Journal of Applied Microbiology 1997, **82**, 405–410

Phenotypic and phylogenetic description of an Italian isolate of "*Microthrix parvicella*"

S. Rossetti, C. Christensson[1], L.L. Blackall[1] and V. Tandoi

CNR, Water Research Institute, Rome, Italy, and [1]Centre for Bacterial Diversity and Identification, Department of Microbiology, The University of Queensland, Brisbane, Queensland, Australia

5623/01/96: received 24 January 1996, revised 17 June 1996 and accepted 18 June 1996

S. ROSSETTI, C. CHRISTENSSON, L.L. BLACKALL AND V. TANDOI. 1997. "*Microthrix parvicella*" strain RN1 was isolated from an activated sludge treatment plant in Italy using micromanipulation techniques. The strain grows as thin unbranchedfilaments which are Gram-positive with Neisser-positive granules. The isolate was characterized by analysis of the 16S rDNA which was amplified directly from cell biomass by the polymerase chain reaction and sequenced. "*Microthrix parvicella*" strain RN1 presents a very high similarity (100%) with another "*M. parvicella*" strain recently isolated in Australia, suggesting that this micro-organism, a novel, deep branching member of the actinomycetes subphylum, is the same causing the common events of bulking and foaming phenomena in activated sludge treatment plants throughout the world.

INTRODUCTION

"*Microthrix parvicella*" is the main filamentous micro-organism responsible for bulking and foaming events in activated sludge systems. "*Microthrix parvicella*" is not a valid name and the organism's presence in activated sludge is evaluated according to morphological properties (Eikelboom and van Buijsen 1983 ; Jenkins *et al.* 1993). Its dominance was revealed in many surveys carried out worldwide (Wanner 1994).

Despite intense research into the conditions supporting the growth of "*M. parvicella*" in activated sludge biocenoses, fundamental information about its physiology is still incomplete and reliable biological control methods to prevent its growth in activated sludge systems are not available (Wanner 1994).

The first definitive pure culture strain of "*M. parvicella*" was obtained more than 20 years ago by van Veen (1973). However, filaments studied by Pasveer (1969) and Farquhar and Boyle (1971a,b) were probably the first reports of "*M. parvicella*" and these studies, the first attempts to isolate this organism. The descriptions of filaments that Pasveer (1969) studied were identical to current descriptions of "*M. parvicella*". However, the isolated filaments were identified as *Escherichia coli*, but no detail of the identification method was given. The pure cultures only exhibited filamentation in an atmosphere of 90% CO_2–10% air, at a pH below 6 and when glucose was the carbon source. Farquhar and Boyle (1971a,b) thought that their "*M. parvicella*" filaments resembled 'filament-forming lactic acid bacteria' and their isolation conditions were biased towards such micro-organisms. None of these first reports described difficulties in filament isolation. However, it is unlikely that "*M. parvicella*" was actually isolated.

The descriptions of the "*M. parvicella*" filaments and of the isolation procedure given by van Veen (1973) are very detailed and clearly show that isolation was complicated and time consuming.

Eikelboom (1975) utilized a medium comprising sludge hydrolysate and a complex vitamin mixture to isolate "*M. parvicella*". No indication of the difficulty in obtaining the several pure cultures was mentioned. The organisms grew to 1 mm colonies on the sludge agar within 10 d of subculture. The same medium was later used by Slijkhuis and Deinema (1982). Subsequently, a chemically defined medium was also developed to study the pattern of carbon utilization by "*M. parvicella*" (Slijkhuis 1983a). The Dutch axenic culture study by Slijkhuis (1983b) is the most comprehensive done to date and provides the only physiological data available for "*M. parvicella*". In particular, an unusual carbon metabolism was revealed. In contrast to what was found during the first isolations by van Veen (1973), the isolates of Slijkhuis *et al.* (1984) could not use common carbon sources such as simple sugars and organic acids but required oleic acid or its polyoxyethylenesorbitan ester (Tween 80) as its carbon and energy

Correspondence to : Dr Simona Rossetti, CNR, Water Research Institute, Via Reno 1, 00198 Rome, Italy.

sources. A further typical characteristic of "*M. parvicella*" concerning its requirement for reduced sulphur and nitrogen was also discovered from the Dutch study. In addition, "*M. parvicella*" could store up to 35% of its dry weight as lipid inclusions with a similar chemical composition as the supplied carbon source (Slijkhuis 1983b).

Despite great interest in "*M. parvicella*" shown by sewage treatment plant operators, engineers and scientists, 11 years elapsed between the Slijkhuis study and reports of new information on pure cultures. The "*M. parvicella*" scenario was complicated by papers from Forster and co-workers (Chacin *et al.* 1994; Kerley *et al.* 1994; Kocianova *et al.* 1994) reporting the isolation of "*M. parvicella*" by micromanipulation to the defined medium of Slijkhuis (1983a). However, their isolate showed variable morphology including rod-filament transitions, spore formation, motility and the ability to sometimes be present as Gram-negative rods then be replaced by Gram-positive extended filaments.

Seviour *et al.* (1994) succeeded in isolating several strains of "*M. parvicella*" by employing a complex medium and the Skerman micromanipulator. None of these Australian isolates that actively grew on R2A medium (Reasoner and Geldreich 1985) was able to grow on the chemically defined medium of Slijkhuis (1983a).

A definitive answer about the identity of an Australian isolate of "*M. parvicella*" was obtained by employing modern taxonomic tools. Blackall *et al.* (1994) sequenced the 16S rDNA of their strain (DAN1-3) and placed it as a novel, deep-branching member of the actinomycetes subphylum.

The specific requirement by "*M. parvicella*" for long-chain fatty acids as carbon and energy sources as reported by Slijkhuis (1983a) was not found to be a prerequisite by any other "*M. parvicella*" researchers. In addition, spore formation and motility were only observed by Kocianova *et al.* (1994).

Table 1 reports media, isolation methods and conditions utilized by the researchers who reported isolation of "*M. parvicella*". The only feature common to all the studies listed in Table 1 is the difficulty in adequately storing this filamentous micro-organism. It seems most likely that the early cultures of this organism including those isolated and studied by Slijkhuis and Eikelboom are no longer viable.

Activated sludge personnel experience little difficulty in identifying "*M. parvicella*" in situ when it appears in its characteristic irregular and winding filamentous morphology. However, there have been three types of "*M. parvicella*" pure culture studies. The first comprises studies where the wrong organism has likely been isolated (Pasveer 1969; Farquhar and Boyle, 1971a,b; Kocianova *et al.* 1994). The second type of study includes those of van Veen (1973), Eikelboom (1975), Seviour *et al.* (1994) and Blackall *et al.* (1994) in which pure cultures of the correct organism seem to have been obtained.

Table 1 The main characteristics of the growth media and methods utilized for the isolation and maintenance of "*Microthrix parvicella*"

Media	Carbon source	Nitrogen source	Sulphur source	pH	Vitamins added	Isolation	Maintenance	Reference
I	Glucose	Ammonium sulphate	Sulphate	NR	Vitamin B12 and thiamine	Dilution and plating	Slant cultures at 4°C	van Veen 1973
H	Sludge hydrolysate	NR	NR	NR	Vitamin complex	Dilution and plating	Slant cultures at 4°C	Eikelboom 1975
H	Sludge hydrolysate	NR	NR	NR	Vitamin complex	NR	NR	Slijkhuis and Deinema 1982
R2A	Complex medium*	Organic nitrogen	Organic sulphur and/or sulphate	7·2	†	Micromanipulation	NR (storage at −80°C in glycerol failed)	Seviour *et al.* 1994
NTM modified	Succinate and peptone	Ammonium sulphate and organic nitrogen	Organic sulphur	8·0	Vitamin B12 and thiamine	Micromanipulation	Slant cultures at 4°C	Blackall *et al.* 1994
R2A	Complex medium*	Organic nitrogen	Organic sulphur and/or sulphate	7·2	†	Micromanipulation	Slant cultures at 4°C	This study

NR, Not reported.

* Contains: yeast extract, proteose peptone, casaminoacids, glucose, soluble starch and sodium pyruvate.

† Contained in yeast extract.

This can be deduced from cellular descriptions and photographs of the pure cultures. The studies of van Veen (1973), Seviour *et al*. (1994) and Blackall *et al*. (1994) report difficulty in obtaining pure cultures of "*M. parvicella*". The studies of Slijkhuis (1983b) reporting the physiological characteristics including the requirements for long-chain fatty acids as carbon and energy sources, and reduced forms of sulphur and nitrogen in defined media, comprise the third type of study. Although from descriptions and photographs, the correct organism was probably isolated, no difficulty in obtaining isolates was mentioned and the culture conditions reported were not confirmed by other pure culture studies. Possibly, a variant of "*M. parvicella*" was studied by Slijkhuis (1983b). Clearly more intensive "*M. parvicella*" studies are required to resolve the global diversity of this organism, its growth characteristics and ultimately, its control in activated sludge plants.

Although the phylogenetic position of one isolate of "*M. parvicella*" has been determined, it needs to be confirmed with other isolates from different parts of the world. This is particularly important because of the difficulty in comparing any two independent studies involving "*M. parvicella*" (see Table 1). The knowledge of the correct taxonomic position of the different isolates could lead the researchers to a more appropriate approach to the study of its physiology which would assist in solving what was defined by Eikelboom (1994) as the "'*Microthrix parvicella*" puzzle'. With this aim, we successfully isolated a "*Microthrix parvicella*" strain from an activated sludge plant in Italy, sequenced its 16S rDNA and obtained its first phenotypic characteristics.

MATERIALS AND METHODS

Activated sludge with "*M. parvicella*" as the dominant filamentous micro-organism was obtained from the full scale conventional treatment plant of Rome, Italy. This plant treats domestic wastewater and is operated at a sludge age greater than 10 d.

The isolation to pure culture of "*M. parvicella*" was achieved by manipulating single filaments to freshly prepared R2A medium (Reasoner and Geldreich 1985), using the Skerman micromanipulator (Skerman 1968). The filaments were manipulated at $\times 320$ magnification which allows confident identification of the selected filament as "*M. parvicella*". Furthermore, contaminating single-celled bacteria can be observed and avoided. The plates were incubated at 20°C and the single filaments growing on the agar surface were regularly checked to monitor the slow growth of the manipulated filaments free of contaminating cells of other bacteria.

The Gram and Neisser staining of the isolate were carried out according to previously published methods (Jenkins *et al*. 1993). The Nile Blue staining procedure was used to highlight lipid granules in the cells (Rees *et al*. 1992).

The pure culture was maintained on R2A slants at 4°C and currently, alternative long-term methods of maintenance have not yet been tested.

The amplification and sequencing of the 16S rDNA, and the comparative sequence analysis were done by the methods described by Blackall *et al*. (1994). The sequence determined for our "*M. parvicella*" strain was deposited in EMBL under accession number X93044.

RESULTS

The micromanipulated filaments, incubated at 20°C on R2A medium, took 5–6 weeks to produce macroscopically visible colonies with a diameter that never exceeded 1 mm. The colony morphology is irregular, non-pigmented and shows a filamentous margin. The isolate, known as strain RN1, was successfully subcultured many times on this solid medium.

The "*M. parvicella*" isolate strain RN1 grows better in R2A liquid medium, taking approximately 3 weeks to show visible growth with an increase in filament numbers by two orders of magnitude. At time 0, filament length and numbers were estimated microscopically, and this was repeated at days 20–30. Initial filament numbers were approximately 200 ml^{-1} and at days 20–30 were approximately 20 000–70 000 ml^{-1}. Because of the paucity of growth, we were unable to determine dry weight or ATP content. Strain RN1 grows in pure culture with the same main characteristics that this micro-organism usually exhibits in mixed liquor samples. It maintains the filamentous morphology and when observed in phase contrast microscopy the filaments are unbranched and it is impossible to distinguish the cellular septa. The filaments are dark, with a twisted/coiled appearance and sometimes empty cells inside the trichoma are visible (Fig. 1a). The filament diameter varies between 0·5 and 0·8 μm and the length is 50–300 μm but it can also reach 500 μm. The isolate is Gram-positive but some parts of the filaments are Gram-variable with Gram-positive granules (Fig. 1b) and when Neisser-stained, positive granules are visible. The filaments contain many large fluorescent inclusions of lipid material with Nile Blue staining (Fig. 1c). These inclusions are visible during growth but disappear in old cells. We did not observe single cells, motile forms or spores in RN1, but the trichoma appear to fragment into shorter filaments (about 50–150 μm length) with culture age.

A near complete 16S rDNA sequence for "*M. parvicella*" RN1 (1374 nucleotides) was obtained. The phylogenetic position of "*M. parvicella*" RN1 as a deep-branching member of the actinomycetes subphylum can be seen in the evolutionary distance tree (Fig. 2).

DISCUSSION

The characteristics of the RN1 isolate fit well the morphological descriptions of "*M. parvicella*" reported in identi-

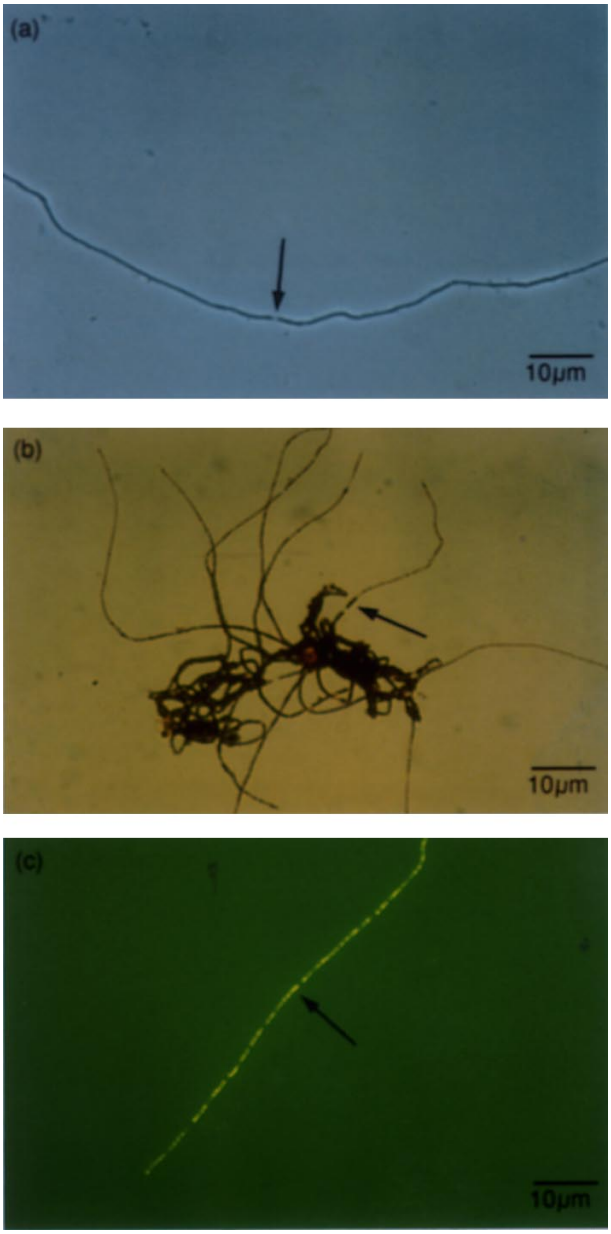

Fig. 1 "*Microthrix parvicella*" isolate, strain RN1 : (a) appearance of unstained filaments when examined by phase contrast microscopy—arrow shows empty cell ; (b) filaments after Gram staining—arrow shows Gram-positive granules ; (c) lipid granules in cells after Nile Blue staining (arrow)

fication manuals (Eikelboom and van Buijsen 1983 ; Jenkins *et al*. 1993). Strain RN1 accumulates lipid material and the ability of "*M. parvicella*" to accumulate intracellular storage material was often observed by the presence of electron-transparent granules and described by van Veen (1973), Eikelboom (1975) and Slijkhuis (1983b).

Strain RN1 appears very similar to the others described in Table 1. What was observed by Kocianova *et al*. (1994) concerns a bacterium quite different from "*M. parvicella*". Their work has to be considered with the knowledge that other organisms with the same morphology as "*M. parvicella*" could be present in the sludge. Therefore, knowledge of morphological properties is insufficient for identification or for understanding properties of the organisms causing the common bulking problems.

In comparison with the 16S rRNA sequence "*M. parvicella*" strain DAN1-3, the sequence of RN1 is 100% identical. Consequently, it has been unequivocally shown that two isolates (RN1 and DAN1-3), independently obtained from two very distant countries (Australia and Italy), have identical 16S rDNA sequences. The morphology of these two isolates is also identical and closely matches that of the organisms in activated sludge plants. This is the first study to show that independently obtained strains of "*M. parvicella*" are isolates of the same species, because the conserved 16S rRNA genes of these organisms are identical. We have been unable to obtain the "*M. parvicella*" strain reported by Kocianova *et al*. (1994) and all other "*M. parvicella*" strains for which there are published data, are no longer viable. It has been suggested that the strain in the study of Kocianova *et al*. (1994) was a *Bacillus* sp. and not "*M. parvicella*" (Blackall *et al*. 1994). Indeed, *Bacillus* species have previously been implicated in bulking in activated sludge plants (Trick *et al*. 1984). There are many phenotypic discrepancies between the isolates shown to be actinomycetes (DAN1-3 and RN1), the isolate of Kocianova *et al*. (1994) and the Slijkhuis (1983b) isolates. However, there is agreement in the morphological descriptions of the isolates in this study and those of van Veen (1973), Eikelboom (1975), Slijkhuis (1983b), Seviour *et al*. (1994) and Blackall *et al*. (1994).

Further work that will be done with the RN1 isolate includes sequencing the 23S rDNA and the determination of chemotaxonomic information. The region of the 23S rDNA between helices 54 and 55 is particulary interesting because Embley and Stackebrandt (1994) have indicated that an insertion element is present in all members of the order *Actinomycetales* (a unique molecular synapomorphy) but absent in *Atopobium*. This latter group is the most deeply branching in the actinomycetes subphylum (Fig. 2). However, the *Microthrix* group (Fig. 2) lies between the *Atopobium* group and the order *Actinomycetales*. There is no 23S rDNA data for any members of the *Microthrix* group.

Recently, further 16S rDNA sequence data for another Australian isolate of "*M. parvicella*" and two clones from a 16S rDNA clone library prepared from a foam dominated by "*M. parvicella*" have been reported (Blackall *et al*. 1996). All Australian and Italian "*M. parvicella*" 16S rDNA data are

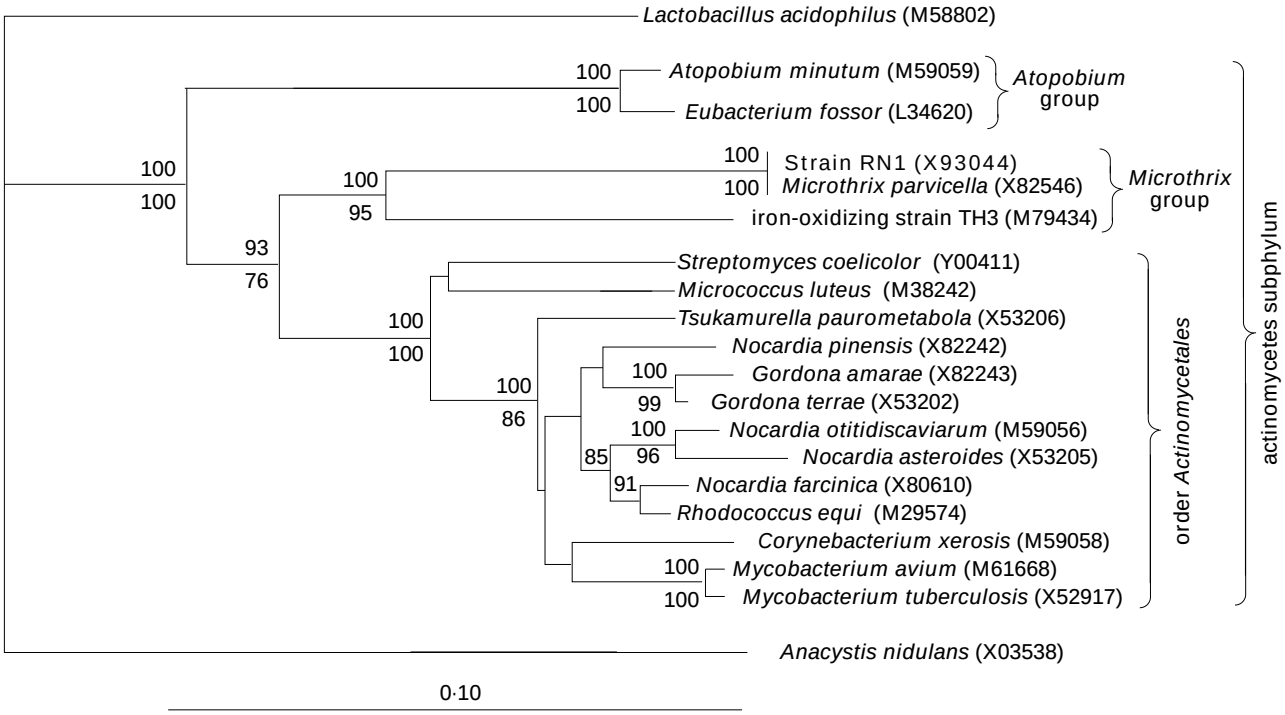

Fig. 2 Evolutionary distance tree of "*Microthrix parvicella*" strain RN1 and its phylogenetic relatives including a range of bacteria reported from activated sludge sewage treatment plants. The tree is based on the comparative analysis of 1126 nucleotide positions. Database accession numbers are in parentheses after species or strain names. Bootstrap values greater than 74% (100 bootstrap re-samplings) from distance (upper) and parsimony (lower) analyses are presented at the nodes. The outgroup used in the analyses was *Anacystis nidulans*. The bar represents 0·1 estimated changes per nucleotide position

>99·2% similar. Additionally, it was suggested to elevate "*M. parvicella*" to *Candidatus* status, but to retain "*Microthrix parvicella*" as the vernacular name, which we have adopted.

ACKNOWLEDGEMENTS

The authors thank Jenny Cassady and Emma Puttick (DNA Sequence Analysis Facility, The University of Queensland) for assisting with DNA sequencing, Franco Duranti for general laboratory assistance, and Magnus Christensson for assistance with preparation of Fig. 2.

REFERENCES

Blackall, L.L., Seviour, E.M., Cunningham, M.A., Seviour, R.J. and Hugenholtz, P. (1994) "*Microthrix parvicella*" is a novel, deep branching member of the actinomycetes subphylum. *Systematic and Applied Microbiology* **17**, 513–518.

Blackall, L.L., Stratton, H., Bradford, D., Sjörup, C., Del Dot, T., Seviour, E.M. and Seviour, R.J. (1996) "*Candidatus Microthrix parvicella*—a filamentous bacterium from activated sludge sewage treatment plants. *International Journal of Systematic Bacteriology* **4**, 344–346.

Chacin, E., Kocianova, E. and Forster, C.F. (1994) Foam formation, anaerobiosis and *Microthrix parvicella*. Journal of the Institution of Water and Environmental Management **8**, 534–537.

Eikelboom, D.H. (1975) Filamentous organism observed in activated sludge. *Water Research* **9**, 365–388.

Eikelboom, D.H. (1994). The *Microthrix parvicella* puzzle. *Water Science and Technology* **29–7**, 271–279.

Eikelboom, D.H. and van Buijsen, H.J.J. (1983) *Microscopic Sludge Investigation Manual*, 2nd edn. Delft, The Netherlands : TNO.

Embley, T.M. and Stackebrandt, E. (1994) The molecular phylogeny and systematics of the actinomycetes. *Annual Review of Microbiology* **48**, 257–289.

Farquhar, G.J. and Boyle, W.C. (1971a) Identification of filamentous microorganisms in activated sludge. *Journal of Water Pollution Control Federation* **43**, 604–622.

Farquhar, G.J. and Boyle,W.C. (1971b) Occurrence of filamentous microorganisms in activated sludge. *Journal of Water Pollution Control Federation* **43**, 779–798.

Jenkins, D., Richard, M.G. and Daigger, G.T. (1993) *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*. New York : Lewis Publishers.

Kerley, S., Kocianova, E. and Forster, C.F. (1994) A biochemical characterisation of *Microthrix parvicella*. Process Biochemistry **29**, 581–586.

Kocianova, E., Forster, C.F. and Foot, R.J. (1994) What is *Microthrix parvicella*? *Journal of Applied Bacteriology* **76**, 301–306.

Pasveer, A. (1969) A case of filamentous activated sludge. *Journal of Water Pollution Control Federation* **41**, 1340–1352.

Reasoner, D.J. and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* **49**, 1–7.

Rees, G.N., Vasiliadis, G., May, J.W. and Bayly, R.C. (1992) Differentiation of polyphosphate and poly-β-hydroxybutyrate granules in an *Acinetobacter* sp. isolated from activated sludge. *FEMS Microbiology Letters* **94**, 171–174.

Seviour, E.M., Williams, C., DeGrey, B., Soddell, J.A., Seviour, R.J. and Lindrea, K.C. (1994) Studies on filamentous bacteria from Australian activated sludge plants. *Water Research* **28**, 2335–2342.

Skerman, V.B.D. (1968) A new type of micromanipulator and microforge. *Journal of General Microbiology* **54**, 287–297.

Slijkhuis, H. (1983a) *Microthrix parvicella*, a filamentous bacterium isolated from activated sludge : cultivation in a chemically defined medium. *Applied and Environmental Microbiology* **46**, 832–839.

Slijkhuis, H. (1983b) The physiology of the filamentous bacterium *Microthrix parvicella*. PhD Thesis, Agricultural University, Wageningen, The Netherlands.

Slijkhuis, H. and Deinema, M.H. (1982) The physiology of *Microthrix parvicella*, a filamentous organism isolated from activated sludge. In *Bulking in Activated Sludge : Preventative and Remedial Methods* ed. Chambers, B. and Tomlinson, E.J. pp. 75–83. Chichester : Ellis Horwood Ltd.

Slijkhuis, H., van Groenestijn, J.W. and Kylstra, D.J. (1984) *Microthrix parvicella*, a filamentous bacterium from activated sludge : growth on Tween 80 as carbon and energy source. *Journal of General Microbiology* **130**, 2035–2042.

Trick, I., Salcher, O. and Lingens, F. (1984) Characterization of filament forming *Bacillus* strains isolated from bulking sludge. *Applied Microbiology and Biotechnology* **19**, 120–124.

van Veen, W.L. (1973) Bacteriology of activated sludge, in particular the filamentous bacteria. *Antonie van Leeuwenhoek* **39**, 189–205.

Wanner, J. (1994) *Activated Sludge Bulking and Foaming Control.* Pennsylvania : Technomic Publishing.