16S rRNA Sequence Analysis of Bacteria Present in Foaming Activated Sludge

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Introduction

The performance of a sewage treatment plant depends upon the microbial community of the activated sludge (GRAY, 1990). The analyses of the microbial community in activated sludge is important for the understanding and possible control of separation problems in sewage treatment plants (SCHUPPLER *et al*., 1995). Foaming is one of the solid separation problems experienced in activated sludge systems that has compelled researchers to examine the biological component of the mixed liquor of activated sludge (BLACKALL and HUGENHOLTZ, 1999).
Foaming filamentous organisms are differentiated mainly using morphology (JENKINS *et al*., 1993, BLACKALL, 1994). Microorganisms identified in foams are *Nocardia* (now *Gordona*) *amarae* (KLATTE *et al*., 1994), *N. rhodochrous, N. asteriodes, N. caviae, N. pinensis* now called *Skermania piniformis* (CHUN *et al*., 1997), *Streptomyces* spp, *Microthrix parvicella, Micromonospo-ra, Type 0675* and *Rhodococcus* (BLACKALL *et al*.,1989; GODDARD and FORSTER, 1987; LECHEVALIER and LECHEVALIER, 1974, 1975; LEMMER and KROPPENSTEDT, 1984; PUJOL et al., 1991; SEVIOUR et al., 1990; SEZGIN and KARR, 1986; SEZGIN et al., 1988). *Nostocoida limicola* and Type 0041 can also cause foaming (GODDARD and FORSTER, 1987; WANNER and GRAU, 1989). Microorganism morphology is a poor descriptive attribute that can vary widely depending upon nutritional conditions. Because of their inadequate characterisation, most of the filamentous bacteria in activated sludge are not included in the widely used and generally accepted phenetic classifications (SEVIOUR and BLACKALL, 1999).
Employing molecular biological methods provide an alternative approach for the detection of microorganisms that are difficult to identify by conventional culture techniques or microscopy (BLACKALL, 1994). The analysis of 16S rRNA genes, aided by using PCR to amplify target sequences in environmental samples, has enabled microbial ecologists to identify and characterise microorganisms in a natural community, like activated sludge, without prior cultivation (SCHUPPLER *et al*., 1995).
The taxonomic position of an organism can be determined by comparing the sequence with those of other bacteria (AMANN et al., 1995). Analysis of such data has helped to resolve the taxonomic position of some of the Eikelboom filaments (SEVIOUR and BLACKALL, 1999). Many of the filaments still remain to be characterised in this way and further extensive phenotypic characterisation is still needed before the organisms can be identified.
Molecular methods as mentioned above provide microbiologists with the tools to study the ecology and population dynamics of these filaments in activated sludge plants.
In this study foaming activated sludge from a water purification plant in Gauteng, South Africa was examined. The organisms in the foam were identified using molecular techniques and phylogenetically placed using the Clustal X software program.

Materials and Methods

Sampling
Activated sludge foam was collected from the aerobic zone of the Centurion Wastewater treatment plant in Gauteng, South Africa.

Microorganisms present
The filaments in the foam sample from Centurion WWTP occurred as Gram positive, irregularly coiled filaments that were found coiled in and around the floc. Filaments were 0.6–0.8 µm in diameter and 100–400 µm in length (not illustrated). No sheath or attached growth was present and no branching occurred. Cellular inclusions were common which gave a "beaded" appearance. According to Jenkins *et al*. (1993) these are characteristics true to "*Microthrix parvicella*".

Sample preparation
Volumes of 100 ml foam were homogenised for 10 min using 70g glass beads. The foam was pelleted by centrifugation for 10 min at 7000 rpm in a Hermle 360 K centrifuge.

DNA extraction and purification (Modified from EHLERS, 1995)
After centrifugation, the supernatant was discarded. The pellet was resuspended in volumes of 40 ml Sodium-Tris-

EDTA buffer (STE, 4 °C, pH 8) (10 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA) and 2 ml lysosyme (3 mg/ml of a freshly made 50 mg/ml stock solution). This was incubated on ice for 1 h. SDS (20%) and proteinase K (100 µg/ml) were added to the cell suspension. The cell suspension was incubated overnight at 50 °C with slight agitation. Two gentle extractions with equal volumes phenol:chloroform/isoamylalcohol were carried out. The suspension was mixed for 30 min in a shake incubator at 100 rpm. Centrifugation was carried out at 7000 rpm for 10 min to separate the organic and aqueous phases. The aqueous DNA-containing top layer was removed with a "wide bore pipet" without disturbing the white protein-rich interface. This step was repeated until there was no more protein visible. A final extraction step with equal volumes chloroform:isoamylalcohol was carried out by centrifugation for 10 min at 7000 rpm in order to remove the residual phenol from the DNA suspension. The aqueous DNA phase was aliquoted into sterile microcentrifuge tubes, and adjusted to 0.3 M sodium acetate (NaOAc) with a 3 M NaOAc stock solution (pH 5.2). Two volumes of cold (–20 °C) absolute ethanol was added on top of the aqueous layer and mixed. The DNA was precipitated overnight at –20 °C. The solution was centrifuged at 10000 rpm for 10 min at 0 °C to pellet the DNA. The supernatant was discarded and the tubes were inverted on towelpaper and left to air dry. The dried pellets were resuspended in 200–300 µl Tris-EDTA (TE) buffer. The DNA was concentrated. A volume of 1 ml cold (-20 °C) 100% ethanol was added again and the salt precipitated for 2 h at –20 °C. The DNA was pelleted again at 10000 rpm for 10 min. A wash step to desalt the pellets, with 70% ice cold (–20 °C) ethanol, was carried out and the pellets were left to air dry. TE buffer was added to the dried and desalted pellets. The purity and concentration of the DNA was determined with a CARY 1E UV-Visible spectrophotometer of Varian (Varian Australia) at A_{260} and A_{280}.

PCR amplification of 16S rDNA

The 16S rRNA genes were amplified from total DNA using two universal bacterial primers, forward primer 27 and reverse primer 1492r (Table 1) (BLACKALL, 1994). The numbering of the primers is based on the *Escherichia coli* 16S rRNA gene. These primers amplify approximately 1400 to 1500 base pairs (bp) (BLACKALL, 1994). The primers were synthesised at ROCHE at a scale of 50 nmol. The (50 µl) PCR mixture contained 25 pmol of each primer, 2.5 mM of each dNTP (dATP, dGTP, dTTP, dCTP [Takara]), 10 X Reaction buffer (100 mM Tris/HCl, 500 mM KCl, 15 mM $MgCl_2$) (Takara), 0.5 unit of *Taq* DNA polymerase (Takara) and 10–50 ng of genomic bacterial DNA. The PCR was performed on a Perkin Elmer GeneAmp 2400 system. The DNA and ddH_2O were subjected to a denaturation step of 98 °C for 2 min followed by addition of the rest of the PCR mix and 30 cycles of 93 °C for 1 min, 48 °C for 45 s, 72 °C for 2 min; a final re-annealing at 48 °C and extension at 72 °C for 10 min (BLACKALL, 1994). Following thermal cycling, the PCR products were visualised by agarose gel electrophoresis and UV illumination (SAMBROOK *et al*., 1989). Sizes of the amplicons were assessed by comparison with a 1 kb marker (Gibco, BRL) run in the agarose electrophoresis.

PCR product purification

The amplification mix was purified using the High Pure PCR Product Purification Kit following the standard protocol as supplied by the manufacturer (Boehringer Mannheim). The purification involved adding binding buffer to the PCR mix and centrifuging it through filter tubes. After this the unincorporated nucleotides were removed by adding wash buffer and centrifugation at 10 000 rpm for 2 min. The PCR products were eluted using elution buffer and centrifugation.

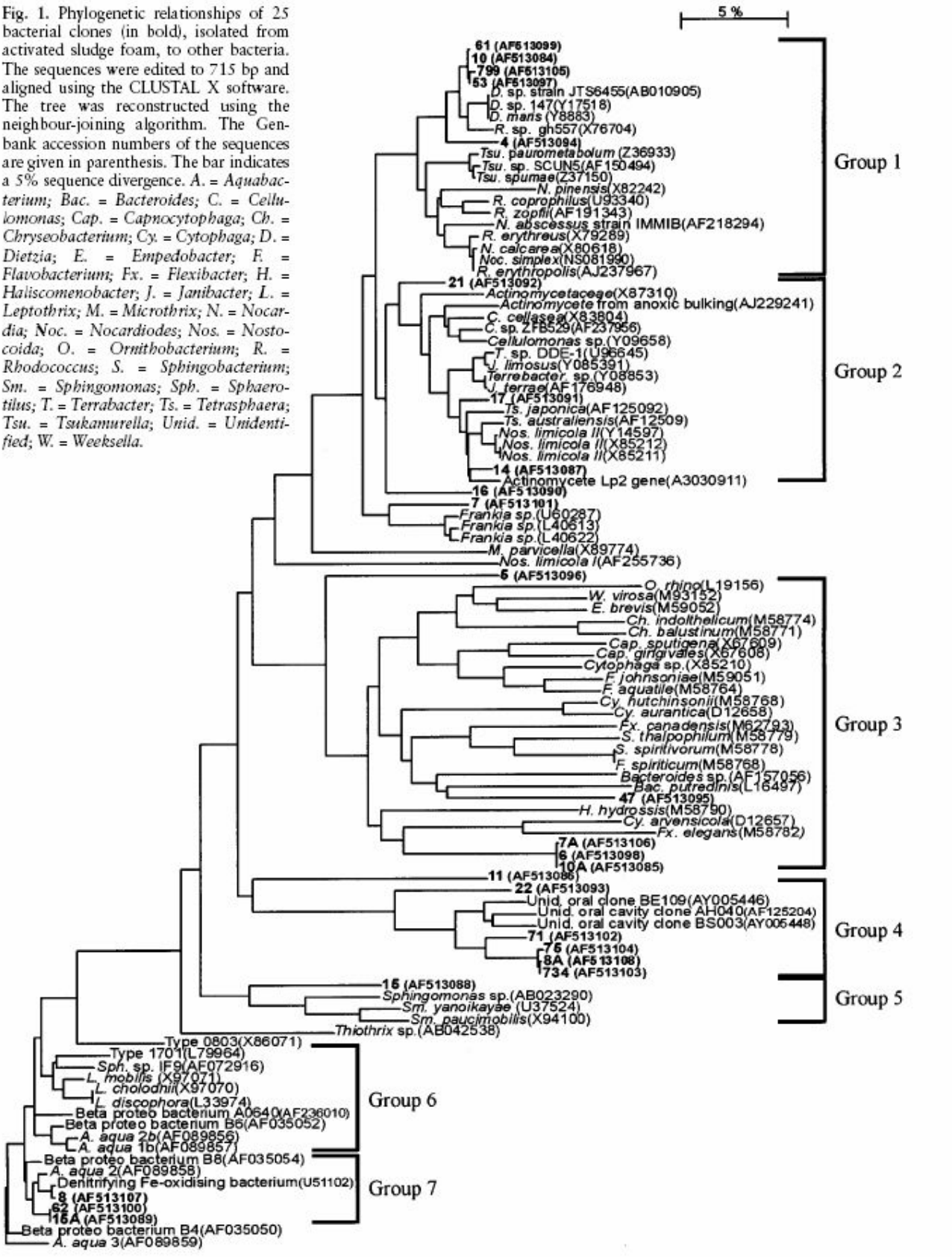
Clone library construction

Clone libraries from purified PCR products were constructed using the PGemT-easy cloning kit following the standard protocol supplied by the manufacturer (Promega). Ligation of the PCR product to the vector was carried out using T4 DNA ligase, ligation buffer and a vector, all supplied by the manufacturer. The ligation was carried out at 4 °C overnight. Transformation was carried out using high efficiency competent cells and recombinant colonies were selected using blue/white colour screening.

Purification of plasmids

Each of the clones was resuspended in a volume of 5 ml Luria Burtani Broth containing ampicillin (10g/L NaCl, 10g/L Tryp-tone, 5g/L Yeast extract) and incubated at 37 °C overnight. The cells were centrifuged at 10 000 rpm for 1 min after which the supernatant was discarded. Each pellet was resuspended in 400 µl lysis buffer (50mM Glucose, 2mM Tris/HCl, 10mM EDTA) and vortexed for 5–10 s. This was incubated at room temperature for 10 min. To this, 400 µl freshly prepared NaOH/SDS (0.2M NaOH, 20% SDS) was added and mixed thoroughly but carefully. This was left on ice for 10 min. Volumes of 300 µl cold (4 °C) 7.5 M NH_4OAc (pH 7.6) was added to each pellet and mixed thoroughly but carefully. This was incubated on ice for 10 min and afterwards centrifuged for 10 min at 10 000 rpm at room temperature. An aliquot of 1000 µl of the supernatant was collected in a new microcentrifuge tube and 650 µl isopropanol was added to this and mixed. The mixture was left at room temperature for 10 min and then centrifuged for 10 min at 10 000 rpm. The supernatant was discarded and 100 µl of 2 M NH_4OAc (pH 7.4) was added to the pellet and mixed thoroughly. This was left on ice for 10 min and then centrifuged for 10 min at 10 000 rpm at room temperature. The supernatant was collected and to this 110 µl isopropanol was added. This was left at room temperature for 10 min. The suspension was centrifuged at 10 000 rpm for 10 min at room temperature and the supernatant discarded afterwards. The pellet was washed with 1 ml 70% ethanol and then dried in the desiccator. Afterwards the pellet was dissolved in 25 µl dH_2O. The purified plasmids were screened for the correct sized insert using restriction enzyme digestion with EcoR1 for 1 h at 37 °C, and agarose gel electrophoresis.

Fig. 1. Phylogenetic relationships of 25 bacterial clones (in bold), isolated from activated sludge foam, to other bacteria. The sequences were edited to 715 bp and aligned using the CLUSTAL X software. The tree was reconstructed using the neighbour-joining algorithm. The Genbank accession numbers of the sequences are given in parenthesis. The bar indicates a 5% sequence divergence. *A.* = *Aquabacterium*; *Bac.* = *Bacteroides*; *C.* = *Cellulomonas*; *Cap.* = *Capnocytophaga*; *Ch.* = *Chryseobacterium*; *Cy.* = *Cytophaga*; *D.* = *Dietzia*; *E.* = *Empedobacter*; *F.* = *Flavobacterium*; *Fx.* = *Flexibacter*; *H.* = *Haliscomenobacter*; *J.* = *Janibacter*; *L.* = *Leptothrix*; *M.* = *Microthrix*; *N.* = *Nocardia*; *Noc.* = *Nocardiodes*; *Nos.* = *Nostocoida*; *O.* = *Ornithobacterium*; *R.* = *Rhodococcus*; *S.* = *Sphingobacterium*; *Sm.* = *Sphingomonas*; *Sph.* = *Sphaerotilus*; *T.* = *Terrabacter*; *Ts.* = *Tetrasphaera*; *Tsu.* = *Tsukamurella*; *Unid.* = *Unidentified*; *W.* = *Weeksella*.



Amplification of 16S rRDNA

PCR's were performed on the recombinant clones using the vector primers SP6 and T7 (Table 1) to amplify the insert. The PCR mix consisted of 40 pmol of each primer, 2.5 mM of dNTPs, 10X Reaction buffer (Takara), 0.5 U of Taq DNA poly-merase (Takara), 10–50 ng of genomic DNA and ddH2O to make up the volume of the reaction to 25 µl. The PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim). The protocol for the kit was followed. Products were visualised with ultra-violet illumination after agarose gel electrophoresis (SAMBROOK *et al*., 1989).

Sequencing

Sequencing of PCR amplified 16S rDNA was carried out using the ABI PRISM dye terminator cycle-sequencing ready-reaction kit (Perkin Elmer) and an ABI Prism 377 sequencer, according to the manufacturers protocol. The sequencing mix – 4 µl Terminator ready reaction Mix, 50 ng PCR product, 1.6 pmol primer – was placed in the thermal cycler. The thermocycling protocol employed was one cycle of 96 °C for 5 s, and 25 cycles of denaturation at 96 °C; reannealing at 50 °C for 5 s and extention at 60 °C for 4 min. The vector primers SP6 and T7 (PROMEGA) was used as end primers and the internal primers used were reverse primer ii, forward primer iv and the 530 forward primer (ROCHE) (Table 1). The excess dye terminators were removed from the reaction mixture by adding a mixture of 50 µl of 100% ethanol and 2 µl 3M sodium acetate (pH 5.2) to the sequencing reaction. This mixture was left on ice for 20 min, after which it was centrifuged at 10 000 rpm for 20 min at 4 °C. The supernatant was tipped off, and 250 µl 70% ethanol was added, and tipped off again. The pellet was gently flicked and the tube centrifuged for 2 min at 10 000 rpm. The excess fluid was carefully dried with a tissue, and the products vacuum dried (ca. 10 min). For analysis, the purified products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer (PE Applied biosystems), denatured for 2 min at 90 °C and loaded onto the ABI Prism model 377 DNA sequencer gel.

Phylogenetic analysis

The sequences obtained were trimmed of uncomparative data so that only from nucleotides 28-1491 were aligned and compared. Sequences were compiled using the Sequence Navigator software package (Applied Biosystems) and compared to available databases by the use of the Basic Local Alignment Search Tool (BLAST) (ALTSCHUL *et al*., 1997) to determine approximate phylogenetic affiliations. The compiled sequences were aligned using the Clustal X software program (THOMPSON *et al*., 1997). The sequences determined in this study were phylogenetically placed by comparative analysis (715 nucleotides) with 16S rDNA sequences of various other bacteria including members of the *Cytophaga-Flavobacter-Bacteroides* group (Table 2). Sequences (from the BLAST search) with the greatest similarity to the clone sequences were selected. These sequences were retrieved from the GenBank database. The phylogenetic tree (Fig. 1) was calculated using the neighbour-joining algorithm (SAITOU and NEI, 1987).

Results and Discussion

Filaments in the foam sample

The filamentous microorganism most frequently observed and with high abundance (approximately 75%), in the Centurion WWTP foam sample was *"Microthrix parvicella"*. Other filaments were so infrequently observed that no positive identification could be made and these occurred as rod shaped cells in chains and filaments that could resemble Type 0041.

PCR amplification of 16S rRNA genes

The 16S rRNA genes of the bacteria in the foaming sample were amplified with the 27f and 1492r primers (Table 1), and PCR produced a single band of about 1500 bp.

Clone library

After screening 47 clones, 25 clones contained the correct sized insert and were submitted to automated cycle sequencing.

Automated cycle sequencing

The nucleotide sequences of the 16S rRNA genes of the selected clones were determined with automated sequencing on the ABI PRISM 377 model and sequences of between 1400bp and 1464bp were obtained.

Phylogeny of the clones

The 25 clone sequences obtained (GenBank accession numbers AF513084 to AF513108) from the foam sample, were aligned and phylogenetically placed with sequences from various other bacteria that showed identity of 90–99% to the clone sequences in the BLAST search (Fig 1). Where similarity less than 90% were found the sequences with the greatest similarity to the clone sequence were used. The clone sequences were edited to a length of 1463 bp. After aligning these sequences to the sequences obtained from GenBank, all of the sequences were trimmed to 715 bp to accommodate shorter sequences from GenBank.

The dendrogram indicated 7 genotypic clusters (Fig. 1).
In Group 1, clones 61, 10, 799, 53 and 4 showed similarities greater than 95% with *Dietzia maris* which was

Table 1. Sequences of the primers used in amplification and sequencing reactions.

Name and Sequence	Sequence 5' – 3'	Reference
27 forward	GAGTTTGATCCTGGCTCAG	BLACKALL, 1994
1492 reverse	TACGGYTACCTTGTTACGACTT	BLACKALL, 1994
SP 6	TTTAGGTGACACTATAGAATAC	PROMEGA
T 7	TAATACGACTCACTATAGGCGA	PROMEGA
530 forward	GTGCCAGCMGCCGCGG	LANE, 1991
Forward no. II	GTGTAGCGGTGAAATGCGTAG	KUNHERT et al., 1996
Reverse no. VII	CTTGCGACCGTACTCCCCAGGC	KUNHERT et al., 1996

reclassified from *Rhodococcus maris* by RAINEY et al. (1995). *D. maris* has been isolated as a dominant microbe from activated sludge foam (SEZGIN et al. 1988).

In Group 2 clones 14 and 17 grouped with *Nostocoida limicola II* often associated with foaming activated sludge (SEVIOUR and BLACKALL, 1999). Clone 17 showed more than 95% similarity to the *Terrebacter* and *Janibacter* spp. Clone 21 seemed to be similar to the *Actinomycetaceae* species within Group 2. Clone 16 grouped alone, but had less than 4% sequence divergence from Group 2 that included *Nostocoida limicola* II, also a well known foam forming filamentous microorganism (SEVIOUR and BLACKALL, 1999).

Although clone 47 falls into group 3, which comprises members of the *Cytophaga-Flavobacter-Bacteroides* (CFB*)* group, it only had a 90% similarity with *Flavobacterium* sp.(Fig. 1). Clones 7A, 6 and 10A had less than 90% similarity to the CFB group.

Group 4 included a number of unidentified bacterial clones from oral cavities. Clones 71, 75, 8A and 734 formed a cluster with this group of clones. Clone 22 also showed greater than 91% similarity with these clones. One of the unidentified clones from an oral cavity (AH040) was resolved into the third subdivision of the candidate division TM7 (HUGENHOLTZ et al., 2001). This subdivision includes bacterial clones from foaming activated sludge (HUGENHOLTZ et al., 2001).

Although clone 15 showed similarities of 96% and more with the organisms in Group 5, when the sequence was compared with those of other bacteria, it also indicated a 97% homology with members of Group 7 (Fig. 1).

Group 7 indicated that the sequences of clones 8, 62 and 15A were more than 95% similar to that of a denitrifying Fe-oxidising bacterium as well as *Aquabacterium aqua* and Beta-proteobacterium B8 (Fig 1). Group 7 had less than 1% sequence divergence from Group 6 which involved the filamentous bacteria Eikelboom Type 1701 and *Sphaerotilus natans* strain IF9, both associated with activated sludge bulking and foaming.

Clone 5 did not fall into any of the groups in the dendogram (Fig. 1). However, a nucleotide sequence of approximately 440 nucleotides showed 97% similarity to members of group 1 i.e. *Dietzia*, *Rhodococcus* and *Nocardia* spp. that form part of the filamentous bacteria associated with activated sludge foam (SEVIOUR and BLACKALL, 1999).

Clone 11 was not closely associated to any of the groups of bacteria examined in the study (Fig. 1). This clone showed 88% similarity to an uncultured bacterium SJA-68 (not indicated in Fig. 1).

Clone 7 had a 93% similarity with members of the genus *Frankia* (Fig. 1).

The sequences from the clones obtained indicate a wide variety of organisms present in the foam sample without prior cultivation of the bacteria present in the sample. Although only a small number of clones were se-quenced, the results indicated that some of the clones isolated did group with the filamentous organisms associated with foaming activated sludge.

"*Microthrix parvicella*" was not identified in the study and this is contrary to the fact that it was established by microscopy that "*M. parvicella*" was the dominant filamentous organism in this sample. The DNA extraction protocol might not have been efficient enough to break up all Gram-positive cells such as "*M. parvicella"*. Another possible explanation for the discrepancy of not finding a 16S rDNA sequence for *"M. parvicella*" could be that the primer set that was used in this study targeted a too narrow range of bacteria. Cloning can also introduce bias in that not all of the clones obtained are sequenced and in this way the organism expected could be missed. The fact that only a few (25) positive clones were obtained in this study could have contributed to "*Microthrix parvicella*" not being among those organisms identified from the sample.

The reason that no clear homology or no definite identification could be made in this study could be that the existing database could still be limited in terms of the sequences available for the filamentous organisms. Hence the importance of further studies like this to expand the database on the filamentous microorganism sequences.

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