Phylogenetic relationships of filamentous sulfur bacteria (*Thiothrix* spp. and Eikelboom type 021N bacteria) isolated from wastewater-treatment plants and description of *Thiothrix eikelboomii* sp. nov., *Thiothrix unzii* sp. nov., *Thiothrix fructosivorans* sp. nov. and *Thiothrix defluvii* sp. nov.

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The relationship of mixotrophic and autotrophic *Thiothrix* species to morphologically similar chemoorganotrophic bacteria (e.g. *Leucothrix* species, Eikelboom type 021N bacteria) has been a matter of debate for some years. These bacteria have alternatively been grouped together on the basis of shared morphological features or separated on the basis of their nutrition. Many of these bacteria are difficult to maintain in axenic culture and, until recently, few isolates were available to allow comprehensive phenotypic and genotypic characterization. Several isolates of *Thiothrix* spp. and Eikelboom type 021N strains were characterized by comparative 16S rRNA sequence analysis. This revealed that the *Thiothrix* spp. and Eikelboom type 021N isolates formed a monophyletic group. Furthermore, isolates of Eikelboom type 021N bacteria isolated independently from different continents were phylogenetically closely related. The 16S rRNA sequence-based phylogeny was congruent with the morphological similarities between *Thiothrix* and Eikelboom type 021N. However, one isolate examined in this study (Ben47) shared many morphological features with the *Thiothrix* spp. and Eikelboom type 021N isolates, but was not closely related to them phylogenetically. Consequently, morphology alone cannot be used to assign bacteria to the *Thiothrix*/type 021N group. Comparative 16S rRNA sequence analysis supports monophyly of the *Thiothrix*/type 021N group, and phenotypic differences between the *Thiothrix* spp. and Eikelboom type 021N bacteria are currently poorly defined. For example, both groups include heterotrophic organisms that deposit intracellular elemental sulfur. It is therefore proposed that the Eikelboom type 021N bacteria should be accommodated within the genus *Thiothrix* as a new species, *Thiothrix eikelboomii* sp. nov., and three further new *Thiothrix* species are described: *Thiothrix unzii* sp. nov., *Thiothrix fructosivorans* sp. nov. and *Thiothrix defluvii* sp. nov.

Keywords: *Thiothrix*, Eikelboom type 021N, *Leucothrix mucor*, filamentous sulfur bacteria

INTRODUCTION

Members of the genus *Thiothrix* are colourless, filamentous bacteria that oxidize reduced sulfur compounds (Williams *et al.*, 1987). They also produce gliding gonidia, form rosettes and have the ability to deposit intracellular sulfur granules (Larkin & Strohl, 1983; Williams & Unz, 1985a, b; Williams *et al.*, 1987). *Thiothrix* species are found in a number of habitats, ranging from sulfide-containing natural waters (Brigmon *et al.*, 1994a; Jones *et al.*, 1982; McGlannan & Makemson, 1990) and irrigation systems (Ford & Tucker, 1975) to activated-sludge wastewater-treatment plants, where their presence in large numbers contributes to the problem of filamentous sludge bulking (Brigmon *et al.*, 1994b; Eikelboom, 1975; Tandoi *et al.*, 1994; Unz & Williams, 1989; Wagner *et al.*, 1994; Williams & Unz, 1985a). The involvement of *Thiothrix* species as causative organisms of filamentous sludge bulking in wastewater-treatment plants has led to studies of their physiology (Richard *et al.*, 1985; Williams & Unz, 1985a, b; Tandoi *et al.*, 1994), ultrastructure (Brigmon *et al.*, 1994b; Williams *et al.*, 1987), growth characteristics (Unz & Williams, 1989; Williams & Unz, 1989) and interactions with heavy metals (Shuttleworth & Unz, 1991, 1993).

It has been suggested that members of the genus *Thiothrix* may be amenable to characterization by morphology alone, due to holdfast formation and their ability to form characteristic rosettes, properties which have been argued to be defining features of the genus (Polz *et al.*, 1996). However, some filamentous bacteria designated Eikelboom type 021N (Eikelboom, 1975) and *Leucothrix mucor* also form rosette-like structures resembling those produced by *Thiothrix* species and produce gonidia (Brock, 1992; Williams & Unz, 1985a). As a result, there has been debate regarding the relationship between these bacterial taxa and the genus *Thiothrix* (Brock, 1974, 1992; Eikelboom, 1975; Harold & Stanier, 1955; Larkin & Strohl, 1983). Woese (1987) has stressed that morphological characteristics are of little use as indicators of phylogenetic relationships among the majority of bacteria and, in axenic cultures of *Thiothrix* species, the morphology of filaments is known to change (Brigmon *et al.*, 1995; Shuttleworth & Unz, 1991, 1993). However, *in situ* morphology and staining reactions have been used extensively to characterize filamentous bacteria associated with activated-sludge bulking and foaming (Eikelboom, 1975), but, because these characteristics can vary depending upon environmental conditions, they are singularly of limited diagnostic value.

The genera *Thiothrix* and *Leucothrix* have been alternatively grouped together in the family *Leucotrichaceae* on the basis of morphology (Brock, 1974) and separated on the basis of physiology (Reichenbach & Dworkin, 1981). Furthermore, bacteria termed '*Leucothrix cohaerens*' (Pringsheim, 1957; Cyrus & Sladká,

1970) and *Thiothrix* sp. forms I and II (Farquhar & Boyle, 1971) have been equated with Eikelboom type 021N bacteria (Eikelboom, 1975). Clearly, much remains to be resolved regarding the relationships among these important bacteria, and the taxonomic status of Eikelboom type 021N bacteria and *Thiothrix* isolates remains unclear.

Only three named *Thiothrix* species, *Thiothrix nivea*, '*Thiothrix ramosa*' and '*Thiothrix arctophilia*' (excluding species *incertae sedis*), have been studied extensively. These have been isolated in pure culture and subjected to phenotypic and genotypic characterization (Lane *et al.*, 1992; Larkin & Shinabarger, 1983; Odintsova & Dubinina, 1990; Dul'tseva & Dubinina, 1994; Polz *et al.*, 1996; Teske *et al.*, 1996). Of these, *Thiothrix nivea* alone appears in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980). Although *Bergey's Manual of Systematic Bacteriology* lists six other *Thiothrix* species (Larkin, 1989), the phylogenetic relationships between these organisms are not known.

Recently, molecular biological approaches based on rRNA sequence analysis have allowed some of the deeper relationships between morphologically distinct sulfide-oxidizing bacteria to be unravelled (Head *et al.*, 1996; Polz *et al.*, 1996; Teske *et al.*, 1996). In this study, the phylogeny of a number of *Thiothrix* isolates [including three of those listed by Larkin (1989)] was investigated in relation to Eikelboom type 021N bacteria isolated from different geographical locations, using 16S rRNA sequence analysis.

METHODS

Bacterial strains. *Thiothrix* and Eikelboom type 021N strains used in this study were obtained from the ATCC, Manassas, VA, USA, and from our own cultures (Table 1). *Thiothrix* sp. strain A1^T, Eikelboom type 021N bacteria strains AP3^T, Ben44–Ben48 and *Thiothrix* sp. form I Ben57^T were isolated on GS medium and *Thiothrix* sp. strains I and Q^T were isolated on LT medium (Williams & Unz, 1985a; Hudson *et al.*, 1994). Eikelboom type 021N strains Ben49 and Ben50 were isolated on EGG medium (Kohno, 1988). All Ben strains were grown on their respective isolation media and all other strains were grown in LTH medium (Williams & Unz, 1985a, 1989). It should be noted that *Thiothrix* sp. form I is a particular bacterial morphotype identified in sewage sludge (Farquhar & Boyle, 1971; Eikelboom, 1975) and is distinct from *Thiothrix* sp. strain I, which was also analysed in this study.

DNA extraction. DNA was extracted either directly from cryopreserved biomass or from cultures grown in LTH medium. Biomass placed in a 1.5 ml microcentrifuge tube was washed three times in 1 ml sterile TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and resuspended in 0.1 ml TE buffer. TE buffer containing 3% (w/v) SDS (0.2 ml) was added and the cells were vortexed for 3 min. The cell lysate was extracted three times with TE-buffered phenol and three times with chloroform. The aqueous phase was transferred to a fresh microcentrifuge tube and 2 vols ice-cold absolute ethanol (98% v/v) was added. The DNA was precipitated

Table 1. Bacterial strains used in this study

AS, activated sludge; HN, high-nitrogen; HS, high-sulfur; Lab., isolated from a lab bioreactor; NA, not applicable.

Organism	Strain(s)	New designation	ATCC no.	Isolation	Reference
<i>Thiothrix</i> sp.	A1 ^T	<i>T. unzii</i>	49747 ^T	AS, USA	Williams & Unz (1985a)
<i>Thiothrix</i> sp.	Q ^T	<i>T. fructosivorans</i>	49748 ^T	AS, USA	Williams & Unz (1985a)
<i>Thiothrix</i> sp.	I	<i>T. fructosivorans</i>	49749	AS, USA	Williams & Unz (1985a)
<i>Thiothrix</i> sp. form I	Ben57 ^T	<i>T. defluvii</i>	NA	AS, Australia	This study
Eikelboom 021N	AP3 ^T	<i>T. eikelboomii</i>	49788 ^T	AS, USA	Williams & Unz (1985a)
Eikelboom 021N	Ben44 (YJ1)	<i>T. eikelboomii</i>	NA	HN AS, Japan	Hudson <i>et al.</i> (1994)
Eikelboom 021N	Ben45 (YJ2)	<i>T. eikelboomii</i>	NA	HS AS, Japan	Hudson <i>et al.</i> (1994)
Eikelboom 021N	Ben46 (YJ4)	<i>T. eikelboomii</i>	NA	Lab. AS, Japan	Hudson <i>et al.</i> (1994)
Eikelboom 021N	Ben47 (YJ5)	–	NA	Lab. AS, Japan	Hudson <i>et al.</i> (1994)
Eikelboom 021N	Ben48 (Bathurst)	<i>T. eikelboomii</i>	NA	AS, Australia	This study
Eikelboom 021N	Ben49 (Carrum 92)	<i>T. eikelboomii</i>	NA	AS, Australia	This study
Eikelboom 021N	Ben50 (Carrum 95)	<i>T. eikelboomii</i>	NA	AS, Australia	This study

overnight at -20°C and pelleted by centrifugation (13000 r.p.m., 15 min, MSE Microcentaur). The DNA was redissolved in 75 μl sterile TE buffer and used directly for PCR. For the Ben strains, DNA was not extracted from the cellular biomass that was obtained from colonies on agar plates and used directly as material for amplification (Blackall, 1994).

Amplification and sequencing of 16S rRNA genes. 16S rRNA genes were amplified using primers pA and pH' (Edwards *et al.*, 1989) and 'touchdown' PCR (initial annealing temperature 65°C , final annealing temperature 55°C ; Don *et al.*, 1991) using Dynazyme DNA polymerase (Flowgen). PCR products were purified using SpinBind cartridges (Flowgen) and sequenced using a Dye Deoxy cycle sequencing kit and an ABI 373A automated DNA sequencer (Applied Biosystems). The primers of Edwards *et al.* (1989) were used for DNA sequencing. 16S rDNA sequence data for the Ben strains were obtained by the methods described by Blackall (1994). Approximately 1300 bp of 16S rRNA sequence was obtained from *Thiothrix* sp. strain A1^T, *Thiothrix* sp. strain Q^T, *Thiothrix* sp. strain I, *Thiothrix* sp. form I strain Ben57^T and Eikelboom type 021N strain AP3^T. The length of sequence obtained from Ben44 to Ben50 ranged from 308 to 413 bp.

Phylogenetic analysis. The SIMILARITY-RANK programme of the Ribosomal Database Project (RDP) database was used to assess the closest relatives of each sequence obtained. A subset of 16S rRNA sequences, showing highest similarity to the sequences determined here, was used in phylogenetic analyses. Sequences obtained from the RDP (Maidak *et al.*, 1994) and GenBank were aligned with the sequences from this study using the GDE sequence editor (Smith *et al.*, 1994). Sequence alignments were corrected manually by comparison with the secondary structure of *Dichelobacter nodosus* (γ -Proteobacteria) 16S rRNA. A subset of 40 bacterial 16S rRNA sequences from the γ -Proteobacteria, including the sequence from the type strain of *Leucothrix mucor*, DSM 2157^T (Ludwig *et al.*, 1995), was used for phylogenetic analyses with near-complete 16S rRNA sequences. The shorter aligned dataset contained 23 sequences. Sequences from *Myxococcus xanthus* DK1622 and *Desulfobacter postgatei* DSM 2034^T (δ -Proteobacteria) were included as outgroups. The final alignment of near-complete

sequences consisted of 1148 unambiguously aligned positions corresponding to *Escherichia coli* positions 112–203 and 228–1314. The shorter alignment consisted of 300 positions corresponding to *E. coli* positions 595–895. Distance analyses using the Jukes and Cantor correction (Jukes & Cantor, 1969) were performed with the TREECON package (Van de Peer & De Wachter, 1994) and trees were generated from distance matrices by the neighbour-joining method (Saitou & Nei, 1987). Parsimony analyses were accomplished with the DNAPARS program (Felsenstein, 1989) and maximum-likelihood analyses (Felsenstein, 1981) were done using fastDNAm1 (Olsen *et al.*, 1994). Sequence data for distance matrix and parsimony analyses were subjected to bootstrap resampling (100 replicates). For distance analysis, this was done using TREECON and for parsimony analysis, bootstrapped datasets were generated using the SEQBOOT program and consensus trees were constructed with the CONSENSE program from the PHYLIP package (Felsenstein, 1989). Preliminary analysis of some of the sequence data has been presented previously (Howarth *et al.*, 1998).

RESULTS AND DISCUSSION

The PCR was used to amplify 16S rRNA genes from pure cultures of *Thiothrix* sp. and Eikelboom type 021N isolates. Preliminary analysis of short sequence datasets indicated that Eikelboom type 021N strains AP3^T, Ben44, Ben45, Ben46, Ben48, Ben49 and Ben50 exhibited a mean 16S rRNA sequence homology of 98.7% (range 97.3–100%) and a near-complete sequence was determined only for strain AP3^T. All of the isolates studied fitted the appropriate morphological and phenotypic descriptions of the genus *Thiothrix* or Eikelboom type 021N bacteria (Hudson *et al.*, 1994; Williams & Unz, 1985a).

Phylogeny of filamentous sulfur bacteria related to *Thiothrix* species and placement of Eikelboom type 021N bacteria in the genus *Thiothrix*

All of the *Thiothrix* isolates and all but one of the Eikelboom type 021N bacteria investigated were mem-

Fig. 1. Phylogenetic tree derived from 16S rRNA sequences of members of the γ -Proteobacteria related to *Thiothrix*. The alignment comprised 1148 unambiguously aligned nucleotides. Figures at nodes represent percentage bootstrap values. Figures above the nodes are from distance analysis those below the nodes are from parsimony analysis. Bootstrap values below 50% have been omitted. Sequences from *Myxococcus xanthus* and *Desulfobacter postgatei* DSM 2034^T (δ -Proteobacteria) were used as an outgroup to root the tree.

bers of the γ -Proteobacteria (Figs 1 and 2). On the basis of comparative 16S rRNA analysis, these organisms formed a monophyletic group with strong bootstrap support (100% distance and parsimony). This finding concurs with the work of Polz *et al.* (1996) and Teske *et al.* (1996), which indicated that the genus *Thiothrix* formed a deeply branching lineage of the γ -Proteobacteria most closely related to sulfide-oxidizing symbionts of marine invertebrates and *Thiomicrospira* species.

However, the taxonomic status of the Eikelboom type 021N bacteria is unclear (Williams & Unz, 1985a, 1989) and it has been commented that they were unlikely to belong to the genus *Thiothrix* (Eikelboom, 1975). Eikelboom type 021N strains share some phenotypic characteristics with both *Thiothrix* and *Leucothrix* and it has been suggested that a new genus should be created for Eikelboom type 021N bacteria (Williams & Unz, 1985a, 1987; Ziegler *et al.*, 1990; Brock, 1992). Many Eikelboom type 021N

Fig. 2. Phylogenetic tree derived from 16S rRNA sequences of selected members of the α -Proteobacteria and members of the γ -Proteobacteria related to *Thiothrix*. The alignment consisted of 300 unambiguously aligned nucleotides. Figures at nodes represent percentage bootstrap values; values below 50% have been omitted. Sequences from *Myxococcus xanthus* and *Desulfobacter postgatei* DSM 2034^T (δ -Proteobacteria) were used as an outgroup to root the tree.

bacteria resemble *Thiothrix* species in that they form long trichomes and deposit internal sulfur (Hudson *et al.*, 1994; Williams & Unz, 1985a). Unlike many *Thiothrix* isolates, Eikelboom type 021N bacteria do not produce a sheath. However, sheath formation has not been observed in all *Thiothrix* strains (Williams & Unz, 1985b), e.g. *Thiothrix* sp. strains A1^T and A3 (Williams & Unz, 1985a; Williams *et al.*, 1987), and thus the absence of a sheath cannot be used to differentiate Eikelboom type 021N bacteria from members of the genus *Thiothrix*. Furthermore, recognition of a sheath using light microscopy can be problematic and the absence of a sheath may only be determined confidently by electron microscopy (Williams *et al.*, 1987). A number of Eikelboom type 021N strains have also been observed to form rosettes and gliding gonidia similar to those produced by *Thiothrix* species (Eikelboom, 1975; Hudson *et al.*, 1994; Williams & Unz, 1985a; Ziegler *et al.*, 1990). However, strain AP3^T, examined in the current study, was not noted to form rosettes (Williams & Unz, 1985a), yet this isolate was closely related to rosette-forming Eikelboom type 021N isolates (e.g. Ben50). Heterotrophic growth has also been considered as a feature that distinguishes Eikelboom type 021N bacteria from *Thiothrix* species. Although most Eikelboom type 021N strains can utilize a wider range of organic substrates than *Thiothrix* species, heterotrophic *Thiothrix* isolates, including strains I and Q^T from this study, are known (Williams & Unz, 1985a; Larkin, 1989).

Although they occupy the deepest branches within the *Thiothrix* clade, strains AP3^T and Ben57^T fall within these phenotypic constraints and thus can be accommodated as distinct species in the genus *Thiothrix*.

Furthermore, the 16S rRNA from all of the strains in the *Thiothrix*/type 021N group had a characteristic deletion in a stem-loop structure corresponding to positions 455–477 (*E. coli* numbering). Within the γ -Proteobacteria, this is unique to the *Thiothrix* clade, although this secondary structure motif has been identified in some members of the ϵ -Proteobacteria (Lane *et al.*, 1992; Polz *et al.*, 1996). It is clear that there are phenotypic differences between strains AP3^T and Ben57^T and the other organisms studied here. However, we would argue that these are not sufficiently diagnostic to warrant the designation of a new genus or genera to accommodate these organisms. The strong support for monophyly of this group and the shared morphological and phenotypic characteristics are compatible with inclusion of *Thiothrix* sp. strain A1^T, strain I, strain Q^T, form I Ben57^T, *T. nivea* JP2^T, '*T. ramosa*' and the Eikelboom 021N bacteria within a single genus. Nevertheless, as more phenotypic and genotypic information on organisms related to Ben57^T and AP3^T becomes available, this may require revision.

Description of new *Thiothrix* species from wastewater-treatment systems

Delineation of taxa at the level of species on the basis of 16S rRNA sequence similarities is not straightforward (Stackebrandt & Goebel, 1994). It has been shown that, at rRNA sequence similarities below 97.5%, it is unlikely that two organisms exhibit greater than 60–70% reassociation of genomic DNA and at values below 97.0%, DNA reassociation is unlikely to be above 60% (Stackebrandt & Goebel, 1994). Thus, strains with less than 97.5% rRNA similarity probably represent distinct species. On this basis, *Thiothrix* sp.

Table 2. 16S rRNA sequence identity of *Thiothrix* species, Ben57^T, 021N strain AP3^T and *L. mucor* DSM 2157^T

The data were generated from 1148 unambiguously aligned positions and did not take into account insertions or deletions.

Organism	1	2	3	4	5	6	7	8
1. <i>T. nivea</i> JP2 ^T	100							
2. ' <i>T. ramosa</i> '	94.1	100						
3. <i>Thiothrix</i> sp. A1 ^T	94.7	94.1	100					
4. <i>Thiothrix</i> sp. I	94.0	97.0	95.1	100				
5. <i>Thiothrix</i> sp. Q ^T	94.4	97.4	95.4	99.4	100			
6. 021N type AP3 ^T	91.8	91.8	91.5	91.7	92.2	100		
7. Ben57 ^T	90.2	89.9	89.2	89.7	89.9	91.0	100	
8. <i>L. mucor</i> DSM 2157 ^T	87.3	87.5	87.0	88.0	88.4	86.6	86.9	100

strain A1^T, *Thiothrix* sp. form I (Ben57^T) and Eikelboom type 021N strain AP3^T clearly represent distinct species. *Thiothrix* sp. strains I and Q^T are also distinct from all other described *Thiothrix* species but share a high degree of 16S rRNA sequence identity (99.4%).

The 16S rRNA sequence identity of *Thiothrix* sp. A1^T to *T. nivea* JP2^T and '*T. ramosa*' was 94.7 and 94.1%, respectively, clearly delineating strain A1^T as a distinct species. Phenotypic characteristics also distinguished strain A1^T from named *Thiothrix* species and the other isolates examined in the current study. For example, like *T. nivea* JP2^T, strain A1^T required reduced sulfur for growth and utilized a limited range of organic carbon sources, but filaments of strain A1^T did not possess a sheath (Williams & Unz, 1985a; Larkin, 1989). Its inability to utilize fructose, sucrose or melezitose for growth distinguished it from strains I and Q^T and similarly it can be distinguished from Eikelboom type 021N isolates that can utilize a broad range of organic substrates (Williams & Unz, 1985a). We propose the name *Thiothrix unzii* sp. nov. for strain A1^T.

Comparative 16S rRNA sequence analyses suggested that strains Q^T and I were most closely related to '*T. ramosa*', a facultatively autotrophic strain (Fig. 1). The 16S rRNA sequences from strains I and Q^T were 97.0% (strain I) and 97.4% (strain Q^T) identical to the '*T. ramosa*' sequence, indicating that they were unlikely to be '*T. ramosa*' isolates. Moreover, *Thiothrix* sp. strains I and Q^T are heterotrophs and do not require reduced sulfur for growth (Larkin, 1989), whereas '*T. ramosa*' is a facultative chemolitho-autotroph (Odintsova *et al.*, 1993), further supporting the separation of *Thiothrix* sp. strains I and Q^T from '*T. ramosa*'. 16S rRNA identity between *Thiothrix* sp. strains I and Q^T was 99.4% (Table 2). High rRNA sequence identity does not always indicate a relationship at the species level (Fox *et al.*, 1992) and it is not possible to draw firm conclusions on the basis of rRNA sequence data alone. However, strains I and Q^T are phenotypically extremely similar and have identical

carbohydrate utilization patterns and temperature and pH optima for growth and were isolated from the same activated-sludge plant (Williams & Unz, 1985a). Cells of strains I and Q^T also have similar dimensions (1.2–2.5 × 1.2–2.5 µm) that are distinct from other *Thiothrix* species (e.g. *Thiothrix* sp. strain A1^T; 0.7–3.0 × 0.7–1.5 µm) (Williams & Unz, 1985a). Thus, *Thiothrix* sp. strains I and Q^T are likely to belong to a single species, which we have designated *Thiothrix fructosivorans* sp. nov. since, unlike *T. nivea* JP2^T and *Thiothrix* sp. strain A1^T, it is capable of growth on fructose as a carbon and energy source (Williams & Unz, 1985a; Larkin, 1989).

Although it is apparent that the phenotypic characters frequently used to differentiate Eikelboom type 021N bacteria from *Thiothrix* species are not totally reliable, the 16S rRNA sequence of Eikelboom type 021N strain AP3^T had lower identity to the *T. nivea* JP2^T sequence (91.8%) than did the sequences from the named *Thiothrix* strains A1^T, Q^T and I examined in this study (94.0–94.7%). Likewise, five strains of Eikelboom type 021N bacteria studied by Kanagawa *et al.* (1998) had a mean 16S rRNA identity to *T. nivea* JP2^T of 91.1% and the G+C content of the DNA from three of their isolates was in the range 44–45 mol% (Kanagawa *et al.*, 1998). This is distinct from the G+C content of *Thiothrix* species (51–55%; Dul'tseva & Dubinina, 1994). Despite the relatively low 16S rRNA sequence identity and differences in G+C content between *Thiothrix* and Eikelboom type 021N isolates, there was strong support from bootstrap analysis (100%, distance and parsimony) for grouping Eikelboom type 021N strain AP3^T and *Thiothrix* sp. form I Ben57^T within the genus *Thiothrix* (Fig. 1). Maximum-likelihood analysis also supported this conclusion (data not shown). *Thiothrix* sp. form I has been equated with Eikelboom type 021N bacteria (Eikelboom, 1975), but it was considered that these organisms did not deposit large amounts of intracellular elemental sulfur and thus it was concluded that they were not members of the genus *Thiothrix* (Eikelboom, 1975). Nonetheless, many Eikelboom type 021N bac-

teria and *Thiothrix* sp. form I deposit intracellular elemental sulfur (Farquhar & Boyle, 1971; Williams & Unz, 1985a) but do not require reduced sulfur for growth (Williams & Unz, 1985a, b). Moreover, of six *Thiothrix* species examined in one study, only two required a source of reduced inorganic sulfur for growth (Williams & Unz, 1985b).

Analyses of shorter sequence datasets, which included sequences from eight morphologically identified Eikelboom type 021N bacteria and *Thiothrix* sp. form I Ben57^T, supported their relationship with the genus *Thiothrix* (Fig. 2). However, the Eikelboom type 021N isolates formed a distinct cluster of closely related sequences (97.3–100% identity) and Ben57^T was affiliated with the Eikelboom type 021N isolates (Fig. 2). Since phenotypic characters do not clearly distinguish Eikelboom type 021N bacteria or *Thiothrix* sp. form I from other *Thiothrix* species, we propose that they be designated as new species within the genus *Thiothrix*. We propose the name *Thiothrix eikelboomii* sp. nov. for organisms closely related to Eikelboom type 021N strain AP3^T and *Thiothrix defluvii* sp. nov. for *Thiothrix* sp. form I, Ben57^T, which was isolated from a sewage-treatment plant in Australia.

Eikelboom type 021N bacteria isolated from different continents are genetically closely related

An important finding from the current study was that, despite a number of the Eikelboom type 021N isolates being obtained independently from three different continents, they were phylogenetically rather homogeneous. There was apparently no indication that clusters within the Eikelboom type 021N group were delineated on a geographical basis. For instance, strains isolated from Australia (Ben48, Ben49, Ben50) were interspersed with Japanese isolates (Ben44, Ben45, Ben46) in our phylogenetic tree (Fig. 2), which, in some cases, had identical 16S rRNA sequences to Australian isolates.

One isolate (strain Ben47), however, which was identified as an Eikelboom type 021N bacterium on the basis of rosette formation and typical morphological features of trichomes (Eikelboom, 1975; Hudson *et al.*, 1994), was most closely affiliated with the α -*Proteobacteria*, phylogenetically distant from the *Thiothrix*/type 021N group (Fig. 2). This casts doubt on the diagnostic utility of morphological characters for the identification of Eikelboom type 021N bacteria (*T. eikelboomii*) since, clearly, organisms unrelated to *Thiothrix* may also produce rosettes with a morphology normally associated with *Thiothrix* species. The limited diagnostic utility of cellular morphology for identifying filamentous bacteria in wastewater-treatment plant micro-organisms is supported by recent observations based on *in situ* whole-cell hybridization. In some cases, cells morphologically identified as Eikelboom type 021N or *Thiothrix* did not hybridize with probes targeting *T. nivea* or Eikelboom type 021N strains (Pernelle *et al.*, 1997), and Nielsen *et al.*

(1998) also found filaments morphologically identified as Eikelboom type 021N that bound the TNI probe specific for *T. nivea* but not an Eikelboom type 021N-specific probe.

The relationship of *L. mucor* DSM 2157^T to *Thiothrix* species and Eikelboom type 021N bacteria

An interesting observation made in the current study, which warrants further investigation, was that *L. mucor* DSM 2157^T may be related to the genus *Thiothrix*. Like *Thiothrix* species and some Eikelboom type 021N strains, *Leucothrix* species can form rosettes and gonidia, although filaments are not ensheathed. In general, *Leucothrix* species do not deposit intracellular sulfur, but isolates recently assigned to the genus *Leucothrix* accumulated elemental sulfur when incubated with thiosulfate or sulfide and were capable of lithoheterotrophic growth (e.g. '*Leucothrix thiophila*' strain 6WS^T; Dul'tseva *et al.*, 1996). Contradictory results concerning sulfur accumulation by *Leucothrix* species have been reported previously (Brock, 1992). Oxidation of sulfide and accumulation of elemental sulfur are characters shared with *Thiothrix* species and many Eikelboom type 021N bacteria isolated from activated sludge (Hudson *et al.*, 1994; Williams & Unz, 1985a). Moreover, the sulfur-oxidizing bacteria isolated by Dul'tseva *et al.* (1996) and assigned to the genus *Leucothrix* produced swollen cells within filaments and knots were observed in some filaments. These are all features noted in both Eikelboom type 021N bacteria (Williams & Unz, 1985a) and *L. mucor* (Brock, 1992). It is often argued that the genus *Leucothrix* is exclusively marine, whereas *Thiothrix* species have been isolated from both marine and freshwater habitats (Larkin, 1989; Brock, 1989, 1992). It is clear, however, that many non-marine *Leucothrix*-like bacteria also exist (Williams & Unz, 1985a, 1989), and habitat is not a useful defining feature for *Leucothrix* species. The phenotypic differences between *Leucothrix*, *Thiothrix* and Eikelboom type 021N are therefore rather poorly defined. Consequently, genotypic analyses are required to establish the relationships between these organisms more confidently.

16S rRNA analysis placed *L. mucor* DSM 2157^T (Ludwig *et al.*, 1995) as a deeply branching lineage at the base of the *Thiothrix* clade (Fig. 1). Thus, *L. mucor* DSM 2157^T not only has many morphological features in common with *Thiothrix* species and Eikelboom type 021N isolates, but may also share a common ancestry with these organisms. This relationship was well supported by comparative sequence analysis using a distance method (95% bootstrap support) and by maximum-likelihood analysis (data not shown), but was less well supported by parsimony analysis (77% bootstrap support). On the basis of comparative 16S rRNA analysis, '*L. thiophila*' 6WS^T was reported to be related to *L. mucor* DSM 2157^T and more distantly related to *T. nivea* JP2^T and '*T. ramosa*' (Dul'tseva *et al.*, 1996). However, no phylogenetic tree or 16S rRNA

sequence identity data including '*L. thiophila*' 6WS^T was presented (Dul'tseva *et al.*, 1996) and the 16S rRNA sequence of '*L. thiophila*' 6WS^T is not currently available from the public databases. Additional 16S rRNA sequence data from five Eikelboom type 021N strains reported by Kanagawa *et al.* (1998) were also not publicly available. It was therefore not possible to include '*L. thiophila*' 6WS^T or the Eikelboom type 021N data from Kanagawa *et al.* (1998) in our analyses and to conduct a more definitive assessment of the relationship between *Leucothrix* and *Thiothrix*. However, although comparative 16S rRNA sequence analysis suggested a relationship between these taxa, *L. mucor* DSM 2157^T lacks the 16S rRNA secondary structure motif that is characteristic of *Thiothrix* species. When more 16S rRNA sequence data become available for members of the genus *Leucothrix*, it will be possible to test the hypothesis that *Thiothrix* and *Leucothrix* share a common ancestry.

Emended description of the genus *Thiothrix* Winogradsky 1888, 39^{AL}

Thiothrix (Thi'o.thrix. Gr. n. *thium* sulfur; Gr. n. *thrix* hair; M.L. fem. n. *Thiothrix* sulfur hair).

Cells are rods, 0.7–2.6 µm in diameter and 0.7–5.0 µm in length, that exist in multicellular filaments. Gliding gonidia are produced from the end of the filaments. Gram-negative or Gram-variable. No flagella are present but a tuft of fimbriae may be present at the end of the gonidium. A sheath may be produced but is not present in all species. Rosettes may be produced and a holdfast may be present, but not in all species. Aerobic or microaerophilic. Optimum temperature 15–30 °C; maximum 32–37 °C; minimum 4–10 °C. pH range for growth 6.0–8.5. Facultatively autotrophic, mixotrophic and heterotrophic species have been isolated. Simple organic compounds are utilized as growth substrates by facultatively autotrophic and mixotrophic strains; some strains, particularly heterotrophic strains, can also utilize a range of sugars. Sulfur globules are deposited within invaginations of the cytoplasmic membrane when cells are grown in the presence of a reduced inorganic sulfur compound. Some, but not all, species have a requirement for reduced sulfur.

Found in sulfide-containing water and in wastewater-treatment systems. The G + C content of the DNA is in the range 44–55 mol% (*T_m*). The type species is *Thiothrix nivea* Winogradsky 1888, 39^{AL}.

Description of *Thiothrix unzii* sp. nov.

Thiothrix unzii (un'zi.i. M.L. gen. n. *unzii* of Unz; named for R. F. Unz, who has made important contributions to the study of filamentous bacteria in wastewater).

Cells are rods, 0.7–1.5 µm in diameter and 0.7–3.0 µm long, and form multicellular filaments. Gliding gonidia

are produced from the end of the filaments. Gram-negative. No flagella are present but a tuft of fimbriae is present at the end of the gonidium. A sheath is not present. Rosettes and a holdfast are formed. Volutin inclusions, sudanophilic granules and poly-β-hydroxybutyrate are present within cells. Growth occurs in the temperature range 4–33 °C and pH range 6.5–8.5, but there is no growth at 37 °C. Oxidase-positive and catalase-negative. Requires reduced inorganic sulfur for growth and is believed to be mixotrophic. Sulfur globules are deposited within invaginations of the cytoplasmic membrane. Gelatin and casein are hydrolysed. Starch, Tween 80 and urea are not hydrolysed. Pyruvate, succinate, acetate, lactate and propionate are utilized as sole carbon sources if sodium thiosulfate is present. Weak growth occurs with malate. No growth occurs with mannoheptulose, glucose, galactose, mannose, sorbose, fructose, rhamnose, fucose, ribose, xylose, arabinose, glyceraldehyde phosphate, maltose, sucrose, lactose, trehalose, melibiose, gentiobiose, cellobiose, melezitose, raffinose, starch, inulin, salicin, aesculin, amygdalin, mannitol, inositol, glycerol, erythritol, sorbitol, gluconate, glucuronate, galacturonate, Tween 80, tributyrin, methanol, ethanol, *n*-propanol, *n*-butanol, isoamyl alcohol, *n*-amyl alcohol, phenol, citrate, 2-oxoglutarate, formate, butyrate, hydroxybutyrate, valerate, caproate, oleate, benzoate, *m*-toluate or glycollate. Ammonia, nitrate, asparagine, glutamine, aspartate, glutamate and glucosamine are used as sole nitrogen sources. No amino acids serve as sources of both carbon and nitrogen. Nitrate is reduced to nitrite. Sulfide and thiosulfate serve as sole sulfur sources.

Isolated from activated-sludge plants. The type strain of *Thiothrix unzii* is A1^T (= ATCC 49747^T).

Description of *Thiothrix fructosivorans* sp. nov.

Thiothrix fructosivorans (fruc.to.si.vo'rans. M.L. neut. n. *fructosum* fructose; L. part. pres. *vorans* eating; M.L. adj. *fructosivorans* fructose-eating).

Cells are rods, 1.2–2.5 µm in diameter and 1.2–2.5 µm long, and form multicellular filaments. Gliding gonidia are produced from the end of the filaments. Gram-negative. No flagella are present but a tuft of fimbriae is present at the end of the gonidium. A sheath is present. Rosettes and a holdfast are formed. Volutin inclusions, sudanophilic granules and poly-β-hydroxybutyrate are observed within cells. Growth occurs in the temperature range 4–28 °C and pH range 6.5–8.5, but there is no growth at 33 °C. Oxidase-positive and weakly catalase-positive. No requirement for reduced inorganic sulfur for growth and is heterotrophic. Sulfur globules are deposited within invaginations of the cytoplasmic membrane when cells are grown in the presence of a reduced inorganic sulfur compound. Gelatin is hydrolysed. Starch, casein, Tween 80 and urea are not hydrolysed. Fructose, sucrose, melezitose, pyruvate, succinate, malate, ace-

tate, lactate and propionate are utilized as sole carbon sources. No growth occurs with mannoheptulose, glucose, galactose, mannose, sorbose, rhamnose, fucose, ribose, xylose, arabinose, glyceraldehyde phosphate, maltose, lactose, trehalose, melibiose, gentiobiose, cellobiose, raffinose, starch, inulin, salicin, aesculin, amygdalin, mannitol, inositol, glycerol, erythritol, sorbitol, gluconate, glucuronate, galacturonate, Tween 80, tributyrin, methanol, ethanol, *n*-propanol, *n*-butanol, isoamyl alcohol, *n*-amyl alcohol, phenol, citrate, 2-oxoglutarate, formate, butyrate, hydroxybutyrate, valerate, caproate, oleate, benzoate, *m*-toluate or glycollate. Ammonia, nitrate, proline, cysteine, asparagine, glutamine, aspartate, glutamate and glucosamine are used as sole nitrogen sources. Nitrite, alanine, glycine, phenylalanine, lysine, cystine, leucine or methionine cannot serve as sole nitrogen sources. No amino acids serve as sources of both carbon and nitrogen. Nitrate is reduced to nitrite.

Isolated from activated-sludge plants. The type strain of *Thiothrix fructosivorans* is Q^T (= ATCC 49748^T).

Description of *Thiothrix eikelboomii* sp. nov.

Thiothrix eikelboomii (ei.kel.boom'i.i. M.L. gen. n. *eikelboomii* of Eikelboom; named for D. H. Eikelboom, who pioneered morphological identification keys for filamentous bacteria in wastewater).

Cells are rods and form multicellular filaments. Base-to-tip differentiation in filaments is observed. Cell morphology in filaments is highly variable, and cuboidal, barrel-shaped, cylindrical, discoid and bead-like cells are often observed. Discoid cells at the base of filaments can range from 1.0 to 3.0 µm in diameter and 0.4 to 0.7 µm in length. Apical cells are 0.6–0.8 µm in diameter and 1.0–5.0 µm in length and form bead-like strings. Knots may be observed in filaments. Gram-negative or Gram-variable. A sheath is absent. Rosettes and a holdfast are formed in some but not all strains. Gliding gonidia are produced from the end of the filaments only in rosette-forming strains. No flagella are present but a tuft of fimbriae is present at the end of the gonidium. Volutin inclusions, sudanophilic granules and poly-β-hydroxybutyrate are observed within cells. Growth occurs in the temperature range 10–33 °C and pH range 6.5–8.5, but there is no growth at 37 °C. Oxidase-positive. No requirement for reduced inorganic sulfur for growth and is heterotrophic. Sulfur globules are deposited within invaginations of the cytoplasmic membrane when cells are grown in the presence of a reduced inorganic sulfur compound. Gelatin is hydrolysed. Starch, casein, Tween 80 and urea are not hydrolysed. Glucose, fructose, maltose, sucrose, trehalose, melezitose, mannitol, pyruvate, succinate, malate, acetate, lactate and propionate are utilized as sole carbon sources. No growth occurs with mannoheptulose, galactose, mannose, sorbose, rhamnose, fucose, ribose, xylose, arabinose, glyceraldehyde phosphate, lactose, melibiose, gentiobiose, cellobiose, raffinose, starch, inulin, salicin,

aesculin, amygdalin, inositol, glycerol, erythritol, sorbitol, gluconate, glucuronate, galacturonate, Tween 80 or tributyrin. Ammonia, arginine, tryptophan, proline, cysteine, phenylalanine, methionine, valine, asparagine, glutamine, aspartate, glutamate, isoleucine, tyrosine, histidine, leucine and alanine are used as sole nitrogen sources. Some isolates may also use nitrate, nitrite or urea as sole nitrogen sources. Tyrosine, histidine and glutamate are used as carbon and nitrogen sources. Nitrate is reduced to nitrite.

Isolated from activated-sludge plants. The type strain of *Thiothrix eikelboomii* is AP3^T (= ATCC 49788^T).

Description of *Thiothrix defluvii* sp. nov.

Thiothrix defluvii (de.flu'vi.i. L. neut. n. *defluvium* sewage; L. gen. n. *defluvii* of sewage).

Cells are rods, which exist in multicellular filaments. Base-to-tip differentiation of filaments is observed with basal cells being 2.0–4.0 µm in diameter and 0.5–2.0 µm in length and cells at the tips of filaments being 1.0–2.0 µm in diameter and 5.0–10 µm long. Cell shape can be irregular. Cells can be cylindrical or barrel-shaped and elongate swollen cells are often present. Gram-negative. A sheath is absent. Rosettes, a holdfast and gliding gonidia and knots in filaments may be formed but these are not features of all strains. Cells deposit intracellular elemental sulfur when grown in the presence of reduced inorganic sulfur compounds. No volutin granules are present and poly-β-hydroxybutyrate is not deposited. Growth occurs in the temperature range 10–30 °C and there is no growth at 4 or 37 °C.

Isolates of this bacterium are extremely slow growing and it has not been possible to determine the biochemical properties of this organism. The type strain is *Thiothrix defluvii* Ben57^T.

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