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The Filamentous Bacterial Morphotype *'Nostocoida limicola'* I Contains at least Two Previously Described Genera in the Low G+C Gram Positive Bacteria

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Summary

Isolates of eight bacterial filaments fitting the published morphological description of *'Nostocoida limicola'* I were obtained from the mixed liquor of four different Australian and one Czech Republic activated sludge plants by micromanipulation. On the basis of their near complete (Ben 200 and Ben 201), or partial (Ben 77, Ben 78, Ben 202, Ben 203, Ben 204 and Ben 205) 16S rRNA gene sequences, six of these isolates were 99.3–100% similar to *Lactosphaera pasteurii* and *Trichococcus flocculiformis,* a bulking filament only reported previously in Germany. The other two (Ben 203 and Ben 204) were 99.9% similar to *Streptococcus suis.* Hence, all are in the low mol % G+C Gram-positive bacteria division of the *Bacteria.* On this evidence *'N. limicola'* I is phylogenetically unrelated to *'Nostocoida limicola'* II, which is now known to be in the Actinobacteria, even though these two filamentous bacteria appearing in activated sludge systems have been considered to be closely related to each other historically.

Key words: *Nostocoida limicola* – activated sludge – *Trichococcus flocculiformis* – *Lactosphaera pasteurii* – *Streptococcus* – low mol% G+C Gram-positive bacteria

Introduction

Most activated sludge plants suffer sporadically from the operational disorders of bulking and foaming, both of which are caused by an assortment of often poorly understood filamentous bacteria (SEVIOUR and BLACKALL, 1999). Many of these filamentous bacteria have not been grown in axenic culture and so have never been characterized or given taxonomically valid names. Among these is the morphotype *'Nostocoida limicola'* which is seen with increasing frequency in bulking and foaming activated sludge plants around the world (eg WANNER et al., 1998). Three morphological variants of this organism have been recognised (EIKELBOOM & VAN BUIJSEN, 1983; JENKINS et al., 1993), although their descriptions in the literature often differ with source and from the original description given by VAN VEEN (1973). Many reports (eg SEVIOUR et al., 1994; WANNER et al., 1998) on their occurrence in activated sludge samples fail to differentiate between these morphotypes. Although all three consist of spherical/discoid cells in chains (SEVIOUR and BLACKALL, 1999), they are distinguished from each other primarily on the basis of their individual cell dimensions and the general appearance of the filaments (JENKINS et al., 1993). All stain Neisser positively, indicating the presence of intracellular polyphosphate granules, but the Gram stain reaction is variable.

Recently several strains of *'Nostocoida limicola'* II were isolated and described from activated sludge plants in Australia and Italy (BLACKALL et al., 2000), and on the basis of their 16S rDNA sequences, all formed a distinct cluster in the high mol% G+C gram-positive bacteria or Actinobacteria. *'Nostocoida limicola'* III has also been grown from Australian biomass samples (LIU et al., in the press), and emerged after 16S rRNA gene sequencing as a planctomycete, related to *Isosphaera pallida,* but clearly

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

Isolate	Site of Isolation	Date of Isolation	GenBank Accession Number
Ben77	Prague, Czech Republic	Jan. 98	AF244375
Ben78	Prague, Czech Republic	Jan. 98	AF244370
Ben200	Hobart, Tasmania	Mar. 97	AF244371
Ben201	Gibson Island, Queensland	Jul. 97	AF244372
Ben202	Ballarat, Victoria	Jul. 97	AF244373
Ben203	Bendigo, Victoria	May. 97	AF244376
Ben204	Bendigo, Victoria	May. 97	AF244377
Ben205	Bendigo, Victoria	Jun. 97	AF244374

not to *N. limicola*' II. '*Nostocoida limicola*' I has never been reported to have been grown in axenic culture previously, and so its relationship to the other two '*N. limicola*' morphotypes is currently unknown. Such information is considered important, since as well as being of academic interest, it has practical implications. Strategies for controlling the problems caused by '*N. limicola*' in activated sludge are likely to be of limited value if the collection of organisms currently "identified" as this morphotype, an identification based solely on their recognisable microscopic features, are different bacteria. This paper describes the characterization of eight isolates of bacteria all "identified" unequivocally on their morphological and staining properties following literature descriptions (VAN VEEN, 1973; JENKINS et al., 1993) as '*N. limicola*' I in biomass samples from activated sludge plants in eastern Australia and the Czech Republic. These were isolated by micromanipulation and grown in axenic culture. It reveals that *N. limicola*' I, '*N. limicola*' II and '*N. limicola*' III are phylogenetically quite different to each other, and importantly, that '*N. limicola*' I contains representatives from at least two bacterial genera. Some are very closely related to *Lactosphaera pasteurii* (JANSSEN et al., 1995) and *Trichococcus flocculiformis*, a bulking filament only reported previously in plants in Germany (SCHEFF et al., 1984), while others are members of the genus *Streptococcus*, most probably *S. suis*.

Materials and Methods

Isolation of 'N. limicola' I

'*N. limicola*' I was positively identified in biomass samples on the basis of its distinctive microscopic appearance (SEVIOUR and BLACKALL, 1999). The individual gram-positively staining filaments consisted of small (< 1.0 µm) coccoid cells (Figure 1). These filaments were then micromanipulated (SKERMAN, 1968; BRADFORD et al., 1996) onto a wide range of media. These included R2A (REASONER and GELDREICH, 1985), SCY and HA (EIKELBOOM, 1975), GS (WILLIAMS and UNZ, 1985), and Trypticase Soy Agar, (TSA, Oxoid, Melbourne) containing 0.1% (w/v) of a range of carbon sources. Of those evaluated, the medium which supported best growth of all the isolates described here was freshly prepared (not the Oxoid) R2A agar, although all grew less well on the other media. In some cases, a medium with the same composition as R2A medium, but prepared using filtered final supernatant from the secondary clarifier at the Bendigo wastewater treatment plant instead of tap water, and consequently referred to as SR2A medium, was used (LIU et al., in the press). After incubation of the plates at 25 °C, and daily checks for possible contamination by unwanted closely associated faster growing bacteria (BRADFORD et al., 1996; SEVIOUR et al., 1997), isolates were obtained eventually in axenic culture after repeated streaking onto fresh plates of R2A agar. Visible colonies were apparent after 48–72 h incubation, making this morphotype a relatively easy to isolate and rapidly growing member of the filamentous bacteria seen in activated sludge. Cultures were maintained at –80 °C in 20% glycerol. The sources of these strains are listed in Table 1.

Phenotypic and Phylogenetic Characterisation of 'N. limicola' I

SEM (SEVIOUR et al., 1984) was used for determining cell/filament morphology. Sequence alignments and phylogenetic analyses were performed using methods available in the ARB software package of STRUNK et al., (1998). Trees were constructed using Fast DNAml. Tree topologies were compared with those obtained using neighbour joining (evolutionary matrices prepared using the JUKES and CANTOR (1969) parameter model) and maximum parsimony, and very similar groupings were obtained with all three trees presented in Figures 3 and 4.

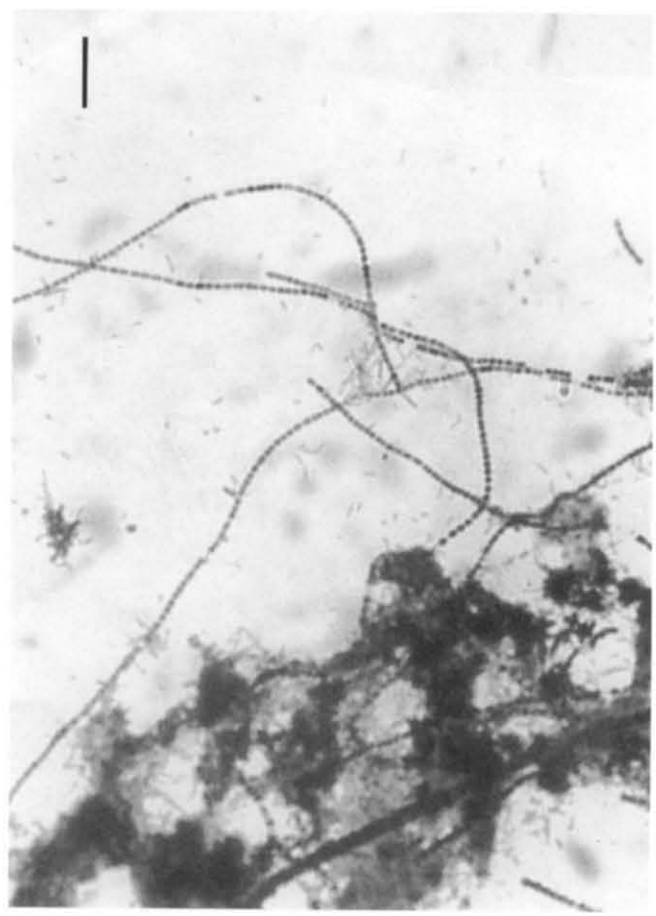

Fig. 1. Appearance of '*Nostocoida limicola*' I as chains of cocci in an activated sludge biomass sample. Scale ≙ 5 µm.

For strains Ben 203 and 204, the trees were not identical with the three methods used. Although Ben 203 and 204 always grouped in the same way, these were not supported by high bootstrap values after bootstrap resampling (100x) was carried out. The sequences of the 16S rDNA of the *'N. limicola"* I strains have been deposited in GENBANK with the accession numbers listed in Table 1.

Results

Isolation and description of strains

'N. limicola' I is not seen in many of the samples of biomass examined microscopically in our laboratories, but was readily isolated by micromanipulation from

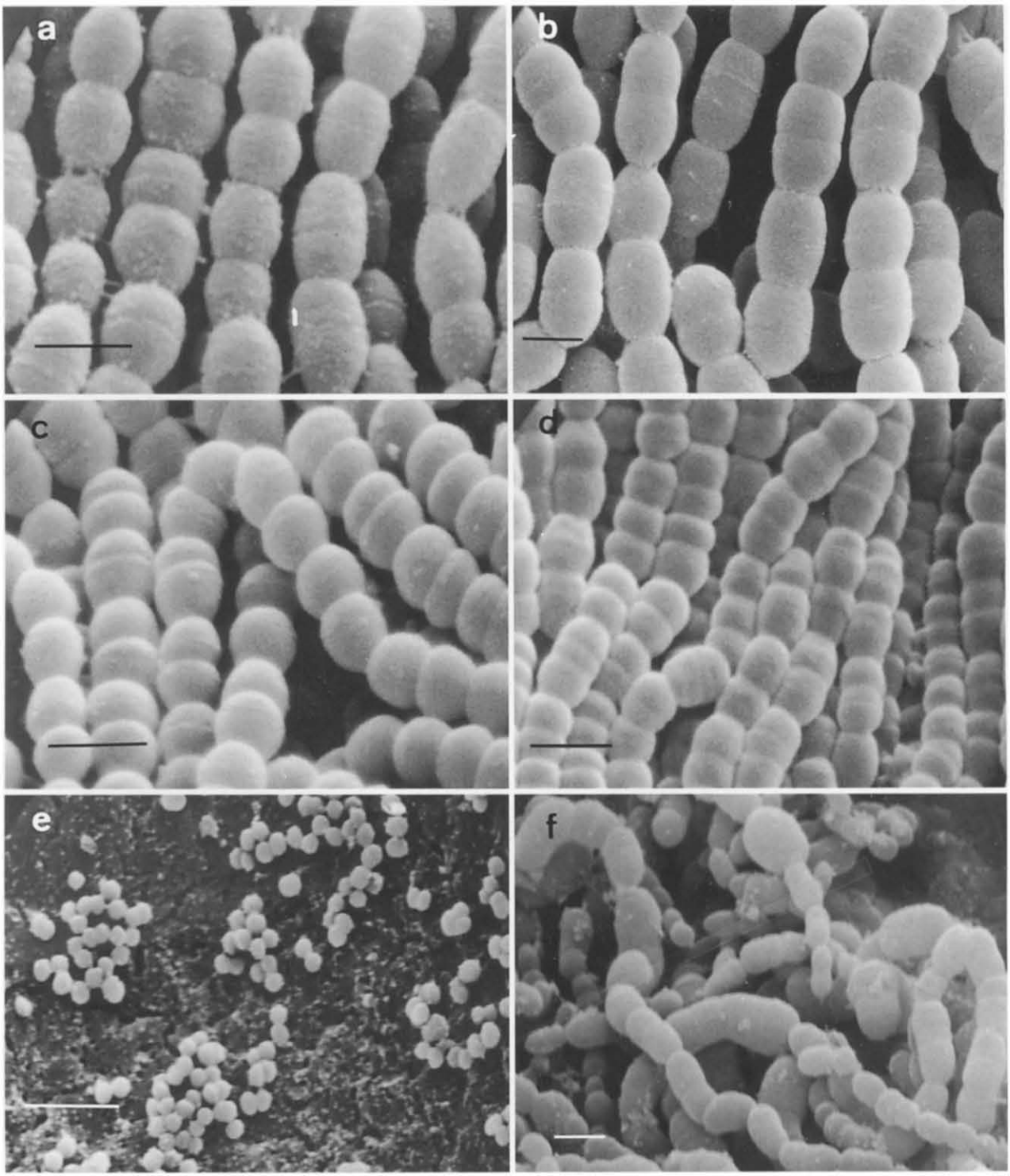

Fig. 2. SEM of pure cultures of Ben 200 (Fig 2a), Ben 202 (Fig 2b), Ben 205 (Fig 2c) and Ben 204 (Fig 2d), all grown on SR2A agar (see text for composition). Bar marker is 1μm in all cases. SEM of Ben 200 grown in R2A broth with (Fig 2e) and without (Fig 2f) shaking. Scale ≙ 2 μm and 4 μm respectively.

those which did contain it. Eight strains were obtained, six from Australian plants and two from the Czech Republic (Table 1). All stained Gram-positively, and in axenic culture their morphology on the SR2A agar was consistent with that for '*N. limicola*' I, with regular cocci in chains (Figures 2a–d). However, in some isolates (eg Ben 77, 78 and 200), cell arrangement and shape varied considerably depending on the culture conditions and medium used. Often these isolates appeared quite differently to what might be expected for '*N. limicola*' I, growing as single or paired cocci in R2A shaken broth (Figure 2e), or for example, on R2A agar as pleiomorphic swollen irregular cells (Figure 2f). No such culture dependent morphological variations were seen with strains Ben 203 and Ben 204, which always grew as long chains of regular cocci under all the conditions used here (Figure 2d). This remarkable morphological variation will be the focus of another publication. The individual cell shapes of these isolates grown on R2A agar were recognisably different, varying from spherical (eg Ben 200) to ovoid cells, as in Ben 203 (Figure 2a, c).

Phylogenetic characterisation of isolates

Almost complete sequences of 1388 and 1422 nucleotide base pairs were obtained for the 16S rDNA of Ben 200 and Ben 201. Only partial sequences were acquired for Ben 77 (691 bp, *E.coli* 574–1265), Ben 78 (692bp, *E.coli* 574–1271), Ben 202 (504 bp, *E.coli* 564–1067) and Ben 205 (441 bp, *E.coli* 604–1044), be-

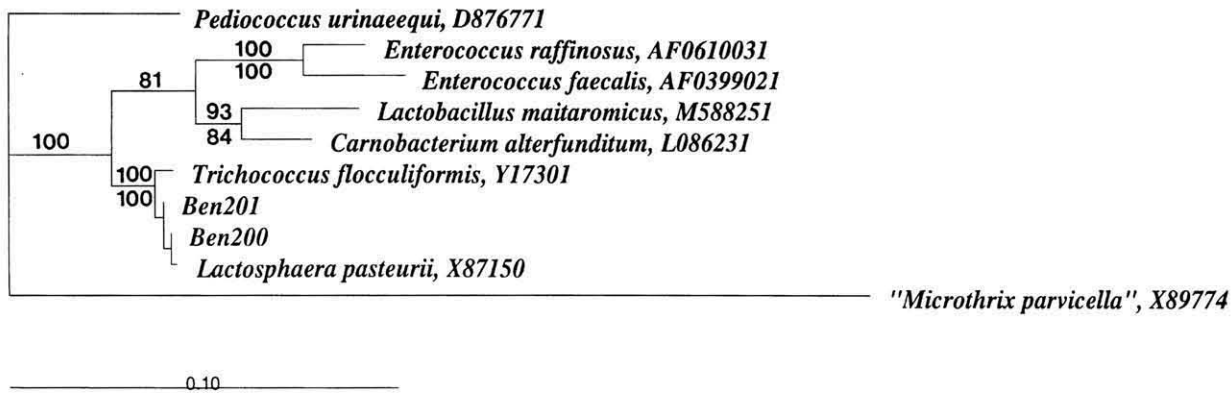

Fig 3. treeN1+3 (5676×2720×2 psd). Dendrogram constructed near complete 16S rRNA gene sequences of strains Ben 200 and 201, showing their phylogenetic position in the Low G+C Gram positive bacteria, using DNAml. Bootstrap values expressed as % of 100 samplings of 70% and above for parsimony and neighbour joining are given above and below nodes respectively. Scale bar (10%) is % of nucleotide substitutions.

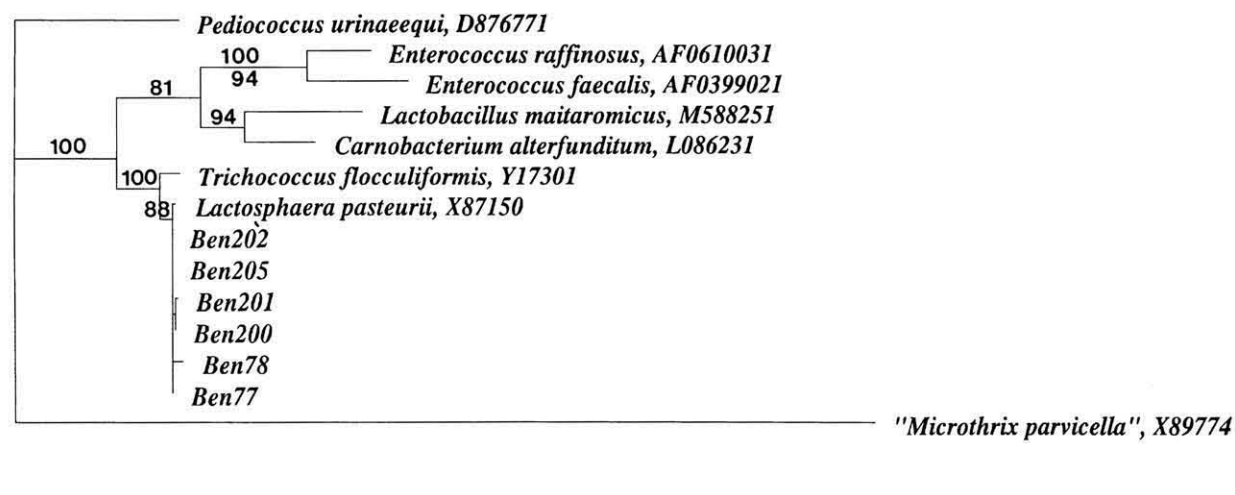

Fig 4. treeN1–2 (5676×2544×2 psd). Dendrogram constructed with DNAml using partial 16S rRNA gene sequences of strains Ben 77, 78, 200, 201, 202 and 205 and the same procedures detailed in Fig 3.

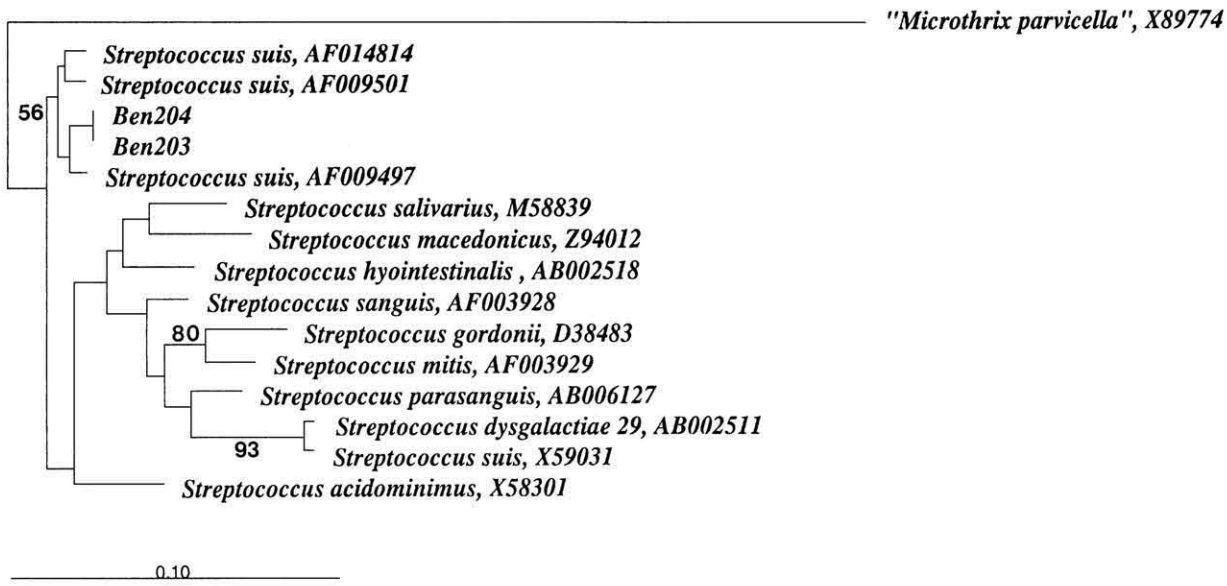

Fig 5. treeN1-1 (5675×2128×2 psd). Dendrogram constructed with DNAml using partial 16S rRNA gene sequences of strains Ben 203 and 204, and the same procedures detailed in Fig 3, except lower bootstrap values are given.

cause these sequences proved to be highly similar (99.6%–99.9%) to those of the corresponding regions of the 16S rDNA from Ben 200 and Ben 201. Phylogenetic analysis of the 1388 bp sequences of Ben 200 and Ben 201 (Figure 3) using the ARB (STRUNK et al., 1998) software package revealed them to be almost identical to each other, and to the previously described bacteria *Lactosphaera pasteurii* (JANSSEN et al., 1996) and *Trichococcus flocculiformis* (SCHEFF et al. 1984; STACKEBRANDT et al., 1999) ie 99.9% in all cases. These relationships were supported by high bootstrap values.

Isolates Ben 203 (436 bp, *E. coli* 452–888) and Ben 204 (542 bp, *E. coli* 438–978) were also only partially sequenced. Phylogenetic analyses of their 16S rDNA sequences (436 nucleotides) revealed they were also members of the low mol% G+C gram-positive bacteria and almost identical to each other (99.9 %). However, both were different to Ben 77, 78, 200, 201, 202 and 205. Instead their closest relatives were *Streptococcus suis* (CHATELLIER et al., 1998; RASMUSSEN and ANDRESEN, 1998) and *Streptococcus sanguis* (KAWAMURA et al., 1995), (ie >99% and 97% respectively), as shown in Figure 4.

Discussion

Descriptions of the microscopic appearance of '*Nostocoida limicola*' vary considerably in the literature, but those of VAN VEEN (1973) and JENKINS et al., (1993) were used here to 'identify' this filamentous morphotype in activated sludge samples for isolation. The data presented here resolve the phylogeny of six Australian and two Czech Republic isolates of '*N. limicola*' I, the filamentous bacterium responsible for episodes of bulking around the world. This organism initially described and invalidly named by EIKELBOOM and VAN BUIJSEN, (1983) on the basis of its microscopic appearance, staining reactions and superficial similarity to the morphotypes '*N. limicola*' II and III, is a member of the low mol% G+C Gram-positive bacteria. The 16S rDNA sequences demonstrate that isolates of this morphotype from several different plants and parts of the world, and indistinguishable from each other when viewed under the microscope, contain members of more than a single known genus. The same situation has been reported for the filamentous activated sludge morphotype, Eikelboom Type 1863, which describes at least three distinct bacterial genera (SEVIOUR et al., 1997). Even though some activated sludge filamentous bacterial morphotypes appear to consist of a single bacterium eg "*Microthrix parvicella*" (ERHARDT et al., 1997), the results presented here emphasise the risks involved in relying solely on morphological characters to "identify" these organisms (SEVIOUR and BLACKALL, 1999). More isolates of '*N. limicola*' I from other countries are required to determine any biogeographical differences which might exist among them, but experiences with other filamentous bacteria (ERHARDT et al., 1997; HOWARTH et al., 1999) and the findings with the Czech Republic isolates, Ben 77 and Ben 78, would suggest that they are likely to differ little in other parts of the world to those described here.

These results also confirm that '*N. limicola*' I , '*N.limicola*' II and '*N. limicola*' III are phylogenetically unrelated bacteria, the latter two now known to be members of the Actinobacteria and the Planctomycetales respectively

(BLACKALL et al., 2000; LIU et al., in the press). Thus, the generic term of '*Nostocoida limicola*' commonly used by microbiologists when surveying the filamentous bacterial populations of activated sludge in attempts to reach "cause and effect" relationships to control bulking and foaming problems (eg JENKINS et al., 1993; WANNER 1994) is clearly misleading. Results presented here demonstrate that several different bacteria, probably with different physiologies and ecological properties, are in fact being described and discussed.

Equally important is that the isolates of '*N. limicola*' I described here are probably not novel organisms, and so they may now be in a position to be validly named. Thus, using 16S rRNA sequence comparisons, Ben 77, 78, 200, 201, 202 and 205 all cluster very closely with *Lactosphaera pasteurii*, an aerotolerant coccus which has never been reported to grow in chains, which was isolated from an anoxic digester sludge (JANSSEN et al., 1995). Subsequently STACKEBRANDT et al. (1999) showed that *L. pasteurii* is itself >99% similar to the filamentous bacterium *Trichococcus flocculiformis* in terms of their 16S rRNA sequences. *T. flocculiformis* was described earlier as growing as cocci in long chains, and validly named by SCHEFF et al., (1984). Some of the Ben isolates (eg Ben 200) showed considerable morphological variation in axenic culture (Fig 2), including an ability to grow as single cells under certain conditions, and regular chain formation by them was rarely seen in this study, except when SR2A medium was used.

This finding is particularly interesting. Although *T. flocculiformis* was viewed as an important bulking organism in Germany, it has been not reported, as far as we know, in any other countries. Consequently it is not listed or described in the manuals widely used in the industry (eg JENKINS et al., 1993; WANNER, 1994; EIKELBOOM, 2000) to identify activated sludge bulking bacteria. SCHEFF et al., (1984) specifically commented that in their opinion their *T. flocculiformis* was not related to *N. limicola*' I, a decision based almost totally on a comparison of cell dimensions, and cell and filament morphologies, and one which can be criticised, especially in the light of our experiences with our Ben isolates and their morphological flexibility. Thus, from the 16S rRNA gene sequence data, *T. flocculiformis* and some '*N. limicola*' I isolates are very closely related (99.9%). Further effort is now needed to resolve their taxonomy and to properly name and speciate these "*N. limicola*" I isolates. Together with *L. pasteurii* they will almost certainly all emerge as members of the genus *Trichococcus*. This work is currently in progress, and all have very similar 16S rRNA gene sequences, DNA G+C mol% values and identical cell wall peptidoglycan types. However, DNA: DNA hybridization values suggest that while the Ben strains and *T. flocculiformis* are the same genomic species, *T. flocculiformis* and *L. pasteurii* belong to different genomic species (PETER SCHUMANN, personal communication). So these data suggest a much wider global distribution for *T. flocculiformis* than has been considered previously.

The two isolates Ben 203 and Ben 204, although morphologically indistinguishable from the others in appearing as long chains of cocci in chains, and being "identified" confidently by us as '*N. limicola*' I in activated sludge samples, emerge after 16S rDNA sequencing as members of the genus *Streptococcus*. They are most closely related to *S. suis* (Figure 3), a serious pathogen of pigs, and a zoonotic bacterium reported to cause meningitis in humans, which has had a confused taxonomic past. Only recently have molecular approaches shown it to be a well-defined species with multiple serotypes (CHATELLIER et al., 1998; RASMUSSEN and ANDRESEN, 1998). The '*N. limicola*' I isolates studied here appear closest to *S. suis* serotypes 22, 24 and 26 (CHATELLIER et al., 1998), all obtained originally from diseased pigs. Both Bendigo and Ballarat (the sites of isolation of Ben203 and 204) are important pig farming areas in Australia. The potential pathogenicity of our isolates was not examined, but it may be that all '*N. limicola*' I filaments should be handled with caution until this is clarified. Probing samples containing morphologically identified '*N. limicola*' I with the low mol% G+C Gram positive 16S rRNA targeted probe of MEIER et al., (1999) confirms them as members of this division (LIU and SEVIOUR, unpublished). It is now possible to use the 16S rDNA sequence data presented here to design more specific RNA targeted probes to determine to which group the Gram-positive cocci occurring in chains in activated sludge, all currently "identified" as '*N. limicola*' I, belong. This should also clarify whether bacteria different to those described here are also members of this morphotype, and assist eventually in determining their population dynamics and developing strategies for their control. These probes have now been designed and are currently being validated.

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