongala showing uneven Gram stain reaction (b). Nomarski optics were used in (a). Bar, 1 μm.

Streptococcus suis (Chatellier *et al*., 1998). Both are in the low-G+C-containing Gram-positive bacteria. On the other hand, pure cultures of '*N. limicola*' II so far available represent novel taxa in the *Actinobacteria* (Blackall *et al*., 2000) and hence are phylogenetically unrelated to '*N. limicola*' I.

The phylogenetic position of '*N. limicola*' III has not yet been reported and neither has its successful isolation and culture from activated sludge. This paper describes a protocol for growing '*N. limicola*' III from Australian activated sludge plants in pure culture, and the 16S rRNA gene sequences of five isolates and their phylogenetic positions. Results show that the strains are phylogenetically quite different from both '*N. limicola*' I and II, being members of the *Planctomycetales*.

Samples of biomass were collected from activated sludge plants and returned to the laboratory immediately for isolation, or kept at 4 °C and processed within 48 h of collection. This delay did not appear to affect the recovery success rate with samples (e.g. from Tongala, see below) where it was possible to investigate its possible influence. Micromanipulation was used to isolate '*N. limicola*' III from biomass samples (Bradford *et al*., 1996) where it appears either as large (ca. 2 μm diameter) spherical cocci in chains (Fig. 1a) or as irregular, discoid, flattened cells (Eikelboom & van Buijsen, 1983; Jenkins *et al*., 1993). It proved to be an extremely difficult organism to grow. Eventually, a medium was developed which supported its growth. This was freshly prepared R2A agar (Reasoner & Geldreich, 1985), made up in filtered effluent water from the secondary clarifier of the local activated sludge plant and either autoclaved or membrane-filtered before use (referred to as SR2A agar). Other effluents from secondary clarifiers in other plants have also been used with equal success. Attempts using biomass containing an enriched culture of '*N. limicola*' III from a plant in Tongala, Victoria, Australia (Fig. 1) treating dairy waste led to an initial success in isolating one strain (Ben 220). The SR2A media tried included glucose, lactose (selected because of the type of waste treated) and L-arabinose, all at 0·05 % (w/v), as carbon sources. However, only L-arabinose supported good growth. Consequently, this medium, ASR2A (L-arabinose supernatant R2A medium), containing 0·05 % (w/v) L-arabinose was used for isolation of all the '*N. limicola*' III studied here. The reasons for its success are not known, but this medium may provide the basis for an isolation protocol for other filamentous bacteria from activated sludge systems, which may produce a much higher success rate than those currently available (Seviour & Blackall, 1999).

A total of five isolates from different plants in eastern Australia was obtained. The source of these isolates, their dates of isolation and the type of plant from which each came are listed in Table 1. Colonies of all five isolates, staining Gram-variable (e.g. Fig. 1b) and

Table 1. Site and date of isolation of '*Nostocoida limicola*' III strains and their accession numbers

Isolate	Site of isolation	Date of isolation	GenBank no.
Ben 200	Tongala (dairy waste), Victoria, Australia	7/1997	AF244748
Ben 222	Bendigo (domestic), Victoria, Australia	11/1997	AF244749
Ben 223	Hunter Valley (domestic), New South Wales, Australia	2/1998	AF244750
Ben 224	Gibson Island (domestic), Queensland, Australia	8/1998	AF244751
Ben 225	Nambour (dairy waste), Queensland, Australia	10/1998	AF244752

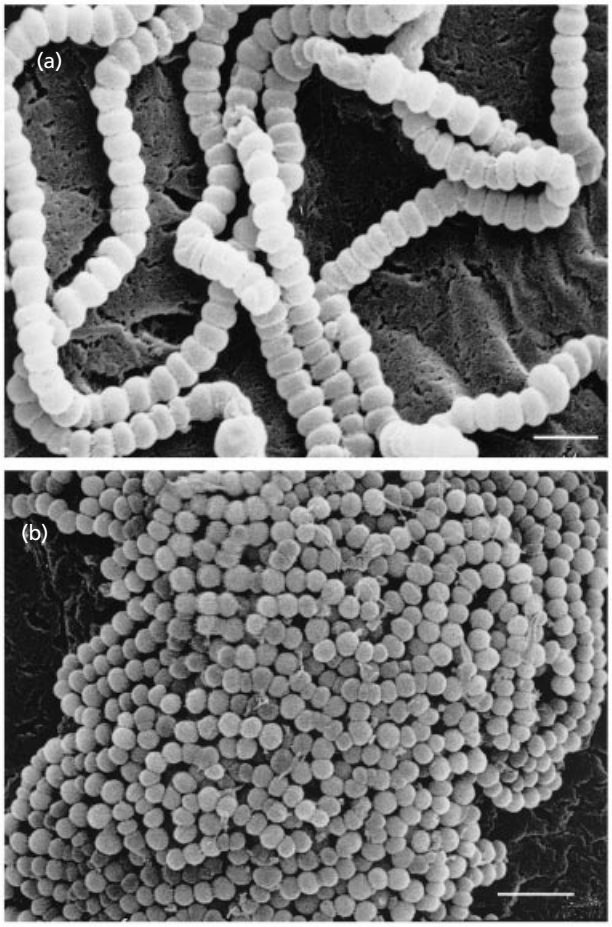

Fig. 2. SEM of pure cultures of Ben 220 (a) and Ben 222 (b) showing filaments with similar morphologies to those seen in activated sludge samples. Cells of Ben 220 often have a more discoid appearance than those of Ben 222. Bars: (a) 2 μm; (b) 5 μm.

unevenly for polyphosphate using methylene blue (data not shown), took several weeks to appear on ASR2A agar and all were pink. However, Ben 220 produced distinctive black colonies and a black diffusible pigment after 3–4 months incubation. Morphologies of all the pure cultures were very similar to those seen in activated sludge biomass (e.g. compare Fig. 1a with Fig. 2a, b), each producing the characteristically large (ca. 2 μm diameter) regular cocci in chains. Subtle differences in cell shape were apparent when pure cultures were examined by SEM (Seviour *et al.*, 1984) as shown in Fig. 2(a, b), and the flattened discoid cells seen in some biomass samples were apparent in some cultures (Fig. 2a). All isolates grew best at 28 °C and none grew above 40 °C on ASR2A agar.

Considerable difficulty was encountered in amplifying the 16S rRNA genes by PCR with all these isolates and, of many protocols used, only the one described here worked reliably. DNA from cultures grown on ASR2A medium was extracted using the method of Bond *et al.* (1995) except that lysozyme was added at 4 mg l^{-1}, followed by five cycles of freeze (liquid N_2 for 3 min)/thawing (heating at 65 °C). Proteinase K (0·4 %, w/v), RNase (0·2 mg l^{-1}) and SDS (0·5 %, w/v) were then added. Extracted DNA was resuspended in water and stored at −20 °C; PCR was performed using the primers 27f and 1525r (Liesack & Stackebrandt, 1992). The reaction mix contained 2·5 U *Taq* (AmpliTaq Gold; Applied Biosystems), 10 × PCR buffer (Applied Biosystems), 200 μm of each of the dNTPs (Roche Biochemicals) and 5 % DMSO in a total volume of 50 ìl. PCR was performed with a GeneAmp 2400 system (Applied Biosystems), with preactivation at 94 °C for 10 min, followed by 30 cycles at the same temperature for 30 s, 1 min annealing at 50 °C and 2 min extension at 72 °C, followed by a final 15 min extension at 72 °C. The PCR products were purified using QIAquick columns (Qiagen), cloned using the pGEM-T easy kit (Promega), and plasmids were recovered with the QIAprep kit (Qiagen). The presence of inserts was determined using restriction analysis with *Eco*RI (Roche Biochemicals). Inserts of the appropriate size were then sequenced with the ABI Prism Big Dye system (Applied Biosystems) on an Applied Biosystems 373A sequencer. Almost complete sequences were obtained from all five isolates and these sequences were analysed using the ARB software (O. Strunk and others; http://www.micro.biologie.tu-muenchen.de). Trees were constructed with FASTDNAML and compared with those obtained with neighbour-joining (Jukes & Cantor, 1969) and maximum-parsimony. Bootstrap resampling (× 100) was carried out.

Analysis of the near-complete sequences of the 16S rRNA gene from Ben 220 (1470 bp), Ben 222 (1467 bp), Ben 223 (1470 bp), Ben 224 (1463 bp) and Ben 225 (1467 bp) revealed that they were all members of the *Planctomycetales*, most closely related to *Isosphaera* species (Fig. 3), a filamentous budding bacterium isolated originally (as *Isosphaera pallida*) from hot springs (Giovannoni *et al.*, 1987). Ben 222 and 225 were almost identical to each other (99·5 %) and Ben 223 and 224 were also closely related to each other (95·6 %), whereas Ben 220 grouped separately and closest (but only 89·4 % similar) to *I. pallida* X64372 (Fig. 3). A high G+C content of the 16S rRNA gene of *Isosphaera*, considered to be characteristic of this genus alone among the planctomycetes (Griepenburg *et al.*, 1999), was also noticed with the '*N. limicola*' III isolates here (i.e. all between 60 and 62 mol %). The taxonomic allocation of these to the *Planctomycetales* received additional support from fluorescence *in situ* hybridization (FISH) studies. All possessed the probe sequence of the 16S rRNA-targeted PLA 46 probe of Neef *et al.* (1998) and gave bright fluorescence with it (Fig. 4). However, although Ben 222, 223, 224 and 225 also contained the PLA 886 sequence of Neef *et al.* (1998), Ben 220 had two mismatches with it.

TEM of the '*N. limicola*' III isolates also supports the phylogenetic evidence that these bacteria are members

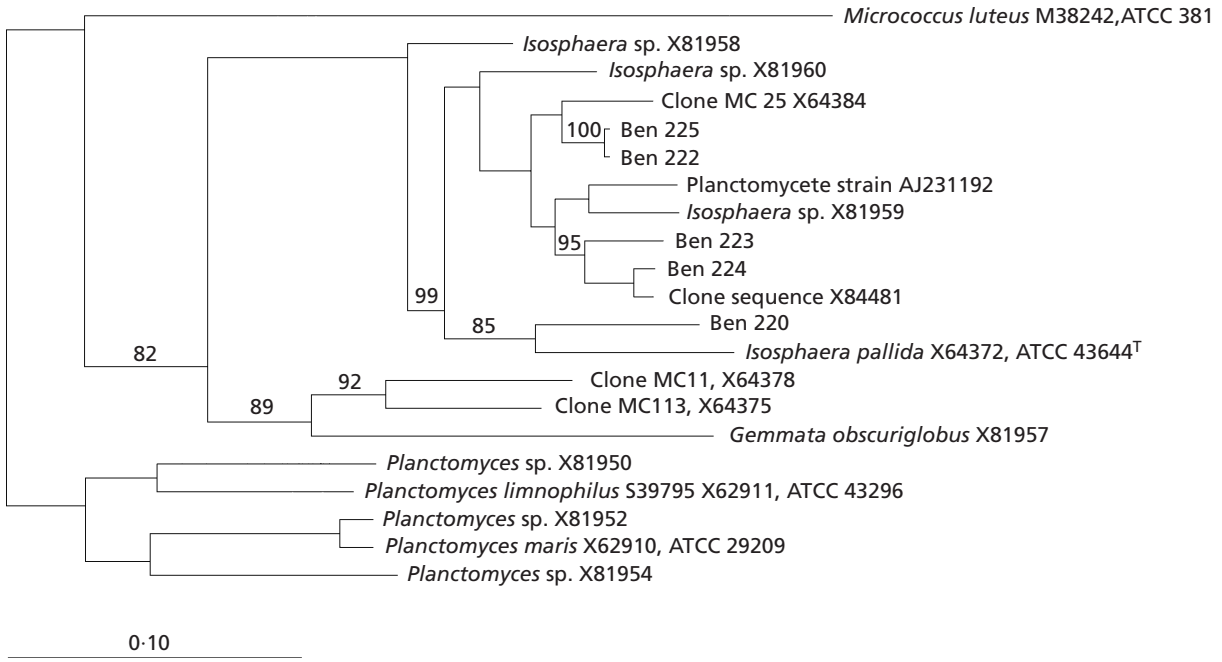

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequence data indicating the phylogenetic positions of five isolates of '*N. limicola*' III-like filaments (Ben 220, 222, 223, 224, 225). The maximum-likelihood algorithm was used and bootstrap values above 75% from 100 resamplings are given. The outgroup used was *Micrococcus luteus*.

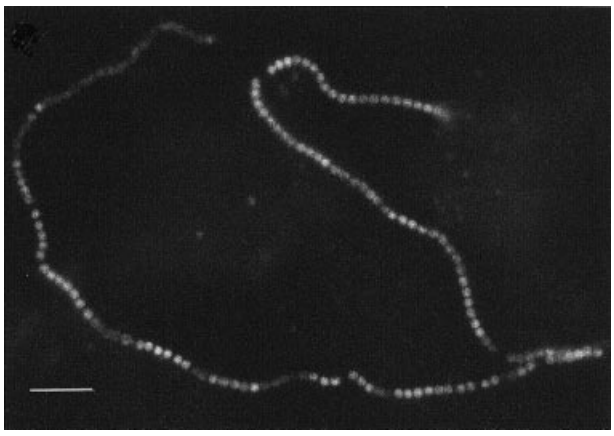

Fig. 4. FISH of Ben 220 in a biomass sample from the Tongala plant using the PLA 46 probe of Neef *et al.* (1998). Uneven fluorescence of cells in the filament can be seen, suggesting that some may be moribund. Bar, 10 μm.

of the *Planctomycetales*. Cell morphology and internal architecture varied depending on whether chemical fixation or cryosubstitution methods were used, as reported earlier for the *Planctomycetales* (Lindsay *et al.*, 1995); representative cryofixed cells with some of these distinctive ultrastructural features are presented in Figs 5, 6 and 7. For example, in some, but not all isolates, it was possible to see what appeared to be a rudimentary intercalary budding of the cocci (Fig. 5a, arrowed) noted previously in *I. pallida* (Giovannoni *et al.*, 1987). Negatively stained cells showed the seemingly uniform distribution of the distinctive crateriform structures (Giovannoni *et al.*, 1987; Staley *et al.*, 1992) on their surface (Fig. 5b, arrows). These are of a similar small size (4–5 nm diameter) to those reported for *I. pallida* (Giovannoni *et al.*, 1987). A typical trilaminar cell wall consisting of an electron-dense layer, an electron-translucent layer and an inner electron-dense layer (Fig. 5c, arrows) was also seen, with no evidence of any associated peptidoglycan material (Giovannoni *et al.*, 1987). Underlying the wall and very closely apposed to it so that it is not always visible with the TEM, is the cytoplasmic membrane, again a typical feature of the *Planctomycetales* (Lindsay *et al.*, 1997). An internal membrane, the intracytoplasmic membrane (ICM), compartmentalizes the cell to produce large membrane-bounded regions ramifying throughout the cytoplasm. These can be quite large and irregularly shaped regions (Fig. 5d; Fig. 6a, b; Fig. 7a) or in the form of tubules. Where the ICM lie close together they can appear as double membranes with a thin layer separating them (e.g. Fig. 5d, arrowed; Fig. 6a, b, arrowed; Fig. 7c). In Ben 225 only, large regions and distinctive tubules with a constant diameter of 70–80 nm were present. These tubules sometimes appeared to form parallel arrays within the cytoplasm (Fig. 7b, c, d), a feature not described previously for any other planctomycete. A double-membrane-bounded DNA-containing nuclear body, as seen in *Gemmata* (Fuerst & Webb, 1991), was

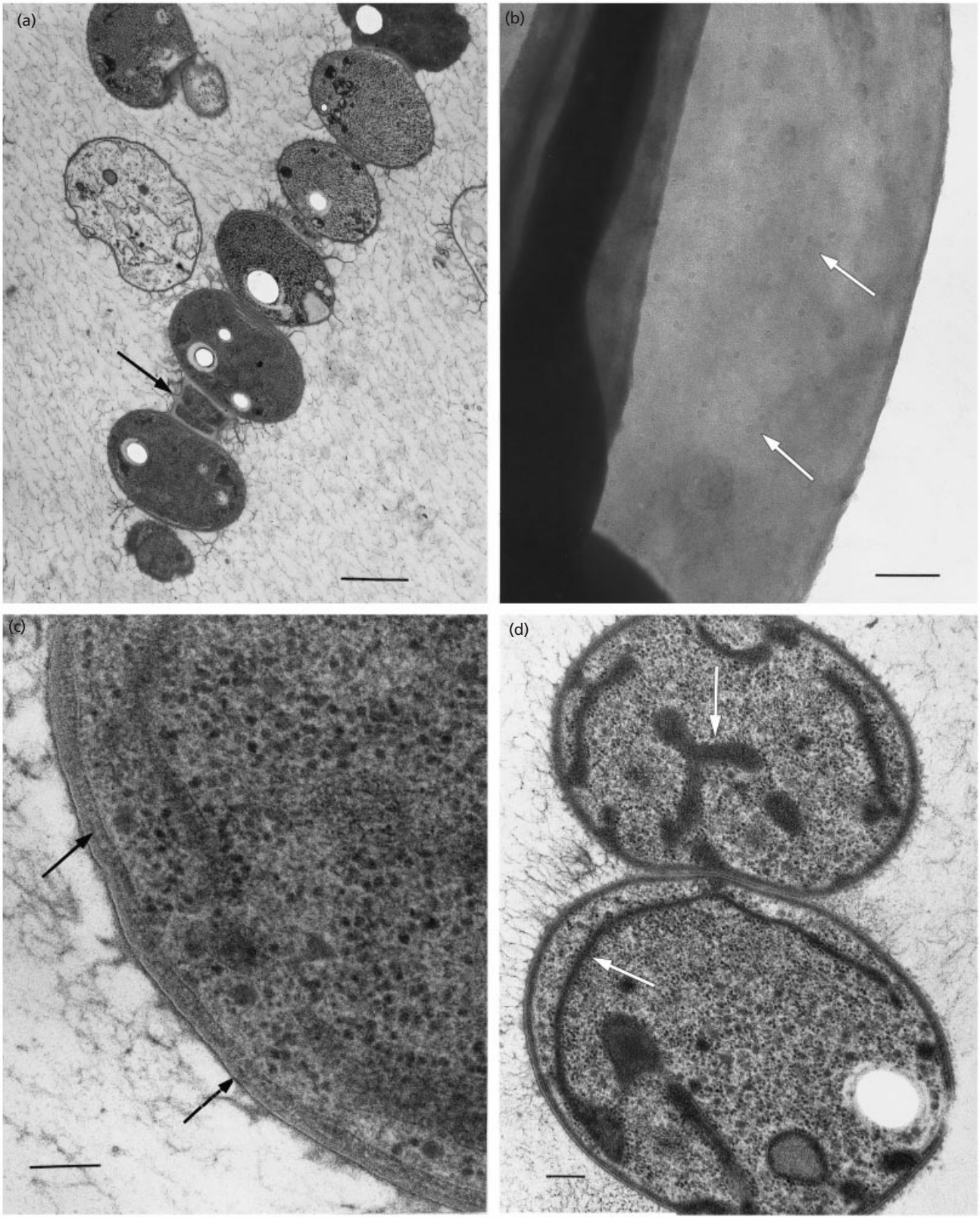

Fig. 5. TEM of cryosubstituted pure cultures of '*N. limicola*' III showing: (a) intercalary buds along a chain of cocci of Ben 220 (bar, 1 μm); (b) negatively stained cells of Ben 223 showing crateriform structures (arrowed) on the cell surface (bar, 100 nm); (c) the trilaminar wall structure of Ben 220 (arrowed) (bar, 100 nm); and (d) the ICM (arrows) compartmentalizing the cell of Ben 220 (bar, 200 nm).

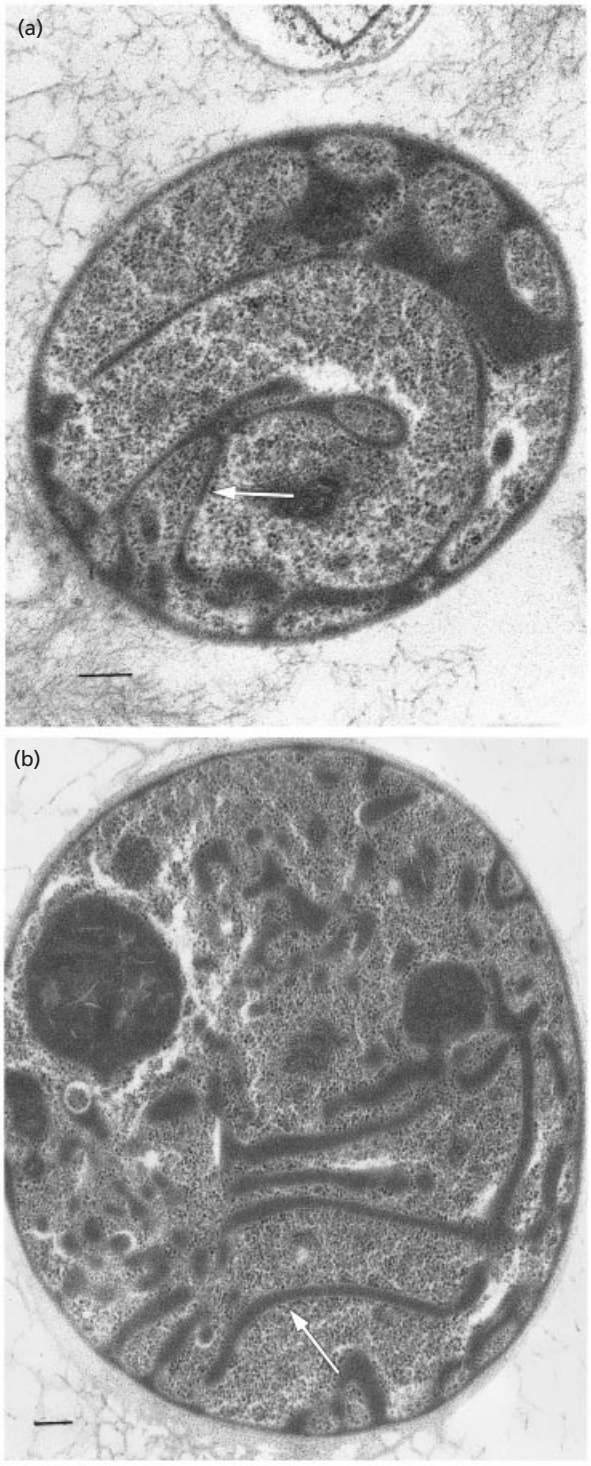

Fig. 6. Ultrastructure of Ben 222 (a) and Ben 223 (b) showing the compartmentalization of the cells. Bars, 200 nm.

not detected in any of these '*N. limicola*' III strains. The nucleoid, typically condensed in *Planctomycetales* (Fuerst, 1995), is shown in Figs 6(a) and 7(c), and multiple nucleoids, possibly from a single folded nucleoid being sectioned more than once, can be seen in Fig. 7(a, arrowed). Ribosomes are frequently visible aligned against the ICM [Fig. 6a, b (arrowed), 7c].

Because our isolates are very slow-growing, it has not been possible to obtain sufficient biomass to perform DNA–DNA hybridization or other genomic fingerprinting studies with them; it is therefore not clear whether they represent several novel species of *Isosphaera* or even new *Planctomycetales* genera. There are obvious morphological similarities between these '*N. limicola*' III isolates and the published description of *I. pallida* (Staley *et al.*, 1992), although none of the isolates obtained here could grow on the 31PY or 1aPY media of Schlesner (1994) used by Griepenburg *et al.* (1999) to culture their isolates of *I. pallida*. Furthermore, none of the isolates grew above 40 °C and none appeared to glide and exhibit phototactic behaviour, determined using the methods described by Giovannoni *et al.* (1987), although most seemed to grow best at low concentrations ($< 0{\cdot}1\,\%$) of L-arabinose in SR2A agar (J.-R. Liu, unpublished). Thus, the 16S rRNA gene sequence data and the limited phenotypic characterization, including the ultrastructural information (e.g. Ben 225), might support the view that some of them (e.g. Ben 220) are previously undescribed *Isosphaera* strains.

A level of diversity greater than previously suspected among members of this genus may be implied from 16S rRNA gene sequence analyses of clones in the culture-independent studies of Ward *et al.* (1995) and several of the novel *Planctomycetales* isolates obtained and characterized by Griepenburg *et al.* (1999). Some of our '*N. limicola*' III isolates showed close sequence similarities to previously described 16S rRNA gene sequences. Thus, Ben 222 and Ben 225 clustered closely with the MC25 clone of Liesack & Stackebrandt, (1992) from soil, and Ben 223 and Ben 224 were very similar to the 16S rRNA sequence (accession no. X84481) obtained by Bond *et al.* (1995) from activated sludge. Clone library work suggests that *Planctomycetales* are prominent populations in activated sludge communities (e.g. Bond *et al.*, 1995; Kämpfer *et al.*, 1996; Ward *et al.*, 1995). Neef *et al.* (1998) could detect large cocci up to 2·5 μm in diameter, (i.e. cells of a size and shape which are distinctive for '*N. limicola*' III) with their *Planctomycetales* probes in most of the biomass samples they examined with FISH. Although none of these cocci appeared in chains, the possibility remains that these may have been non-filamentous forms of '*N. limicola*' III and, if so, might explain why this organism is not 'identified' by microscopic examination more often in surveys of activated sludge plants (Seviour & Blackall, 1999). FISH probes designed more specifically for '*N. limicola*' III are needed to address this question.

Lastly, but equally important, is that the phylogenetic data obtained in this study finally allows the taxonomic relationships between '*N. limicola*' I, II and III to be

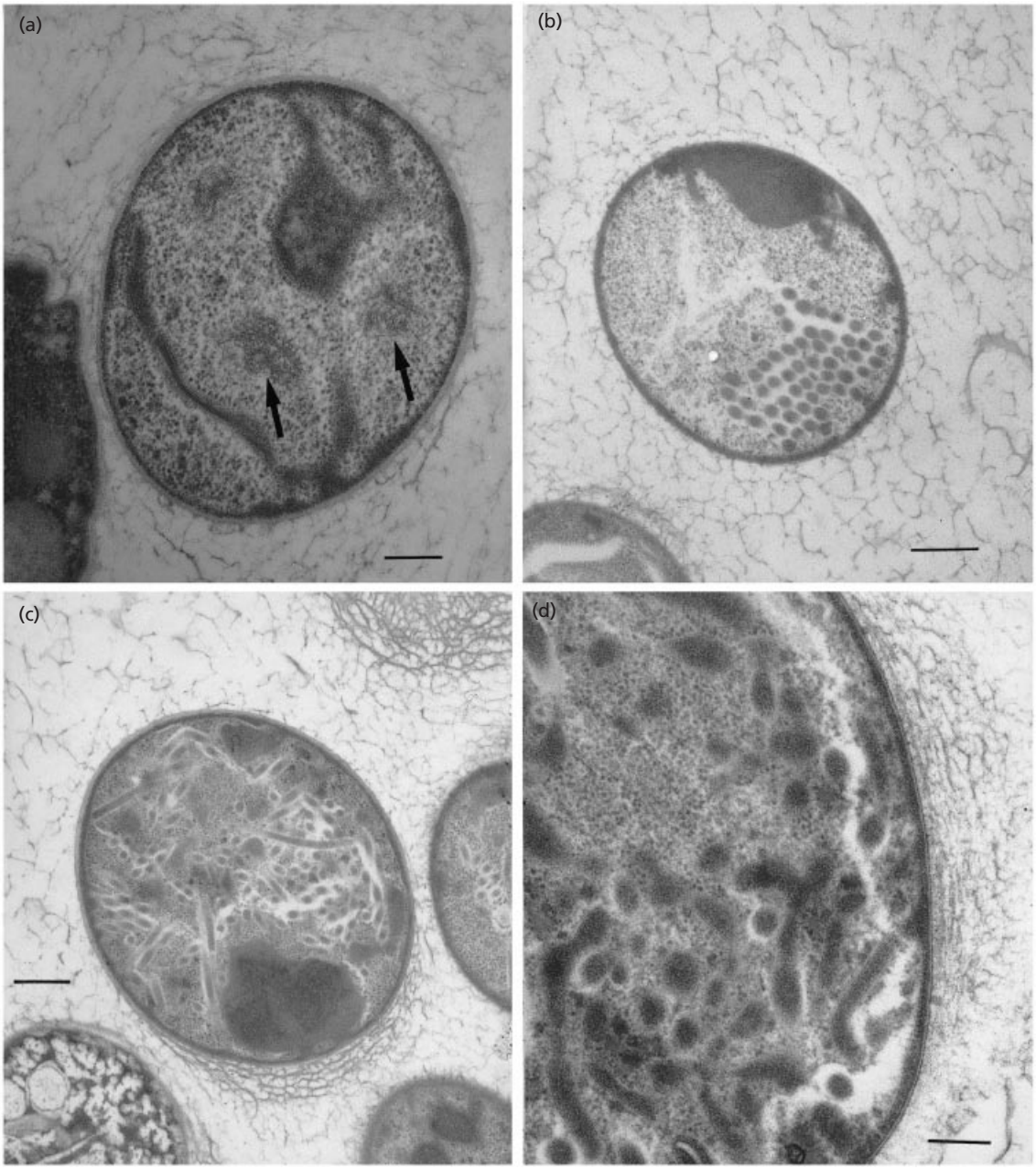

Fig. 7. TEM of cryosubstituted cells of Ben 224 showing compartmentalization and multiple nucleoids (a) and unusual distinctive regular tubules in Ben 225 (b, c, d). Bars: (a), 1 μm; (b), 200 nm; (c), 200 nm; (d), 100 nm.

clarified. These three morphotypes are not morphological variants of a single bacterium, but represent three distinct lineages, the low-G+C-containing Gram-positive bacteria (J.-R. Liu, P. Burrell, E. M. Seviour, J. A. Soddell, L. L. Blackall & R. J. Seviour, unpublished), the high-G+C-containing Gram-positive bacteria (Blackall *et al.*, 2000) and the *Planctomycetales*, respectively. The sequence data available now for these three morphotypes will allow 16S rRNA-targeted probes to be designed against all three and, in combination with microautoradiography (Lee *et al.*, 1999), their behaviour to be compared *in situ* for the first time.

Acknowledgements

We wish to thank the plant operators, especially Mr Chris Andrews, Nestlé, Tongala, Victoria, who supplied activated sludge biomass samples for this study. This study was supported by an ARC Small Research Grant to R.J.S. and an ARC SPIRT Grant with SA Water to R.J.S. and C.P.S. to support C.A.M.

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