NOTE

'Candidatus Nostocoida limicola', a filamentous bacterium from activated sludge

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Five strains of '*Candidatus* Nostocoida limicola' were isolated by micromanipulation from two activated sludge plants. Two (Ben17 and Ben18) were from Sunbury, Victoria, Australia, and three (Ben67, Ver1 and Ver2) were from Verona, Italy. The near complete 16S rDNA sequences were determined for five strains and the phylogenetic location of this important bulking filament in the actinomycete subphylum is reported for the first time. Phylogenetically, the Ben strains formed one group with 99·4% 16S rDNA similarity, and the Ver strains formed another with 99·9% 16S rDNA similarity. The mean similarity between the two groups was 97·4%. By 16S rDNA comparison, the closest relative to all strains was *Terrabacter* sp. strain DPO1361 (95·0–95·5% identical). On R2A medium, all strains generally grew as short filaments or clumps of cocci, whereas on glucose sulfide (GS) medium, all grew as irregular twisting filaments comprising Gram-positive and Gram-negative cells, which is close to their *in situ* morphology. Polyphosphate was stored either as granules (R2A) or throughout the trichomes (GS). None of the strains could grow without added nitrogen, reduce nitrate to nitrogen gas or grow anaerobically, whereas all could grow at 15–30 °C, produce catalase and reduce nitrate to nitrite. All were inactive in the Hugh & Leifson test. This paper describes '*Candidatus* Nostocoida limicola'.

Keywords: activated sludge, bulking sludge, filamentous bacteria, '*Nostocoida limicola*', actinobacteria

The overgrowth of filamentous bacteria between the flocs in activated sludge wastewater treatment plants and on the liquid surfaces of aeration tanks produces the serious solids separation problems known as bulking and foaming (Jenkins *et al.*, 1993). The name '*Nostocoida limicola*' is given to one group of these filamentous bacteria which are conventionally identified by their microscopic morphological attributes (Eikelboom & van Buijsen, 1981; Jenkins *et al.*, 1993). '*N. limicola*' has been isolated previously and the data generated for pure cultures are summarized in Table 1 (Eikelboom, 1975; Nowak & Brown, 1990; van Veen, 1973). Essentially, there are three morphotypes (Eikelboom & van Buijsen, 1981) but this paper is restricted to '*N. limicola*' morphotype II. Recently, the

incidence of '*N. limicola*' in both bulking and foaming events has increased (Wanner *et al.*, 1997), particularly in plants treating wastewater from industrial sources. Therefore, a better description of the filaments is warranted.

Five '*N. limicola*' II filaments were isolated by micromanipulation (Skerman, 1968) from two activated sludge sewage treatment plants (Sunbury, Victoria, Australia and Verona, Italy) to plates of freshly prepared R2A (for isolates Ben17, Ben18, Ver1 and Ver2; Reasoner & Geldreich, 1985) or glucose sulfide (GS) medium (for isolate Ben67; Williams & Unz, 1985) and incubated at 20–22 °C for several weeks until visible colonies were apparent. A morphological variant of strain Ben67 was separately subcultured and called Ben68. When the strains grew on both R2A and GS media (22 °C for 4 d), they generally produced small, round, whitish, shiny colonies. However, the ability of the isolated filaments to be successfully

Table 1. Data pertaining to the six currently described isolates of '*N. limicola*'

Reference (strains)	Isolation and growth medium	Cellular dimensions, morphology and phenotypic characteristics
van Veen (1973) (two isolates)	I* (isolation), 3–8 weeks at 17–20 °C SCY‡	Cells, $0 \cdot 3$–$0 \cdot 6 \times 0 \cdot 7$–$1 \cdot 5$ µm; filaments, $0 \cdot 6$–$0 \cdot 9$ mm Gram-positive; non-motile; filaments of chains of coccoid cells Thylakoids, cysts, other resting cells and photosynthetic pigments not detected Each cell wholly or partially divided by transverse cross wall Occasional filament branching Cell division in one plane Facultative anaerobes or microaerophiles Growth down to pH $5 \cdot 3$ Growth at 11–35 °C, optimum of 30 °C
Eikelboom (1975) (two isolates)	I† (isolation), 2–5 weeks at 20 °C SCY‡ (growth)	Cells, one isolate, $0 \cdot 5$ µm wide, the other, $1 \cdot 2$ µm wide; filaments, seldom greater than 200 µm Gram-positive Ovoid cells divided by cross walls On SCY, white colonies are 2–4 mm diameter in a few days and are composed of filaments
Nowak & Brown (1990) (two isolates of '*N. limicola*' II)	Sludge hydrolysate agar§ (isolation), 7 d at room temp TSA‖ + $0 \cdot 2$ % glucose (growth) TSI¶ (growth)	Filament width: one isolate, $1 \cdot 1$ µm wide; the other $1 \cdot 3$ µm wide Gram- and Neisser-positive Twisted, sheathless, long, unbranched filaments PHB intracellular On TSA and TSI after 5–7 d, colonies were cohesive, slimy and 3 mm in diameter Negative for: nitrate reduction, citrate utilization, oxidase, catalase, urease, protease, VP and MR test Fermentative, facultative anaerobes Good growth on a range of carbohydrates (glucose, lactose, sucrose, mannitol, glycerol, Tween 80) with complex ingredients Poor growth on SCY and where metabolizable substrates concentration was low Requirement for cyanocobalamin and thiamin Growth at 10–34 °C, optimum of 23–28 °C

* The I medium of van Veen (1973) contained (g l^{-1}): glucose, $0 \cdot 15$; $(NH_4)_2SO_4$, $0 \cdot 5$; $Ca(NO_3)_2$, $0 \cdot 01$; K_2HPO_4, $0 \cdot 05$; $MgSO_4 \cdot 7H_2O$, $0 \cdot 05$; KCl, $0 \cdot 05$; $CaCO_3$, $0 \cdot 1$; vitamin B_{12}, 1×10^{-5}; thiamin, 4×10^{-4}; and agar (Oxoid), 10.

† The I medium used by Eikelboom (1975) was the same as that listed in the Methods.

‡ SCY contains (g l^{-1}): sucrose, $1 \cdot 0$; casitone (Difco), $0 \cdot 75$; yeast extract (Difco), $0 \cdot 25$; trypticase soy broth without glucose (BBL), $0 \cdot 25$; vitamin B_{12}, 10^{-5}; thiamin, 4×10^{-4}; and agar (Oxoid), 10.

§ Sludge hydrolysate agar. Domestic activated sludge was thickened to 20 g l^{-1}, 10 ml NaOH was added and the suspension was boiled for 60 min under reflux. The suspension was cooled to room temperature, neutralized with HCl and solids were removed by centrifugation at 20000 ***g*** for 30 min. The dark brown supernatant was solidified with agar and autoclaved.

‖ TSA, Tryptic soy agar.

¶ TSI, Triple-sugar iron.

subcultured was extremely erratic and there was no pattern to the growth either for media or strains.

The Neisser stain (Jenkins *et al.*, 1993) for polyphosphate, the Rees *et al.* (1992) stain for polyphosphate and lipophilic inclusions, and the Gram stain (Jenkins *et al.*, 1993) were applied to all strains from both R2A and GS media. Microscopic observations of stained and unstained preparations were carried out with a Zeiss Axioskop microscope equipped for phase-contrast, bright field and epifluorescence illumination. A Zeiss MC 100 photo-
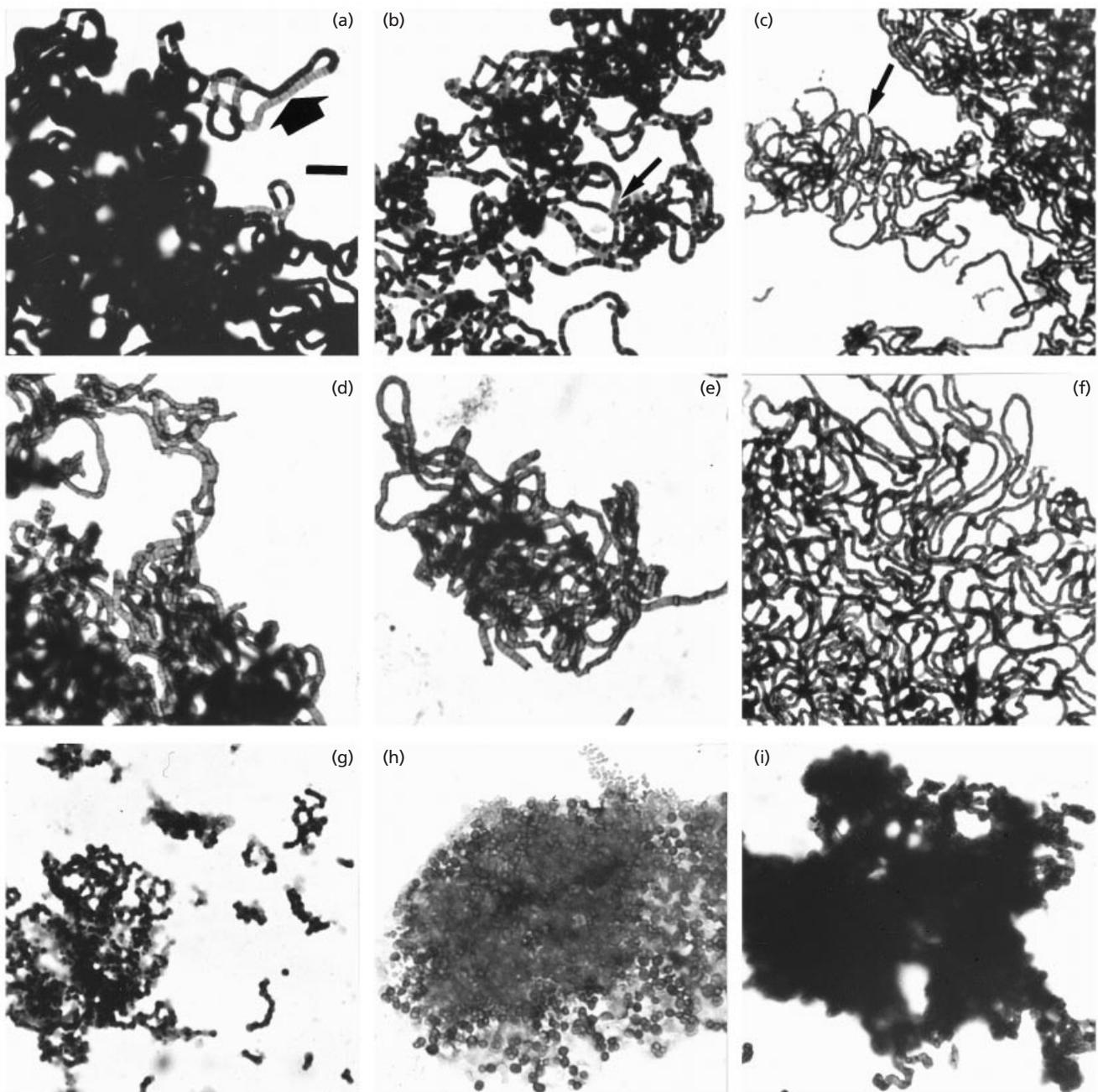
Fig. 1. Light micrographs of '*Nostocoida limicola*' II strains. Arrows in (a)–(c) indicate paler regions of the filaments that corresponded with the Gram-negative portions of the filaments. (a) Ben67 on GS medium, Gram stain; (b) Ben17 on GS medium, Gram stain; (c) Ver2 on GS medium, Gram stain; (d) Ben17 on GS medium, Neisser stain; (e) Ben17 on GS medium, Neisser stain; (f) Ver2 on GS medium, Neisser stain; (g) Ben17 on R2A, Gram stain; (h) Ben68 on R2A, Neisser stain; (i) Ver1 on R2A, Gram stain. Bar in (a) (valid for all micrographs), 5 μm.

micrography system was used to obtain photomicrographs. The filaments of the Ben strains were generally wider than those of the Ver strains (compare Fig. 1a and b with c; and Fig. 1d and e with f). The Gram stain reaction for all strains was extremely variable, as exemplified by the presence of both Gram-positive and Gram-negative cells or parts of cells for strains growing on both media (examples of Gram-negative parts are shown by arrows in Fig. 1a, b and c). Most often, the filaments were evenly positively Neisser stained on GS medium (Fig. 1d, e and f); on R2A, cells were either evenly positive (Fig. 1h) or contained positive granules. Some strains produced lipophilic inclusions, but this feature was not consistent on the different media. The
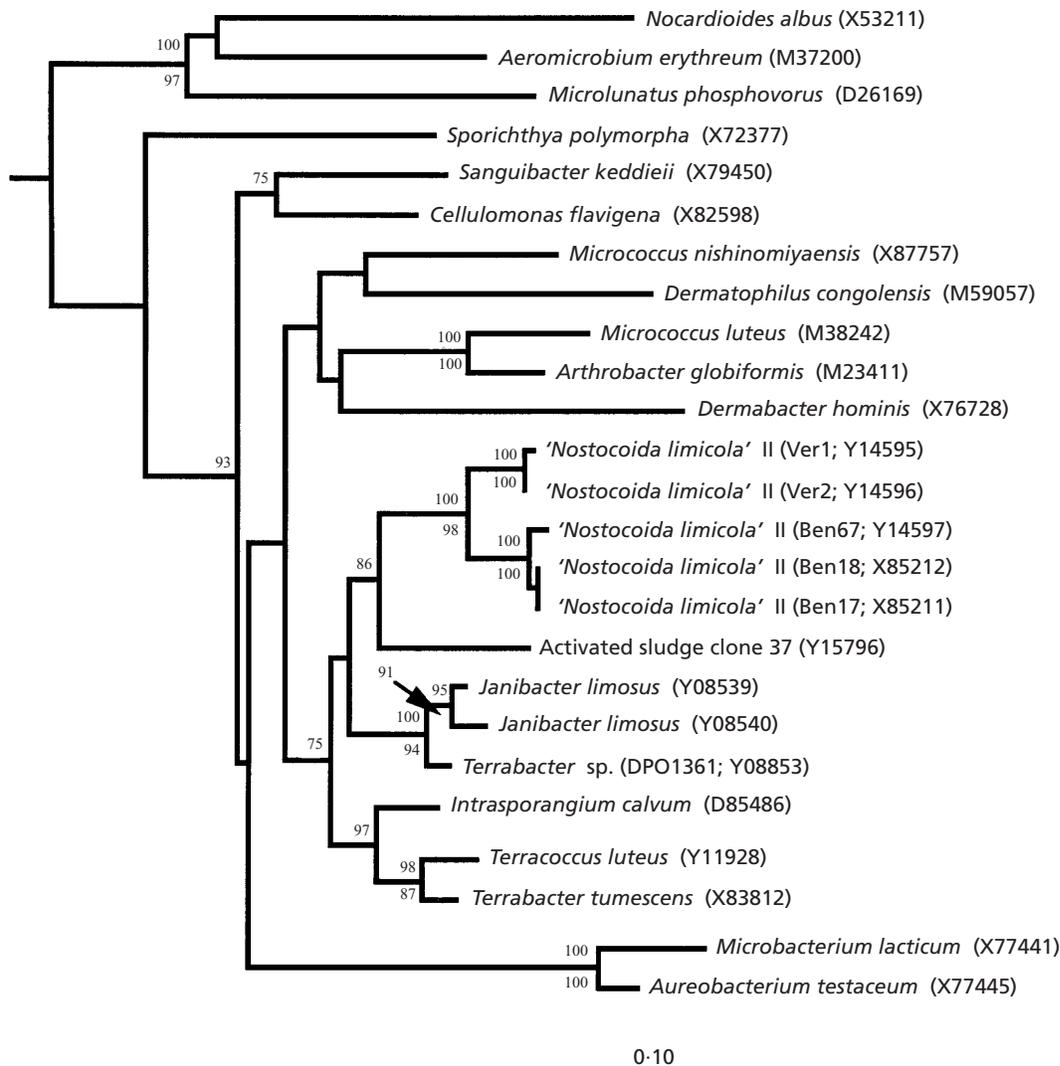
Fig. 2. Evolutionary distance tree of '*Nostocoida limicola*' II strains and their closest relatives in the actinobacterial division based on a comparative analysis of 1161 nucleotides of the 16S rDNA. Bootstrap values greater than 75% from 100 resamplings from distance (above) and parsimonious (below) analyses are presented at the nodes. The outgroup was '*Candidatus* Microthrix parvicella' (not shown).

morphology of the strains was markedly affected by the different media. On GS, they generally grew as regular long filaments and closely resembled the appearance (stained and unstained) of '*N. limicola*' II in activated sludge plants (Fig. 1a–f). On R2A, the strains appeared generally as clumps of swollen cells with extremely short filaments (Fig. 1g and i) and at times, clusters of cocci (Fig. 1h). Fig. 1(c) and (f) show the results for Ver2, but these were the same for Ver1.

The methods used to determine the phenotypic attributes of '*N. limicola*' II were essentially the same as those reported in Blackall *et al*. (1997) and Smibert & Krieg (1994). Strains Ben17, Ben67 and Ver2 were cultured in mineral, salt and vitamins medium (MSV; Williams & Unz, 1989) plus 0·5 g glucose l⁻¹; the

inoculum for all tests was 1%. Briefly, the carbon substrate utilization tests were done in MSV plus 0·5 g carbon source l^{-1}; the nitrogen utilization tests were done in MSV with 0·5 g glucose l^{-1} and 0·05 g N l^{-1} of the different nitrogen sources (ammonium, nitrite, nitrate, urea); growth temperatures were determined in MSV plus 0·5 g glucose l^{-1}; growth under anaerobic conditions was determined in serum bottles with liquid R2A medium; and denitrification ability was assessed in two basal media (liquid R2A and MSV plus 0·5 g glucose l^{-1}) amended with 0·1% KNO_3.

The majority of strains could grow on sucrose as the carbon source, nitrite as the nitrogen source and at 35 °C (two of the three strains). However, most strains could not use ethanol as the carbon source or grow at
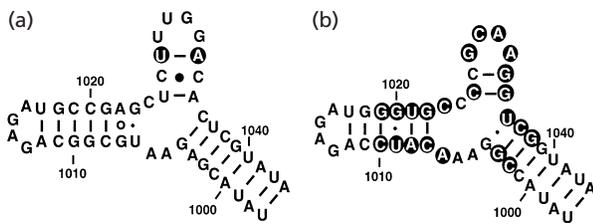
Fig. 3. Secondary structural models for the V6 region in the 16S rRNA of (a) '*Nostocoida limicola*' II strains Ver1 and Ver2; and (b) '*Nostocoida limicola*' II strains Ben17, Ben18, Ben67 and Ben68. Nucleotides in filled-in circles in (a) and (b) show the 24 differences between the two models. Numbering is as for the 16S rRNA of *E. coli*.

8 °C (two of the three strains). All strains could grow on acetate, pyruvate, propionate, glucose, fructose, mannose, lactose, Tween 80, peptone and glycerol as sole carbon sources; none could grow on citrate, succinate, lactate, oleic acid, oleate or propanol as sole carbon sources. All strains could use ammonium, nitrate and urea as their nitrogen source, but none could grow without added nitrogen to a mineral based medium. All strains grew at 15, 20, 25 and 30 °C, but none could grow at 40 °C. No strains could grow anaerobically or with nitrate as the terminal electron acceptor and none produced any reaction in either open or closed Hugh & Leifson tubes. However, they could all reduce nitrate to nitrite and produce catalase.

The near complete 16S rDNA sequences were obtained for all five isolates of '*N. limicola*' II using methods previously described (Blackall, 1994; Blackall *et al.*, 1994). These data for '*N. limicola*' II were phylogenetically analysed in a dataset of 16S rDNA sequences from a small range of close and distant relatives according to basic local alignment search tool (BLAST; Altschul *et al.*, 1990) analyses. Methods for phylogenetic analysis included manual alignment of sequences in ae2 (Ribosomal Database Project), preparation of datasets masked according to the Lane

(1991) mask and analysis by evolutionary distance and parsimony methods in PHYLIP (Felsenstein, 1993). A total of 100 bootstrap resamplings was carried out. A phylogenetic tree of '*N. limicola*' II and other actinomycetes is presented in Fig. 2, which describes for the first time the phylogenetic position of '*N. limicola*' II in the domain *Bacteria* as a member of the actinomycetes division. Our '*N. limicola*' II strains have *Terrabacter* and *Janibacter* species as their closest phylogenetic relatives. The '*N. limicola*' II strains produced a tight cluster (97·4 % identical; > 98 % bootstrap value) divided into two well supported (100 % bootstrap value) subgroups comprising the Ver strains (99·9 % identical) and the Ben strains (99·4 % identical). A clear variability in the region from nucleotides 997–1043 (V6 region, *Escherichia coli* numbering; Brosius *et al.*, 1978) was observed between the strains comprising the two subgroups in Fig. 2. Potential secondary structural models for this variable V6 region for the two subgroups are presented in Fig. 3. The sequence data for the different isolates were deposited in EMBL (Ben17, X85211; Ben18, X85212; Ben67, Y14597; Ver1, Y14595; Ver2, Y14596). A comparison between signature nucleotides of the Ben and the Ver strains and their closest relatives (Table 2) highlights Ben and Ver strain signatures that can be used in determining the affiliation of new strains with one or the other of the '*N. limicola*' II subgroups.

Both van Veen (1973) and Nowak & Brown (1990) reported that their isolates were facultative anaerobes (Table 1), but our isolates could not grow anaerobically when tested in serum bottles or when tested for their mode of attack on glucose according to the Hugh & Leifson test. Nowak & Brown (1990) reported fermentative growth (Table 1) which is not a characteristic of our strains. In addition, our strains could not denitrify nitrate which could be a mechanism of anaerobic growth. Phenotypic similarities between our strains and those in the Nowak & Brown (1990) study were: the inability to reduce nitrate or utilize citrate; the ability to grow on glucose, lactose, sucrose, glycerol and Tween 80; and intracellular storage of lipophilic

Table 2. 16S rDNA signature nucleotides for '*Nostocoida limicola*' II Ben and Ver strains and their close relatives

Position*	Ben strains	Ver strains	*Intrasporangium*	*Terrabacter tumescens* AF005023	*Terrabacter* sp. Y08853	*Terrabacter* sp. U96645	*Janibacter*	*Terracoccus*
140–223	G–C	G–C	G–C	G–C	G–T	A–T	G–T or A–T	G–C
658–748	G–T	G–C	G–T	G–T	G–T	G–T	G–T	G–T
694	A	A	G	G	A	A	A	G
1003–1037	G–C	G–T	G–C	G–C	G–C	G–C	G–C	G–C
1007–1022	C–G	G–C	C–G	C–G	C–T	C–T	C–T	C–G
1133–1141	G–C	G–C	A–T	A–T	A–T	A–T	A–T	A–T

* For all strains, position 30–553 is C–G, 69–99 is G–T, 258–268 is A–T, 630 is C, 659–746 is T–A, 660–745 is G–C, 838–848 is C–G, 839–847 is T–A and 859 is C.

Table 3. Codified record for 'Candidatus Nostocoida limicola'

Feature	'Nostocoida limicola' II
Status	Candidatus
Vernacular epithet	'Nostocoida limicola' II
Phylogenetic lineage	Subphylum, actinomycetes; Gram-positive phylum; domain Bacteria
Cultivation	C, minimal growth
Gram reaction	G+
Morphology	F
Basis of assignment	NAS (Ben17, X85211; Ben18, X85212; Ben67, Y14597; Ver1, Y14595; Ver2, Y14596), M, phenotype
Specific identification of morphotype	Morphological identification, Gram-variable portions of the filaments
Habitat, association or host	FL (activated sludge sewage treatment plant)
Metabolism or unusual features	Aerobic, chemoheterotrophic, non-denitrification, nitrate-> nitrite, non-fermentative, non-anaerobic
Growth temperature range	M, 15–35 °C
Source	From mixed liquors and foams of activated sludge sewage treatment plants
Authors	This manuscript

material. Phenotypic anomalies between strains in the two studies were the result of the anaerobic/fermentative mode of growth (discussed above).

Because of the practical difficulty in obtaining biomass from these filamentous bacteria, all the analyses needed for validly naming them have not been carried out. Hence, essential information like DNA base composition (mol% G+C) and chemotaxonomic data, including cell wall and menaquinone composition, are not yet available. This is a problem common to other slow growing filamentous bacteria from activated sludge like 'Candidatus Microthrix parvicella' (Blackall et al., 1996). DNA homology between the Ben and Ver strains would clarify whether these closely related bacteria are in the same species or not. Nevertheless, nucleotide signature differences (Table 2; Stackebrandt et al., 1997) together with the 16S rDNA sequence data, would support the view that 'N. limicola' II is a novel genus. We believe the 16S rDNA data for 'Nostocoida limicola' II should be made available for other groups interested in activated sludge bacteria to assist them in designing and exploiting in situ hybridization probes and in helping them resolve the taxonomic relationships between the three 'N. limicola' morphotypes (Jenkins et al., 1993). Therefore, it is proposed to name the organisms described here as 'Candidatus Nostocoida limicola' (Table 3).

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II isolates Ben17 and Ben18 and the secondary structural models of the 16S rDNA V6 region.

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