Characterization of filamentous Eikelboom type 021N bacteria and description of *Thiothrix disciformis* sp. nov. and *Thiothrix flexilis* sp. nov.

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The phenotypic and genotypic characteristics of 15 strains of Eikelboom type 021N bacteria isolated from wastewater treatment plants were investigated. The strains shared many characters with *Thiothrix* species. However, the Eikelboom type 021N bacteria had only 88.3–92.0% 16S rDNA sequence similarity to members of the *Thiothrix nivea* group, including *T. nivea*, '*Thiothrix ramosa*', *Thiothrix unzii* and *Thiothrix fructosivorans*, and were differentiated from them in sugar utilization and other properties, suggesting that the Eikelboom type 021N bacteria belong to species distinct from the *T. nivea* group. The 15 Eikelboom type 021N bacteria that were investigated were divided into three distinct groups (I to III) on the basis of their genotypic and phenotypic characteristics. The creation of two novel species is proposed, *Thiothrix disciformis* sp. nov. for the group I strains (type strain B3-1^T = JCM 11364^T = DSM 14473^T) and *Thiothrix flexilis* sp. nov. for the group III strains (type strain EJ2M-B^T = JCM 11135^T = DSM 14609^T). *Thiothrix eikelboomii* AP3^T was included in group II and shared many characters with the other group II strains. The inclusion of all group II strains within the species *T. eikelboomii* is proposed, together with emendation of the description of *T. eikelboomii*.

Keywords: *Thiothrix disciformis*, *Thiothrix flexilis*, Eikelboom type 021N, filamentous bacteria

INTRODUCTION

Various kinds of filamentous bacteria are observed in activated sludge, used worldwide for wastewater treatment. Excessive growth of filamentous bacteria in activated sludge causes poor settlement of activated sludge flocs, creating a severe operational problem in wastewater treatment systems that is referred to as bulking. Eikelboom (1975) classified these filamentous bacteria mainly on the basis of microscope observation. Eikelboom type 021N bacteria have been defined as sheathless, curled, multicellular bacteria that resemble blue-green algae. Eikelboom type 021N bacteria have frequently been described as causative agents of bulking and, therefore, have been studied extensively (Howarth *et al.*, 1998; Kohno, 1988;

Kanagawa *et al.*, 2000; Nielsen *et al.*, 1998; Pernelle *et al.*, 1998; Richard *et al.*, 1985; Wagner *et al.*, 1994; Williams & Unz, 1985a, b, 1989). The phylogenetically nearest neighbour of the Eikelboom type 021N bacteria is the genus *Thiothrix*, members of which share many features with Eikelboom type 021N bacteria (Howarth *et al.*, 1998, 1999; Kohno, 1988; Kanagawa *et al.*, 2000; Williams & Unz, 1985a). Howarth *et al.* (1999) emended the description of the genus *Thiothrix* Winogradsky 1888 (Larkin, 1989) to include the Eikelboom type 021N bacteria. Accordingly, Eikelboom type 021N strain AP3^T was classified as *Thiothrix eikelboomii*. Kanagawa *et al.* (2000) have recently examined the genotypic and phenotypic characteristics of 15 strains of Eikelboom type 021N bacteria isolated from bulking activated sludges and revealed a previously unknown diversity within these bacteria.

In this study, we investigated the detailed characteristics of these 15 strains of Eikelboom type 021N

bacteria. Based on their phylogenetic and phenotypic traits, we propose the creation of two novel species in the genus *Thiothrix* for Eikelboom type 021N bacteria and the emendation of the genus *Thiothrix* to include these Eikelboom type 021N isolates.

METHODS

Bacterial strains and culture media. Eikelboom type 021N group I strains B3-1^T, B4-1, B2-7, SCM-A, B5-1, B2-8 and OS-F, group II strains KR-A, T1-4, T2-1 and COM-A, group III strains SNR-3, EJ1M-B and EJ2M-B^T (Kanagawa *et al.*, 2000; Kohno, 1988) and *T. eikelboomii* AP3^T (= ATCC 49788^T) (Howarth *et al.*, 1998, 1999; Williams & Unz, 1985a, b) were analysed in this study. *T. eikelboomii* AP3^T was purchased from the ATCC. All strains were grown at 30 °C and maintained at room temperature for 3–4 weeks on medium EGGC or EGGC agar plates (Kanagawa *et al.*, 2000). Medium EGGC contained (l⁻¹) 0.3 g sodium acetate, 0.3 g Casamino acids, 0.6 g glucose and small amounts of inorganic salts and vitamins. Medium EGGCH was prepared by adding 10 mM HEPES to medium EGGC. For agar plates, media were solidified with 15 g Bacto agar or AGAR-NOBLE (Difco) l⁻¹.

Pre-culture. Cells were pre-cultured at 30 °C with shaking at 200 r.p.m. for 3–4 days in a test tube (17 × 100 mm) containing 3 ml medium EGGC. After this pre-culture, the caps of the tubes were snapped on tightly and the tubes were shaken vigorously to disperse the filamentous cells.

Electron microscopy. For investigation by transmission electron microscopy, cells were cultured as follows. Strain B3-1^T was grown on medium EGGCH for 5 days at 30 °C, strains SCM-A, AP3^T, T1-4 and COM-A were grown on medium EGGC for 6 days at 30 °C and strain EJ2M-B^T was grown on medium EGGC for 3 days at 30 °C. Cells were centrifuged and fixed with 2.5 % (v/v) glutaraldehyde and 1 or 2 % (v/v) osmium tetroxide. Samples were embedded in Spurr resin and ultrathin sections were prepared with a Reichert–Nissei ultramicrotome. Samples were stained with 2 % uranyl acetate and lead citrate (Reynolds, 1963). After treatment by vapour deposition with carbon, the samples were observed under a Hitachi model H-7000 electron microscope operating at 75 kV.

Phylogenetic analysis. The 16S rDNA sequences of the *Thiothrix* species and the Eikelboom type 021N strains were retrieved from EMBL, GenBank and DDBJ databases. The sequences were aligned and compared by using the CLUSTAL W program version 1.6 (Thompson *et al.*, 1994). A phylogenetic tree was constructed from the evolutionary distance matrix calculated by the neighbour-joining method (Saitou & Nei, 1987) with Kimura's two-parameter method (Kimura, 1980). The neighbour-joining analysis was performed with the MEGA program (Kumar *et al.*, 1994). All gaps in the alignment were excluded to draw the tree. Robustness of the tree topology was evaluated by bootstrap resampling analysis (Felsenstein, 1985) with 100 replicates.

Temperature and pH ranges for growth. For determination of the temperature ranges for growth, cells were cultured at 4, 14, 20, 25, 30, 32, 35, 37 and 42 °C. A 3-ml aliquot of medium EGGC or EGGCH was dispensed into each test tube (17 × 100 mm). Pre-cultured cells were inoculated at 1 % (v/v) and cultured with shaking at 200 r.p.m. (at 14–42 °C) or without shaking (at 4 °C). Growth was examin-

ed after 1 and 2 weeks. The pH range for growth was examined on medium EGGC-A (medium EGGC without sodium acetate). The pH of the medium was adjusted to 5.0, 6.0, 7.0, 7.6, 7.9, 8.8, 9.3, 9.6 and 10.3 by adding sterilized 1 M HCl or NaOH to the autoclaved medium. A 3-ml aliquot of the medium was dispensed into each test tube. Pre-cultured cells were inoculated at 1 % (v/v) and cultured at 30 °C with shaking at 200 r.p.m. for 6 days.

Catalase activity and oxidase activity. Cells grown on EGGC agar plates at 30 °C for 3 days were used for both catalase and oxidase activity tests. Catalase activity was assayed by adding 3 % hydrogen peroxide to cells on a glass slide and observing the formation of bubbles. Oxidase activity was assayed using a BACTOLABO oxidase test (Wako).

Influence of salt concentration. NaCl was added to medium EGGC to give final concentrations of 0–3 % (w/v) and the pH of the medium was adjusted to 7.2. The medium was then autoclaved and 3-ml aliquots were dispensed aseptically into test tubes. Cells grown on EGGC agar plates for 7–14 days at room temperature were inoculated and cultured with shaking at 200 r.p.m. at 30 °C. Growth of cells was examined after 8 and 12 days.

Utilization of substrates. Utilization of substrates was studied on medium EGGC-S agar plates prepared by replacing the carbon source of medium EGGC agar with 0.6 g of various organic acids (sodium salts), alcohols, sugars or amino acids l⁻¹. The pH of the medium was adjusted to 7.2 before autoclaving. The substrate solution was filter-sterilized or autoclaved separately and added to the autoclaved medium. Cells grown on EGGC agar plates for 5 days at 30 °C were streaked onto EGGC-S agar plates (35 × 10 mm) and incubated at 30 °C for 14–40 days. When growth was recognized, the colonies were inoculated onto EGGC-S agar plates of the same substrates to ascertain growth. When growth was not recognizable after more than 14 days, cells grown on EGGC agar plates were again inoculated onto EGGC-S agar plates of the same substrate and growth was examined. Growth on xylose, erythritol, galactose, lactose, melibiose, raffinose, arabinose, rhamnose, ethanol, 1-propanol, sorbitol, citrate, aspartate, alanine, formate, gelatin and starch was examined again for all strains by solidifying the medium with AGAR-NOBLE (Difco) instead of Bacto-Agar to ascertain substrate utilization.

Nitrate reduction. Screw-top test tubes (9 × 100 mm) were filled with sterilized medium EGGC to which filter-sterilized KNO₃ had been added to a final concentration of 0.1 %. Pre-cultured cells (40 µl) were inoculated into the test tubes and incubated statically at 30 °C for 1 week. The same experiments were performed in medium EGGC without the addition of KNO₃ in order to compare the results. Nitrite was detected by adding sulfanilic acid solution and α -naphthylamine solution (Komagata, 1985). When colour was not developed by this test, a small amount of zinc powder was added to the tubes to detect the nitrate remaining. Duplicate examinations were performed.

SDS-PAGE of whole-cell proteins. Cells grown on medium EGGC at 30 °C with shaking at 150 r.p.m. for 7 days were harvested by centrifugation. Preparation of whole-cell proteins was performed as described by Pot *et al.* (1994). SDS-PAGE was performed with Ready Gels J Tris/HCl 5–20 % (Bio-Rad) and a Mini Protean 3 cell (Bio-Rad) at 100 V for 1.25 h.

Cellular fatty acid analysis. Cellular fatty acids were converted to methyl esters by treatment with anhydrous methanolic HCl (100 °C, 3 h) and extracted with hexane (Hanada *et al.*, 2002). The methyl esters were analysed with a Hitachi M7200A GC/3DQMS system equipped with a DB-5 ms capillary column (30 m × 0.25 mm; J&W Scientific) coated with (5 %-phenyl)-methylpolysiloxane (Hanada *et al.*, 2002).

RESULTS

Phylogenetic analysis

We analysed the 16S rDNA sequence similarity among the 15 strains of Eikelboom type 021N bacteria and related strains (Fig. 1). We ascertained that the 15 strains could be subdivided into three distinct groups (groups I to III), as described by Kanagawa *et al.* (2000). The 16S rDNA sequence similarity between the group I and II strains was 94.6–95.6 %. The similarity between group III and the other two groups was 89.7–91.0 %. The *Thiothrix nivea* group, including *T. nivea*, '*Thiothrix ramosa*', *Thiothrix unzii* and *Thiothrix fructosivorans*, showed 88.3–92.0 % sequence similarity to the Eikelboom type 021N strains.

Ultrastructural characteristics

As shown in Fig. 2, individual cells within filaments were clearly separated by cross-walls and a continuous outer cell wall was observed along the length of individual filaments. Sheath material was not observed.

Physiological characteristics

The physiological characteristics of the strains are summarized in Table 1. The temperature range for growth of the strains differed slightly among the groups. The group I and II strains grew at 14–35 °C, with an optimum temperature of 25–30 °C. The group III strains grew at 14–37 °C, with an optimum temperature of 20–30 °C. At 35 °C, the group I and II strains hardly grew, while the group III strains grew slightly better. At 37 °C, the group I and II strains did not grow, while the group III strains grew slightly. The group I and II strains showed less growth at 20 °C than at 25 °C, while the growth of group III strains at 20 °C was same as that at 25 °C.

All strains grew at pH 7.0–7.9 and no growth was observed at pH 5.0, 9.6 or 10.3. At pH 6.0, strains T2-1, COM-A or EJ1M-B did not grow, but the others grew. At pH 9.3, growth of strains B3-1^T and KR-A was observed and, at pH 8.8, strain SNR-3 also grew.

Catalase and oxidase activities were positive in all strains. Vigorous bubble generation was observed soon after hydrogen peroxide was added to cells of the group I strains, while gentle bubble generation was observed after a few seconds with cells of the group II and III strains.

The group III strains showed good growth in the presence of 0–1 % NaCl, while growth of the group I and II strains was inhibited by 0.5 % NaCl. Some

Fig. 1. A phylogenetic tree showing the relationships between Eikelboom type 021N strains and related species based on 16S rDNA sequences. The sequence of *Desulfobacter postgatei* (belonging to the δ -subclass of the *Proteobacteria*) is used to root the tree. Bar, 2 nucleotide substitutions in 100 nucleotides. Bootstrap confidence values from 100 bootstrap replicates are indicated at branching points. Strain accession numbers of the Eikelboom type 021N strains are shown in square brackets. Sequence accession numbers are shown in parentheses.

Fig. 2. Transmission electron micrographs of ultrathin sections of cells of Eikelboom type 021N isolates. The bar in (a) indicates 0.5 μm and magnification was the same for all photos. (a) Group I strain B3-1^T; (b) group I strain SCM-A; (c) group II strain T1-4; (d) group II strain COM-A; (e) group II strain *T. eikelboomii* AP3^T; (f) group III strain EJ2M-B^T.

strains in groups I and II grew slightly with 1 % NaCl. With 2 % NaCl, the growth of the group III strains was inhibited slightly, while the group I and II strains showed no growth. With 3 % NaCl, the group III strains hardly grew.

All strains grew on glucose, fructose, mannose, sucrose, maltose, trehalose, mannitol, succinate, pyruvate, acetate, malate, hydroxybutyrate and glutamate. No strains grew on benzoate, erythritol, galactose, lactose, rhamnose, arabinose, ethanol, 1-propanol, formate, gelatin or starch. With glycerol and butyrate, the group I and II strains grew but the group III strains did not. On lactate, the group II and III strains grew but the group I strains did not.

Nitrate reduction was positive in the group II strains T2-1, COM-A and *T. eikelboomii* AP3^T and all group

III strains. It was negative in the group II strains KR-A and T1-4 and all group I strains.

Cellular fatty acids and whole-cell proteins

The cellular fatty acids of all strains were composed mainly of C_{16:1}, C_{16:0} and C_{18:1} (Table 1).

The SDS-PAGE profiles of whole-cell proteins were analysed for all 15 strains and showed differences among the groups (Fig. 3). The profiles within group I were very similar. In group II, strains KR-A and T1-4 showed similar profiles and strains T2-1 and COM-A also showed similar profiles, but *T. eikelboomii* AP3^T showed a profile different from those of the other group II strains. In group III, the profiles of strains EJ1M-B and EJ2M-B^T were very similar, but the

Table 1. Characteristics of the Eikelboom type 021N strains (groups I–III)

Characteristic	Group I							Group II					Group III		
	B3-1 ^T	B4-1	B2-7	SCM-A	B5-1	B2-8	OS-F	KR-A	T1-4	T2-1	COM-A	AP3 ^T	SNR-3	EJ1M-B	EJ2M-B ^T
Optimum temperature (°C)	25–30	25–30	25–30	25–30	25–30	25–30	25–30	25–30	25–30	25–30	25–30	25–30	20–30	20–30	20–30
Salinity tolerance (%)	0.5	0.5	0.5	0.5	0	0.5	0.5	0	0.5	0	0.5	0.5	2	2	2
Nitrate reduction	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
Utilization of:															
Xylose	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–
Melibiose	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Raffinose	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Lactate	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
Propionate	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
Butyrate	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–
Citrate	+	+	–	+	+	+	+	–	–	–	–	–	+	+	–
Aspartate	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
Alanine	–	+	–	+	+	–	+	+	+	+	+	+	+	+	–
Fatty acid content (%):															
C _{16:0}	18	16	31	33	24	26	19	39	17	42	37	33	32	25	26
C _{16:1}	13	13	13	9	10	9	14	17	27	17	16	13	30	34	30
C _{18:1}	68	71	56	58	66	65	63	44	51	36	45	52	38	41	44

Fig. 3. Whole-cell protein profiles of Eikelboom type 021N strains. Lanes 1 and 12, molecular mass markers; 2, group I strain B3-1^T; 3, group I strain SCM-A; 4, group I strain OS-F; 5, group II strain KR-A; 6, group II strain T1-4; 7, group II strain T2-1; 8, group II strain COM-A; 9, group II strain *T. eikelboomii* AP3^T; 10, group III strain SNR-3; 11, group III strain EJ2M-B^T. The molecular mass markers were aprotinin (6.5 kDa), lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA (66.2 kDa), phosphorylase b (97.4 kDa), β -galactosidase (116.25 kDa) and myosin (200 kDa).

profile of strain SNR-3 showed different intensities in the main bands. The differences among the three groups were greater than those within each group and the position of the bands was different among the groups.

DISCUSSION

We studied 15 isolates of Eikelboom type 021N bacteria and present the phenotypic and genotypic information gained. Phylogenetic analysis clearly showed the differences between the *T. nivea* group and the Eikelboom type 021N strains, with less than 92.0% 16S rDNA sequence similarity between them. All Eikelboom type 021N strains utilized various kinds of

sugars, including glucose, mannose, maltose and trehalose, while members of the *T. nivea* group utilized few or none. The filaments of Eikelboom type 021N bacteria were very long and reached several millimetres in length, while those of members of the *T. nivea* group were less than 1 mm long (Eikelboom, 1975; Kanagawa *et al.*, 2000; Williams & Unz, 1985a). The Eikelboom type 021N strains formed fingerprint-like colonies, a characteristic different from members of the *T. nivea* group (Williams & Unz, 1985a; Kanagawa *et al.*, 2000). The Eikelboom type 021N strains grew heterotrophically without requiring reduced sulfur, while *T. nivea* JP2^T and *T. unzii* A1^T require reduced sulfur for growth (Larkin & Shinabarger, 1983; Williams & Unz, 1985a). The G + C contents of *T. nivea* [52 mol % (T_m); Larkin & Shinabarger, 1983] and '*T. ramosa*' [51–52 mol % (T_m); Odintsova & Dubinina, 1990a] have been reported to be higher than those of the Eikelboom type 021N strains (44–46 mol %; Kanagawa *et al.*, 2000). Moreover, *T. nivea* JP2^T and *T. unzii* A1^T had no catalase activity (Larkin & Shinabarger, 1983; Williams & Unz, 1985a), while the Eikelboom type 021N strains did show catalase activity. *T. nivea*, '*T. ramosa*' and *T. fructosivorans* exhibit a sheath (Larkin & Shinabarger, 1983; Odintsova & Dubinina, 1990b; Williams *et al.*, 1987), while the Eikelboom type 021N strains did not. These differences are summarized in Table 2. Thus, the differences between the Eikelboom type 021N strains and members of the *T. nivea* group are clear and the Eikelboom type 021N strains could reasonably be differentiated from members of the *T. nivea* group by creating novel species.

The Eikelboom type 021N strains we investigated could be divided into three distinct groups (I to III) on the basis of phylogenetic data. The 16S rDNA sequence similarities between the group III strains and the other two groups were 89.7–91.0%. The group III strains were clearly differentiated from the group I and II strains by their very low ability to oxidize sulfur

Table 2. Characteristics that differentiate Eikelboom type 021N strains and the *Thiothrix nivea* group

Data were taken from this study and Kanagawa *et al.* (2000) (type 021N groups I to III), Howarth *et al.* (1999) (*T. eikelboomii*), Williams & Unz (1985a) and Howarth *et al.* (1999) (*T. unzii* and *T. fructosivorans*), Larkin & Shinabarger (1983) (*T. nivea*) and Odintsova & Dubinina (1990a, b) ('*T. ramosa*'). +, Positive; –, negative; +/–, positive in some strains but negative in other strains; ND, not determined.

Characteristic	Type 021N				<i>T. nivea</i> group			
	Group I	Group II	Group III	<i>T. eikelboomii</i>	<i>T. unzii</i>	<i>T. fructosivorans</i>	<i>T. nivea</i>	' <i>T. ramosa</i> '
DNA G+C content (mol%)	43.9–44.7	44.1–46.1	44.0–44.4	ND	ND	ND	52 (T_m)	51.3–52.4 (T_m)
Sheath	–	–	–	–	–	+	+	+
Sudan black stain	+	+	+	+	+	+	+	ND
Neisser stain	–	–	–	+	+	+	ND	ND
Reduced sulfur requirement	–	–	–	–	+	–	+	–
Nitrate reduction	–	+ / –	+	+	+	+	+	ND
Catalase activity	+	+	+	ND	–	+	–	+
Utilization of:								
Glucose	+	+	+	+	–	–	–	–
Fructose	+	+	+	+	–	+	–	ND
Mannose	+	+	+	–	–	–	–	ND
Sucrose	+	+	+	+	–	+	–	–
Maltose	+	+	+	+	–	–	–	–
Trehalose	+	+	+	+	–	–	ND	ND
Xylose	–	+ / –	–	–	–	–	–	–
Melibiose	–	+ / –	–	–	–	–	–	–
Raffinose	–	+ / –	–	–	–	–	–	ND
Glycerol	+	+	–	–	–	–	–	–
Mannitol	+	+	+	+	–	–	ND	+ / –
Lactate	–	+	+	+	+	+	–	+
Succinate	+	+	+	+	+	+	–	+
Propionate	+	+ / –	+	+	+	+	–	ND
Butyrate	+	+	–	ND	–	–	ND	ND
Hydroxybutyrate	+	+	+	ND	–	–	ND	ND
Glutamate	+	+	+	+	ND	ND	–	–
Citrate	+ / –	–	+ / –	ND	–	–	–	–
Aspartate	+	+ / –	+	ND	ND	ND	–	–
Alanine	+ / –	+	+ / –	ND	ND	ND	ND	–

compounds (Kanagawa *et al.*, 2000; Kohno, 1988). The tolerance of 2% NaCl also differentiated the group III strains from the group I and II strains. Also, the wide range of growth temperatures, i.e. growth at 35–37 °C and good growth at 20 °C, was another difference from the group I and II strains. The group III strains were also different from the group I and II strains in their inability to utilize glycerol and butyrate. They were clearly different from the group I strains in their ability to reduce nitrate to nitrite. Thus, the group III strains will be reasonably distinguished from the group I and II strains by designating a novel species in the genus *Thiothrix*. The group III strains were phylogenetically closely related to *Thiothrix defluvii* Ben57^T, with 96.8–97.1% 16S rDNA sequence similarity. However, this species is not a member of Eikelboom type 021N, but *Thiothrix* species form I (Howarth *et al.*, 1999), which grows as shorter filaments (50–500 µm) and deposits many sulfur globules. It is apparent that *T. defluvii* Ben57^T does not belong in the same species as the group III strains. We propose that the group III strains be designated as a novel species in the genus *Thiothrix*, and we propose the name *Thiothrix flexilis* sp. nov. for strains EJ2M-B^T, EJ1M-B and SNR-3.

The 16S rDNA sequence similarities between the group I and II strains were 94.6–95.6%. This suggests that the group I strains and the group II strains are not

members of the same species (Stackebrandt & Goebel, 1994; Hiraishi & Ueda, 1994). The group I strains differed from the group II strains in lactate utilization, poly-β-hydroxybutyrate staining character (Kanagawa *et al.*, 2000) and catalase activity. We propose that the group I strains be designated as a novel species in the genus *Thiothrix*, and we propose the name *Thiothrix disciformis* sp. nov. for strains B3-1^T, B4-1, B2-7, SCM-A, B5-1, B2-8 and OS-F.

Among the five group II strains, including *T. eikelboomii* AP3^T, the ability to reduce nitrate and the utilization of some carbon sources were different. Phylogenetic analysis of the 16S rDNA sequences showed sequence similarities of 98.5–98.7% between *T. eikelboomii* AP3^T and the other four strains. These results suggest that the five group II strains may not belong to a single species. However, these phylogenetic and phenotypic differences would not be definitive in dividing these strains into different species. Therefore, we propose the inclusion of all group II strains, AP3^T, KR-A, T1-4, T2-1 and COM-A, as members of *T. eikelboomii* and the emendation of *T. eikelboomii*.

Here, we propose the creation of two novel species in the genus *Thiothrix* for the Eikelboom type 021N strains and emendation of the species *T. eikelboomii* (Howarth *et al.*, 1999). In order to include all these Eikelboom type 021N strains into the genus *Thiothrix*, we also propose the emendation of the genus *Thiothrix*.

Emended description of the genus *Thiothrix* (Winogradsky 1888) Howarth *et al.* 1999

The formal description remains that given by Howarth *et al.* (1999), with the following modifications to cell size and gonidium production. Cells are 0.7–4.0 µm in diameter and 0.7–5.5 µm in length; gliding gonidia are produced from the end of the filaments in some but not all strains.

Description of *Thiothrix flexilis* sp. nov.

Thiothrix flexilis (fle.xi'lis. L. adj. *flexilis* pliable).

Cells are rod and form slightly bent multicellular filaments. Filaments are more than 0.5 mm long and can reach several millimetres. Colonies are fingerprint-like. Cell morphology in filaments is variable, particularly for length, and discoid to ovoid-shaped cells are often observed. Cells are 1.0–4.0 µm in diameter and 0.5–5.5 µm in length. Elongate and swollen cells are sometimes present in filaments. Septa are clearly visible between individual cells. Gram-negative. A sheath is absent. Rosettes and holdfasts are observed in some but not all strains. Gliding gonidia are produced from the end of the filaments only in rosette-forming strains. Volutin inclusions are not usually observed. Sudanophilic granules are observed within cells. Growth occurs in the temperature range 14–37 °C and pH range 7.0–7.9, but there is no growth at 4 or 42 °C. Optimum temperature 20–30 °C. Oxidase- and catalase-positive. When cells are grown in the presence of a reduced inorganic sulfur compound, only a few sulfur granules are deposited and there is no production or only slight production of sulfuric acid. Sulfur globules are normally not present in trichomes *in situ*. Reduced inorganic sulfur is not required for growth. Glucose, fructose, mannose, sucrose, maltose, trehalose, mannitol, lactate, propionate, succinate, pyruvate, acetate, malate, hydroxybutyrate, glutamate and aspartate are utilized as sole carbon sources. Almost all strains utilize citrate and alanine. No growth occurs with glycerol, butyrate, benzoate, xylose, erythritol, galactose, lactose, melibiose, raffinose, arabinose, rhamnose, ethanol, 1-propanol, sorbitol, formate, gelatin or starch. Good growth occurs in 0–1 % NaCl and growth is inhibited slightly in 2 % (w/v) NaCl. Nitrate is reduced to nitrite. The G+C content of the DNA is 44 mol % (HPLC). The major quinone is UQ-8. The major fatty acids are C_{16:1}, C_{16:0} and C_{18:1}.

Isolated from activated sludge suffering from bulking. The type strain is strain EJ2M-B^T (= DSM 14609^T = JCM 11135^T). Additional strains are EJ1M-B (= JCM 11134) and SNR-3 (= JCM 11130).

Description of *Thiothrix disciformis* sp. nov.

Thiothrix disciformis (dis.ci.for'mis. L. masc. n. *discus* disc; L. n. *forma* shape; N.L. adj. *disciformis* disc-shaped, the main cell form).

Cells are rods and form slightly bent multicellular filaments. Filaments are more than 0.5 mm long and can reach several millimetres. Colonies are fingerprint-like. Cell morphology in filaments is variable, particularly for length, and discoid to ovoid shaped cells are often observed. Cells of the major form are 1.2–3.0 µm in diameter and 0.5–3.0 µm in length. Elongate and swollen cells are sometimes present in filaments. Septa are clearly visible between individual cells. Gram-negative. A sheath is absent. Volutin inclusions are not usually observed. Sudanophilic granules are observed within cells. Growth occurs in the temperature range 14–32 °C and pH range 7.0–7.9, but there is no growth at 4 or 37 °C. Optimum temperature 25–30 °C. Oxidase-positive. Catalase-positive with violent bubble generation when cells are exposed to hydrogen peroxide. When cells are grown in the presence of a reduced inorganic sulfur compound, sulfur globules are deposited within invaginations of the cytoplasmic membrane and sulfuric acid is produced. Reduced inorganic sulfur is not required for growth. Glucose, fructose, mannose, sucrose, maltose, trehalose, mannitol, succinate, pyruvate, acetate, malate, butyrate, hydroxybutyrate, glutamate, glycerol and aspartate are utilized as sole carbon sources. Almost all strains utilize citrate and alanine. No growth occurs with lactate, benzoate, xylose, erythritol, galactose, lactose, melibiose, raffinose, arabinose, rhamnose, ethanol, 1-propanol, sorbitol, formate, gelatin or starch. Growth is inhibited by 0.5 % (w/v) NaCl. Nitrate is not reduced. The G+C content of the DNA is 44–45 mol % (HPLC). The major quinone is UQ-8. The major fatty acids are C_{16:1}, C_{16:0} and C_{18:1}.

Isolated from activated sludge suffering from bulking. The type strain is strain B3-1^T (= DSM 14473^T = JCM 11364^T). Additional strains are B4-1 (= JCM 11365), B2-7 (= JCM 11362), SCM-A (= JCM 11132), B5-1 (= JCM 11366), B2-8 (= JCM 11363) and OS-F (= JCM 11131).

Emended description of *Thiothrix eikelboomii* (Howarth *et al.* 1999)

The formal description remains that given by Howarth *et al.* (1999), with the following modifications. Volutin inclusions are observed in some strains; some strains grow with mannose, glycerol, xylose, propionate, aspartate and alanine; some strains may show slight growth with melibiose, raffinose and sorbitol; nitrate is reduced to nitrite by some strains. The following is added to the description of Howarth *et al.* (1999): Filaments are more than 0.5 mm long and can reach several millimetres. Colonies are fingerprint-like. Elongate and swollen cells are often present in filaments. Elongate cells in some strains sometimes reach 8 µm in length and swollen cells in some strains sometimes reach 8 µm in diameter. Septa are clearly visible between individual cells. Sudanophilic granules are observed densely within cells. Catalase-positive. Sulfuric acid is produced when cells are grown in the

presence of a reduced inorganic sulfur compound. Butyrate, hydroxybutyrate and glutamate are utilized as sole carbon sources. No growth occurs with 1-propanol, ethanol, benzoate, formate or citrate. Growth is inhibited by 0.5% (w/v) NaCl. The G+C content of the DNA is 44–45 mol% (HPLC). The major quinone is UQ-8. The major fatty acids are C_{16:1}, C_{16:0} and C_{18:1}.

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