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16S rRNA Analysis of Isolates Obtained from Gram-Negative, Filamentous Bacteria Micromanipulated from Activated Sludge

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Summary

Individual filaments of the Gram negative, bulking filamentous morphotypes Eikelboom Type 0092, Type 0411, Type 0803 and *Herpetosiphon* sp. were identified in activated sludge mixed liquors and specifically isolated by micromanipulation. Although their isolation and partial phenotypic description have previously been reported, we sought them to determine their phylogenetic position and to compare our data with the previous descriptions. Direct cell lysis procedures and the polymerase chain reaction were used to obtain their 16S rRNA genes. These were sequenced and the data were analysed to phylogenetically place the filaments into their respective lines of descent in the domain *Bacteria*. Type 0803 is a member of the *Rubrivivax* subgroup of the beta proteobacterial subclass; *Herpetosiphon* sp. belongs in the *Chloroflexus* subdivision of the green non-sulfur lineage and its sequence is most similar to that of *H. aurantiacus*; while Type 0092 and Type 0411 belong in the *Flexibacter-Cytophaga-Bacteroides* phylum. The contemporary "identification" of these organisms is ambiguous and relies on subjective morphological criteria and staining reactions. However, the sequence data reported here are being examined for morphotype-specific regions to be exploited for RNA-directed, DNA probes for rapid, unequivocal, *in situ* identification of each filament type.

Key words: Bulking – Activated sludge – Gram negative filaments – Phylogeny

Introduction

Activated sludge sewage treatment plants regularly suffer from problems associated with the separation of the activated sludge biomass (flocs) from the purified water following biological treatment. These problems have been divided into six categories (*Wanner,* 1994). The overgrowth of filamentous bacteria extending beyond the flocs and precluding their gravity sedimentation is known as bulking. In excess of 25 different morphotypes of filamentous bacteria have now been described. These are differentiated and "identified" according to their microscopic morphology and staining reactions (*Eikelboom* and *van Buijsen,* 1983, *Jenkins* et al., 1993). The procedure for morphologically describing filamentous bacteria from activated sludge is steeped in history (*Cyrus* and *Sladká,* 1970, *Farquhar* and *Boyle,* 1971 a, *van Veen,* 1973) and is considered the standard procedure for their differentiation and identification. Although the process requires practice and skill, "identification" of filaments is important since strategies employed for their control are filament-type specific. However, the isolation and taxonomic identification of filamentous bacteria from activated sludge has attracted comparatively little attention. Of the 25 different filamentous bulking bacteria currently recognised, no more than five have valid names (*Eikelboom* and *van Buijsen,* 1983, *Jenkins* et al., 1993). The majority of these filaments are "named" according to a numbering system (*Eikelboom,* 1975) and three of the four described in this

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Table 1. Comparison of rankings of dominant filamentous organisms in surveys of activated sludge plants from different countries

Information or Filament type	South Africa[1]	USA[2]	USA[3]	USA[4]	Netherlands[5]	Germany[2]	Australia[6]	Australia[7]
No. of plants	56	270	167	17	200	315	66	65
No. of different filaments ranked	24	18	24	18	17	10	19	21
Type 0092	1	8	14	16	4	–[8]	3	3
Type 0411	–	–	17	–	–	–	–	19
Type 0803	8	11	8	11	9	10	7	10

[1] *Blackbeard* et al. (1986); [2] *Jenkins* et al. (1993); [3] *Jenkins* et al. (1984); [4] *Williams* and *Unz* (1985); [5] *Eikelboom* (1977); [6] *Seviour* et al. (1990); [7] *Seviour* et al. (1994); [8] not reported

Table 2. Some characteristics of the filaments in this report[1,2]

Filament	Trichome	Cells	Intracellular deposits	Other
Type 0092	0.8–1.0 × 10–60 μm, straight, irregularly-curved or bent filaments, cell septa hardly visible	0.4–0.8 × 1.4–2.0 μm	polyphosphate distributed throughout cells	entire trichome stains grey-black with Neisser stain
Type 0411	50–150 μm, coiled-irregularly bent	0.5–0.8 × 1.5–4.7 μm	–	"identified" as *Flexibacter*
Type 0803	50–150 μm, straight-smoothly-curved	0.6–0.8 × 0.9–3.0 μm	electron-dense areas at septa, volutin globules, almost circular, electron-transparent regions	filaments extending from flocs, sometimes free in bulk solution, sometimes attached to inorganic material
Herpetosiphon	200–500 μm	0.5–1.5 × 3.0–5.0 μm	poly-β-hydroxybutyrate	slow gliding motility, microsheath present

[1] All information from *Eikelboom* (1975), *Pipes* (1978), *Jenkins* et al (1993), and *Trick* and *Lingens* (1984)
[2] Apart from exceptions noted in the table, none of these filaments are sheathed, the cells are not motile, cells attached to the filaments are not prominent, all are Gram negative

publication are "identified" in this manner. Type 0092, Type 0411 and Type 0803 have been found consistently in bulking activated sludge incidents when surveys of this problem have been done (Table 1). *Herpetosiphon* sp. has not been reported in these surveys and is therefore not considered a prominent bulking organism. However, the inadequacy of the *in situ* identification methods may preclude the recognition of *Herpetosiphon* sp. in sludge. Cardinal characteristics of these filaments are presented in Table 2. Information on Type 0092, Type 0411 and Type 0803 has been summarised most recently by *Jenkins* et al. (1993) and the isolation and characterisation of *Herpetosiphon* sp. from bulking activated sludge have been described (*Senghas* and *Lingens,* 1985, *Trick* and *Lingens,* 1984).

Control of the growth of bulking filamentous bacteria in activated sludge plants is important. If their kinetic characteristics were known (e.g., substrate affinity, maximum specific growth rates on a range of substrates), plant operational or design strategies could be manipulated for their control in the same manner as has been achieved with Type 021N (*van Niekerk* et al., 1987), and *Gordona amarae* (*Blackall* et al., 1991, *Cha* et al., 1992). Clearly, the first step in this process is to obtain isolates of the organisms and to characterise them in axenic culture. Although all of the filament types addressed in this publication have been reported as having been isolated in the past (*Eikelboom,* 1975, *Horan* et al., 1988, *Seviour* et al., 1994, *Trick* and *Lingens,* 1984), their cultivation in axenic culture has been very poorly addressed and is considered by most who attempt it, to be a particularly difficult task.

To clarify the phylogenetic position of the Gram negative filaments Type 0092, Type 0411, Type 0803 and *Herpetosiphon* sp., and to prepare morphotype-specific rRNA directed, DNA-hybridisation probes for their *in situ* identification using technique described by *Amann* et al. (1995), we isolated representatives of each of them from activated sludge plants in Australia. We sequenced their 16S rRNA genes for comparative analysis.

Materials and Methods

Isolation and photomicrography of the filaments. The organisms were isolated from the mixed liquor of several treatment plants in Victoria, Australia during the period 1991–1994 (Table 3). Plant samples were collected by the operators and handled as described previously (*Seviour* et al., 1994). Filaments were iso-

Table 3. Isolates of filamentous bacteria obtained in pure culture from activated sludge plants

Filament	Strain No.	Isolation Date	Plant Location and Features[1]	EMBL Accession No.
Type 0092	Ben 30	March, 1992	EBPR Plant, Bendigo, not bulking	X85210
Type 0411	Ben 32	October, 1992	Conventional Plant, Carrum, bulking but not due to this filament	X85209
Type 0803	Ben 04B	July, 1991	Conventional Plant, Benalla, not bulking	X86071
Type 0803	Ben 05B	July, 1991	Conventional Plant, Benalla, not bulking	X86070
Herpetosiphon	Ben 15	May, 1994	EBPR Plant, Bendigo, not bulking	X86447

[1] EBPR – Enhanced Biological Phosphate Removal. All plants are in Victoria, Australia

lated by micromanipulation using a Skerman (1968, 1978) micromanipulator. R2A agar (*Reasoner* and *Geldreich,* 1985) was used and aspects of media dispensing to petri dishes followed the recommendations in *Skerman* (1978). Briefly, a 50 µl aliquot of the sludge sample was placed near the edge of an R2A plate which was tilted so that the liquid ran over the agar surface to the other side of the dish. The liquid was not allowed to contact the edge of the petri dish. The inoculated R2A plates were dried in a biohazard cabinet for *ca.* 5 min. A 32X long working distance, phase contrast objective was used to view the samples and was the objective used for micromanipulation providing a total magnification of 320X. The filaments in the samples on the R2A agar were "identified" using the methods of *Eikelboom* and *van Buijsen* (1983) and *Jenkins* et al. (1993). Then, individual filaments "identified" as Type 0092, Type 0411, Type 0803 and *Herpetosiphon* from the various samples (Table 3) were micromanipulated away from the inoculum to a sterile zone on the petri dish. Filament purity was confirmed by careful microscopic observation and a macroscopically visible square was scored around the filament using the micromanipulator. Subsequently, a region around the scored zone on the petri dish was collected using a sterile scapel and transferred to a fresh plate of R2A which was incubated at 20 °C. Each manipulated filament was viewed at least daily to ensure that the developing colony resulted from the filament and not from unobserved contaminating single cells that could have been co-manipulated. The colonies were subcultured to fresh plates of R2A and pure cultures were prepared for SEM using techniques described earlier (*Seviour* et al., 1984). The reports of *Skerman* (1968, 1978), *Blackall* (1991) and *Tandoi* et al. (1992, 1994) should be consulted for more detailed information and diagrams describing the micromanipulation technique.

Amplification and sequencing of 16S rDNA. The small subunit (16S) rRNA gene was amplified directly from cell biomass using 27f and 1492r primers (*Lane,* 1991). Biomass (*ca.* 1 mm^3) from R2A cultures of each isolate was directly lysed as previously reported (*Blackall* et al., 1994).

The amplification cycling profile and subsequent purification and sequencing of the 16S rDNA were carried out according to methods detailed in *Blackall* (1994). Both strands of the 16S rDNA were sequenced using the primers 27f, 342r, 357f, 519r, 530f, 787r, 803f, 1100r, 1114f, 1241f, 1392r and 1492r (*Dorsch* and *Stackebrandt,* 1992). The sequences determined have been deposited in EMBL (Table 3).

Comparative sequence analysis. Sequence analysis and phylogenetic interpretations were performed as described earlier (*Blackall* et al., 1994). Three datasets were prepared – the *Rubrivivax* beta proteobacterial subclass (for Type 0803); the *Chloroflexus* subdivision of the green, non-sulfur bacteria (for *Herpetosiphon* sp.); and the *Flexibacter-Cytophaga-Bacteroides* phylum (for Type 0092 and Type 0411). If variable regions were excluded from the datasets for analysis, it was done according to the Bacterial mask of *Lane* (1991). Outgroups used for comparison were *Alcaligenes xylosxidans* (for the *Rubrivivax* subgroup of the beta proteobacteria); *Thermus thermophilus* (for the *Chloroflexus* subdivision of the green non-sulfur bacteria); and *Chlorobium limicola* (for the *Flexibacter-Cytophaga-Bacteroides* phylum).

Results

Isolation of the filaments and description of pure cultures

Specific filaments identified as Type 0092, Type 0411, Type 0803 and *Herpetosiphon* sp. and micromanipulated to sterile media, grew to produce colonies. Regular microscopic observation of the developing colonies, particularly in the period immediately after micromanipulation, showed that the colonies were the result of filament growth. We observed that the colonies did not arise from the growth of single celled organisms that could have been co-manipulated with the filaments. The morphology of the filaments growing on R2A agar and observed by SEM are shown in Fig. 1. We tested a wide range of media for their ability to support the growth of all these strains and found that freshly prepared R2A medium (*Reasoner* and *Geldreich,* 1985) gave the best results. Filaments identified as Type 0092 and *Herpetosiphon* grew well on R2A, producing macroscopic colonies within 7 days. Type 0411 and Type 0803 were more slow growing (10–12 days). Type 0092 (Ben 30) produced yellow, shiny, slightly elevated colonies on R2A. The filaments underwent fragmentation to cells of various lengths (0.3–0.6 µm × 0.8–4.3 µm). Type 0411 (Ben 32) produced small (< 2 mm), pink, shiny colonies comprised of filaments of cells (0.3–0.7 µm × 2.6–4.0 µm). Cell septa were very difficult to see. Type 0803 (Ben 04B and Ben 05B) produced small (< 2 mm), colourless colonies of filaments (width 0.4–0.9 µm) whose septa were impossible to observe by light microscopy and were just visible under SEM (data not shown). *Herpetosiphon* sp. (Ben 15) produced orange-pink colonies that readily spread over the surface of the R2A agar plate leaving few discrete colonies. The filaments that com-

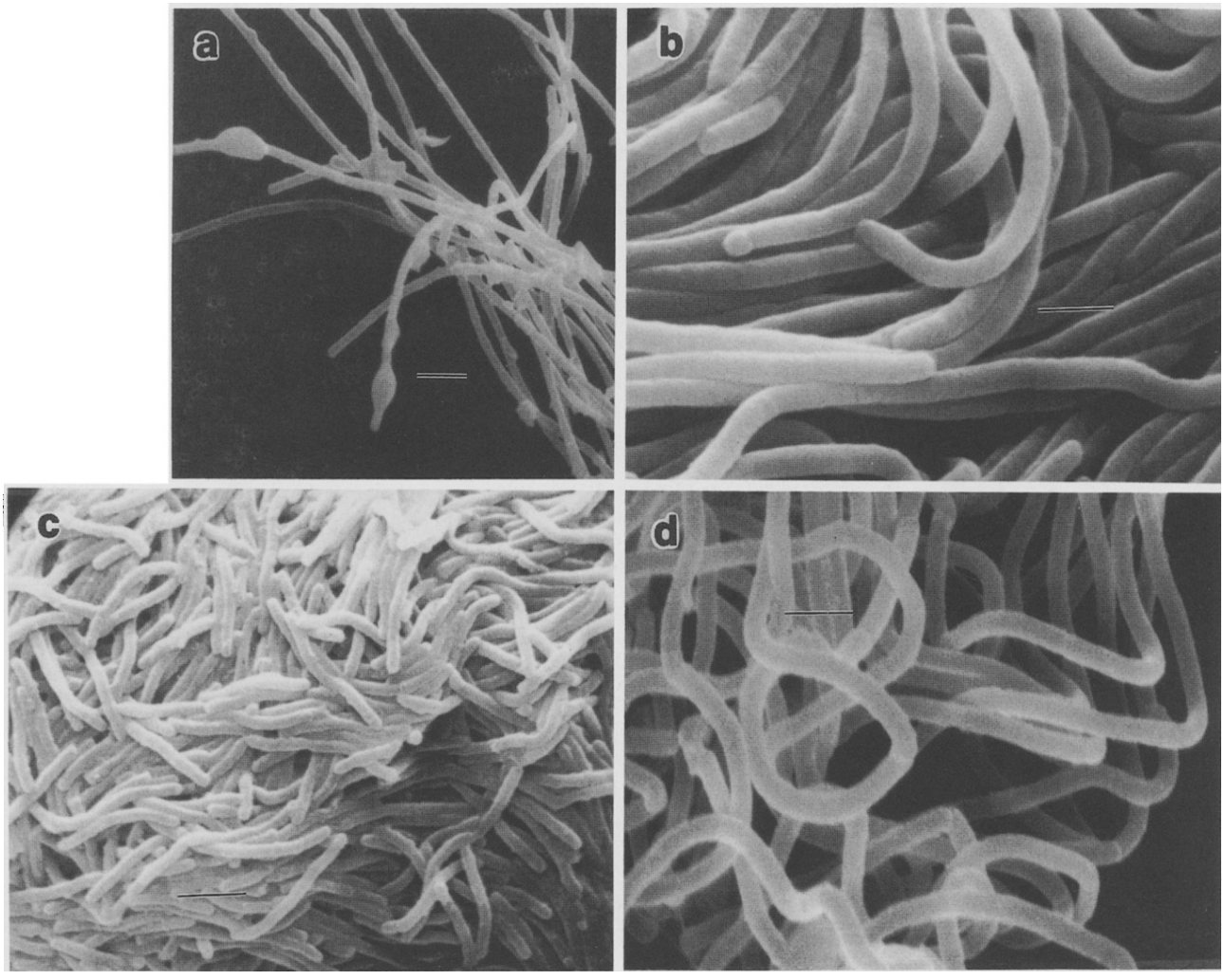

Fig. 1. Micrographs of gram negative filaments. (a) *Herpetosiphon* (bar = 4 μm), (b) Type 0803 (bar = 1 μm), (c) Type 0092 (bar = 2 μm), and (d) Type 0411 (bar = 2 μm).

prised the colonies were 0.7–1.4 μm in diameter and cell septa could not be seen. Knots along the filaments were observed.

Phylogenetic analysis of isolated filaments

Phylogenetic analysis of an unmasked dataset (1407 nucleotides) including the near complete 16S rRNA sequences of Type 0803 (Ben04B – 1430 nucleotides, X86071; Ben05B – 1474 nucleotides, X86070) placed these strains into the *Rubrivivax* subgroup of the beta proteobacterial subclass (Fig. 2). The sequences of the two Type 0803 strains were 99.9% similar. The Type 0803 strains had highest sequence similarity to *Variovorax paradoxus* (D30793, 94.8%), *Rhodoferax fermentans* (D16212 and D16211, 94.2%), *Comamonas testosteroni* (M11224, 94%), and "*Brachymonas denitrificans*" (D14320, 93.7%). The cluster containing Type 0803 and the abovementioned species formed a very well supported group with bootstrap values of 100% (evolutionary distance) and 99% (parsimony). A phylogenetic tree (Fig. 2) from a small dataset is presented. Analysis of an extended dataset of the beta subclass (41 strains, 1003 nucleotides – data not shown) largely verified the groups as defined in the Ribosomal Database Project (RDP) listing (*Larsen* et al., 1993), and clearly showed the coherence of the complete *Rubrivivax* subgroup (bootstrap of 100%, distance analysis) and the closest relatives to Type 0803.

The near complete sequence of *Herpetosiphon* sp. strain Ben 15 (X86447, 1303 nucleotides) was phylogenetically placed by comparative analysis (1046 nucleotides, masked dataset) in the *Chloroflexus* subdivision of the green nonsulfur lineage (RDP listing, Fig. 3) and its sequence was most similar to that of *H. aurantiacus* (M34117, 98.6%). In an evolutionary distance analysis of an extended dataset (11 sequences, 423 nucleotides), the incomplete clone sequences envOS-C2 (X52546) and envOS-4 (M62774) also affiliated with the *Herpetosiphon* group but with an unsupported bootstrap value of 71% (data not shown).

The near complete 16S rRNA sequences of Type 0092 (Ben 30 – 1488 nucleotides, X85210) and Type 0411 (Ben 32 – 1487 nucleotides, X85209) were phylogenetically

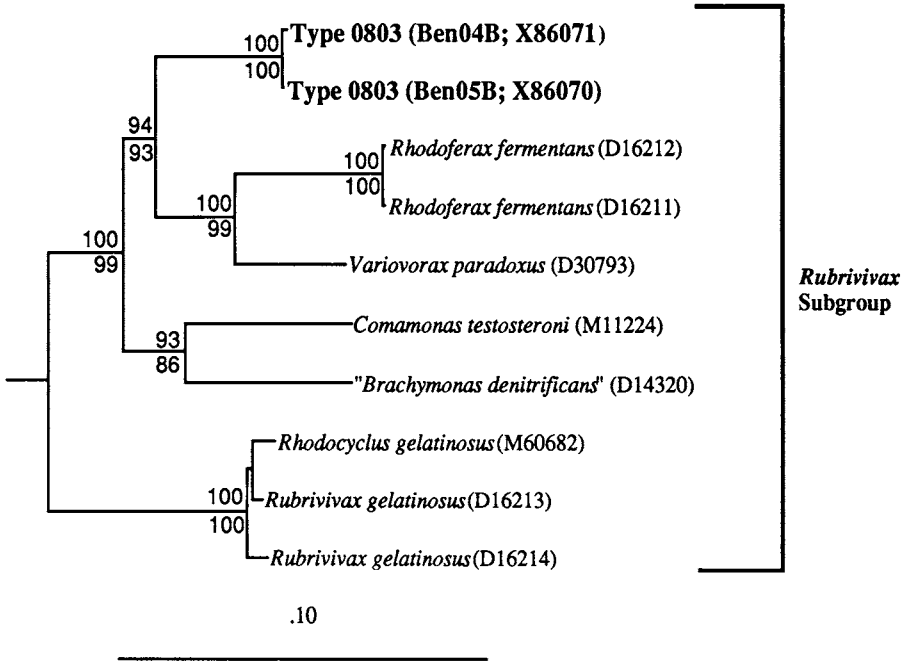

Fig. 2. Evolutionary distance tree of the *Rubrivivax* subgroup of the beta proteobacterial subclass based on the comparative analysis of 1407 nucleotide positions. Database accession nembers are in parenthesis after species or strain numbers. Bootstrap values greater than 75% (100 bootstrap resamplings) from distance (upper) and parsimony (lower) analyses are presented at the nodes. The outgroup used in the analyses but not shown in the tree was *Alcaligenes xylosoxidans*.

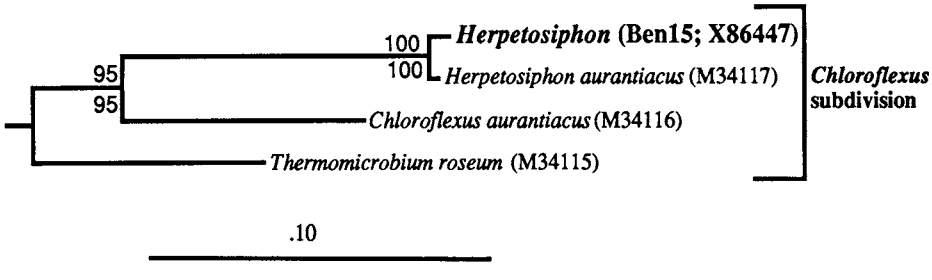

Fig. 3. Evolutionary distance tree of the *Chloroflexus* subdivision of the green non-sulfur phylum based on the comparative analysis of 1046 nucleotide positions. Database accession numbers are in parenthesis after species or strain numbers. Bootstrap values greater than 75% (100 bootstrap resamplings) from distance (upper) and parsimony (lower) analyses are presented at the nodes. The outgroup used in the analyses but not shown in the tree was *Thermus thermophilus*.

analysed in a masked, dataset comprising these filaments and 31 other strains (1130 nucleotides, Fig. 4) which encompassed six subgroups within the *Flexibacter-Cytophaga-Bacteroides* phylum (RDP listing, *Gherna* and *Woese*, 1992, *Larsen* et al., 1993).

Type 0092 (Ben 30, X85210) fell within the *Cytophaga* subgroup and was most closely related by sequence similarity to *Cytophaga columnaris* (D12659, 96.9%), and *Flavobacterium aquatile* (M62797, 93.9%). The *Cytophaga* subgroup in our analysis (9 strains) was well supported by bootstrap resampling (Fig. 4).

The *Flexibacter* subgroup which included Type 0411 was comprised of sequences from 9 strains in our analysis and formed a group not supported by bootstrap resampling. The 16S rRNA sequence of Type 0411 was most similar to that of *Runella slithyformis* (M62786, 93.6%) and both formed a group supported by bootstrap analysis (100%, distance and parsimony analysis).

Similarity matrices for the three datasets analysed in this study are available from the corresponding author.

Discussion

The isolation of filamentous bacteria from sludge samples appears to have been a simpler task in the past than it seems now (*Eikelboom*, 1975, *Farquhar* and *Boyle*, 1971 a, *Pipes*, 1978, *Salcher* et al., 1982, *van Veen*, 1973). These early techniques usually included sample dilution, mixing, and plating onto solidified agar media of differing

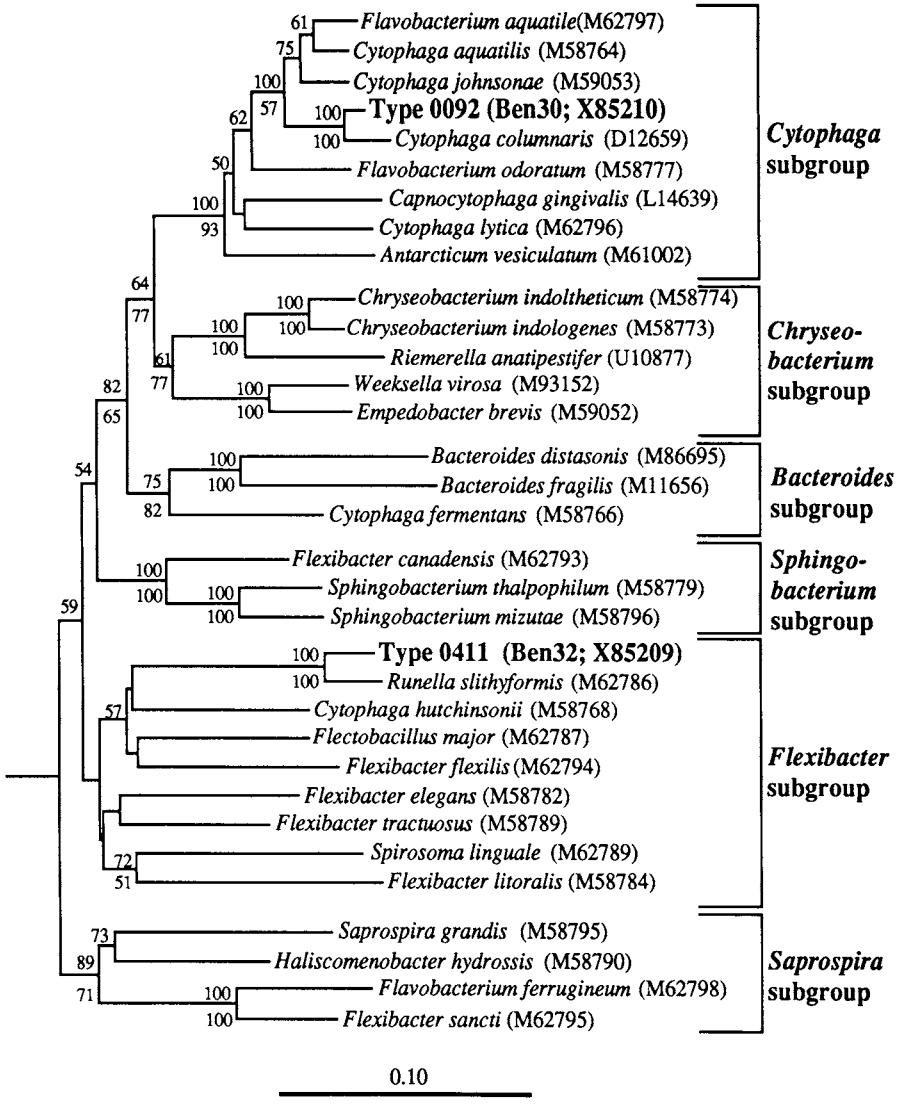

Fig. 4. Evolutionary distance tree of the *Flexibacter-Cytophaga-Bacteroides* phylum based on the comparative analysis of 1130 nucleotide positions. Database accession numbers are in parenthesis after species or strain numbers. Bootstrap values (100 bootstrap resamplings) from distance (upper) and parsimony (lower) analyses are presented at the nodes. The outgroup used in the analyses but not shown in the tree was *Chlorobium limicola*.

composition. Often partial physical selection of the filaments was also included (*van Veen*, 1973). However, morphology of some filaments is known to change under different culture conditions (*Soddell* and *Seviour*, 1994). If filament morphology is different in the treatment plant compared to that seen in axenic culture in the laboratory, determining whether those colonies on the agar arose from the original filaments in the sample becomes an impossible task with this methodology. In our study, filaments were isolated using a heavily biased sampling procedure. They were identified *in situ* according to current standard methods (*Eikelboom* and *van Buijsen*, 1983, *Jenkins* et al., 1993) and then those identified filaments were micromanipulated directly from the samples to sterile growth media. We could readily confirm that the developing colonies were as a result of growth of the specific identified filaments. Therefore, we were not unduly concerned if the morphology of these filaments varied between that seen in activated sludge plants and that during growth on R2A agar in the laboratory. We isolated filaments that were identified as particular morphotypes. Our goal was to phylogenetically place them, and in future experiments, to prepare and evaluate *in situ* hybridisation probes specific for each morphotype. It is possible that each of the morphotypes described by *Eikelboom* and *van Buijsen* (1983) and *Jenkins* et al. (1993) is comprised of more than one genetic entity. However, this possibly can be pursued by employing techniques that we have used here. More isolates of each of the morphotypes reported here are being specifically obtained and studied in the same way.

Comparison of our isolates with previous isolates of the morphotypes

This is the second report of the cultivation to pure culture of Type 0803. *Williams* and *Unz* (1985) isolated Type 0803 from a plant treating domestic/industrial wastewater. Colonies of their Type 0803 were similar to ours and conventional phenotypic tests of their strain gave mostly negative results. Cell, filament and colony descriptions and sizes were also given and these concurred with descriptions and dimensions of our Type 0803. *Williams* and *Unz* (1985) concluded that their single Type 0803 strain appeared most similar to the original morphological description of "*Microthrix parvicella*" which has recently been phylogenetically placed in the actinomycetes subphylum (*Blackall* et al., 1994). Apart from cellular and colony morphology, only two definitive characters (Gram negative reaction and presence of intracellular volutin) were determined for Type 0803 (*Williams* and *Unz*, 1985), and one of these (the Gram reaction by staining) was different to the result for "*M. parvicella*". The supposed similarity between Type 0803 and "*M. parvicella*" relied solely on cellular morphology (*Williams* and *Unz*, 1985) which is partially determined by environmental conditions. Therefore, these organisms could be morphologically similar but unrelated and the phylogenetic placement of our Type 0803 rules out a close relationship with "*M. parvicella*".

Our isolate of *Herpetosiphon* grew from a micromanipulated filament that was identified *in situ* as *Herpetosiphon*. Its colonies resembled those of other strains of *Herpetosiphon* which had been previously isolated from bulking activated sludges (*Senghas* and *Lingens*, 1985, *Trick* and *Lingens*, 1984). According to *Reichenbach* (1992 a), *Herpetosiphon* filaments fragment into shorter pieces and ultimately to single cells depending upon the strain, the growth medium and the age of the culture. Speculation of the role of *Herpetosiphon* in sewage treatment extended beyond their involvement in bulking sludge to degradation of macromolecules in the influent and to the control of other microbial populations (*Reichenbach*, 1992 a).

The remaining two filament types addressed in this paper (Type 0092 and Type 0411) belong to the *Flexibacter-Cytophaga-Bacteroides* phylum in the domain *Bacteria* (Fig. 4).

Eikelboom gave the name Type 0092 to filaments that turn completely grey-black after staining by the Neisser stain (*Eikelboom*, 1975). This is still a distinctive feature of these organisms. *Horan* et al. (1988) reported the only other isolation of Type 0092 to pure culture. Unfortunately, they did not describe their Type 0092 colonies, no micrographs of the filaments were presented, and their phenotypic characterisation was not useful in assisting with an identification. Morphological variability related to growth conditions was also reported (*Buali* and *Horan*, 1989). However, it is not possible to compare our Type 0092 isolate with that of *Horan* et al. (1988).

Eikelboom (1975) described his Type 0411 colonies as almost circular and colourless on a defined medium and as yellow, orange, or red-coloured on media of a higher nutritional value. Although his Type 0411 colonies were slightly larger than ours, differences in the media used may be responsible. Several researchers (*Eikelboom*, 1975, *Pipes*, 1978, *van Veen*, 1973) have suggested that Type 0411 is a *Flavobacterium* or *Flexibacter* sp. We have shown that this original tentative suggestion was correct for our isolate.

Phylogenetic analysis

Two strains of Type 0803, Ben 04B and Ben 05B, were simultaneously obtained from one plant (Benalla, Victoria, Australia) and their overall 16S rRNA sequence similarity is 99.9%. The sequences of both strains of Type 0803 possess all the signature nucleotides for the beta proteobacteria with a C at position 449, a U at position 640 and a G at position 1219 (*Woese*, 1987). They belong in the *Rubrivivax* subgroup of the *Rubrivivax gelatinosus* group of the beta proteobacterial subclass in the domain *Bacteria* (*sensu* RDP, *Larsen* et al., 1993). The phylogenetic analysis of Type 0803 showed that it is not closely related to any other known bacterium with *Variovorax paradoxus* having the most similar 16S rRNA (94.8%). *V. paradoxus* belongs in the *Comamonadaceae*, previously known as the acidovorans rRNA complex (*Willems* et al., 1991) and its members are genotypically highly related but phenotypically diverse. They are common soil and water inhabitants. Other sewage organisms in the beta proteobacterial extended dataset included the well known bulking filamentous bacteria *Sphaerotilus natans* and "*Leptothrix discophora*". Neither of these is considered to belong in the *Comamonadaceae* (*Willems* et al., 1991) but both are members of the *Rubrivivax* subgroup (*Corstjens* and *Muyzer*, 1993). There is a one base mismatch between the SNA (*S. natans*) probe of *Wagner* et al. (1994 a) and our sequence of Type 0803 (at position 662), a two base mismatch between their LDI ("*L. discophora*") probe and Type 0803 (at positions 653 and 658), but there is a perfect match between their CTE probe (specific for *Comamonas testosteroni* and relatives) and our Type 0803 sequence.

The phylogenetic identification of *Herpetosiphon* sp. Ben 15 confirmed its morphological identification as a member of the genus *Herpetosiphon*. The 16S rRNA sequence of Ben 15 exhibits the *Herpetosiphon* signature nucleotides and the truncated loop in the V4 region, characteristic of the green, non-sulfur group (*Woese*, 1987). This group of bacteria is phylogenetically cohesive despite the apparent diversity in phenotypic characters of its few known members. Our 16S rRNA sequence for *Herpetosiphon* Ben 15 is the second one available for members of the genus, although 16S rRNA cataloguing was done for this genus and its presumed close relatives (*Reichenbach* et al., 1986). Their data ruled out any relationship between *Herpetosiphon* and other environmental gliding organisms, like *Vitreoscilla, Beggiatoa* and *Leucothrix* (*Woese* et al., 1985), all of which have been implicated in sludge bulking (*Cyrus* and *Sladká*, 1970, *Farquhar* and *Boyle*, 1971 b, *Pipes*, 1978). The first 16S rRNA sequence obtained for *Herpetosiphon* was for *H.*

aurantiacus (M34117), and the sequence of Ben 15 is 98.6% similar to this, indicating that Ben 15 could be a representative of this species.

The closest neighbour of our Type 0092 is *Cytophaga columnaris,* a bacterium which causes columnaris disease in aquarium fishes typically being found in warm-water (*Reichenbach,* 1992 b). *Cy. columnaris* is a gliding bacterium whose motility has been well studied (*Pate* et al., 1967). Insufficient correlative data exists at present for Type 0092 and *Cy. columnaris* to comment on their possible phylogenetic relationships.

Type 0411 is most closely related to *Runella slithyformis* (M62786) by 16S rRNA sequence similarity (93.6%).These bacteria belong in the *Flexibacter litoralis* subgroup in subdivision II of the phylum (*Larsen* et al., 1993) – in Fig. 4, the *Flexibacter* subgroup. *R. slithyformis* cells are rigid, straight to curved rods that when joined together produce unusual ring -or crescent-shaped structures (*Larkin* and *Borrall,* 1989). Although this cellular morphology is not characteristic of Type 0411, *R. slithyformis* does produce filaments of up to 14 μm in length and the filaments of Type 0411 are usually strongly coiled (*Eikelboom,* 1975) as clearly shown in previously published scanning electron micrographs of this organism (*Seviour* et al., 1994).

Signature nucleotides of the *Flexibacter-Cytophaga-Bacteroides* phylum were obtained from *Woese* (1987) for comparative purposes. These included a U at 570, and A at 995, and a G at 1410 (*E. coli* numbering). Most members of the phylum also have an A at position 1198 and an A at position 1532. Comparative data are presented in Table 4. Most of the signature features from *Woese* et al. (1990) were present in members of the phylum in our analysis. The 1530–1533 Shine-Dalgarno sequence leader was not available for comparison because we used the 1492r primer to amplify the 16S rDNA. All signatures (*sensu Woese,* 1987) were present in the sequence of Type 0092. The sequence of Type 0411 possesses most of the phylum signatures. The phylum-characteristic A at position 995 was a U in Type 0411 and other nucleotides that are characteristic for the flavobacteria (*sensu Woese,* 1987) were also aberrant (Table 4). This was also the case for *R. slithyformis* for all these signatures including the U at 986, except that *R. slithyformis* had the characteristic G at position 809. The CF (cytophaga-flavobacterium cluster) probe of *Wagner* et al. (1994 a) has one mismatch with Type 0411 at position 328. The same mismatch occurs for *R. slithyformis* and *Cy. hutchinsonii.* There is a perfect match between the CF probe and the sequence of Type 0092.

Table 4. Signature nucleotides in the 16S rRNA for the *Flexibacter-Cytophaga-Bacteroides* phylum and their presence in Type 0092 and Type 0411

Position[1]	Bacteroides[2]	Flavobacteria[2]	Consensus[2]	Type 0092	Type 0411
306	G	A	A	A	G
718	U	A	A	A	G
809	A	G	G	G	A
947	A	G	G	G	G
986	C	A/U	A/U	A	U
995	A	A	C	A	U
1198	A	Ag	A	A	G
1224	Ug	Uc	U	U	U
1233	G	G	G	G	G
1234	U	C	C	C	C
1356	C	?	G	G	U

[1] *E. coli* numbering

[2] From *Woese* (1987). Composition upper case – major base; if no other specified, then it accounts for >90% of assayable cases. Composition lower case – minor occurrence base; found in <15% of assayable cases (or in only one species for groups containing seven or less species). ? – no nucleotide could be assigned

Conclusion

In conclusion, we have phylogenetically placed four different bulking filamentous morphotypes. Type 0803 is the only one not closely related to other bacteria that are notably filamentous in their morphology at some stage of their lifecycle. However, since morphology is dependent upon environmental parameters, filamentation may have been overlooked in this group. It was stressed by *Woese* et al. (1990) that morphological characters are of limited use as indicators of phylogenetic relationships. A range of exceptions to this statement were highlighted, including the complex morphology of spirochaetes. Morphogenetic processes could be crucially important in the control of bulking in activated sludge since if the bulking bacteria could be encouraged to grow as shorter filaments, bulking may be alleviated. Morphological variability is also an important consideration in the isolation of bulking filamentous bacteria. Micromanipulation is a method where one can be confident that the isolated bacteria originated from a specific filament in the sample.

The sequences of the four morphotypes are now being examined to identify morphotype-specific regions that could be exploited for preparation of *in situ* hybridisation probes as has been achieved with other bulking filaments (*Wagner* et al., 1994 a, *Wagner* et al., 1994 b) and more recently with *Zoogloea ramigera* (*Rosselló-Mora* et al., 1995) in the activated sludge milieu. For valid taxonomic descriptions of our isolates, further physiological and phenotypic characterization is needed.

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