Thermomonas fusca sp. nov. and *Thermomonas brevis* sp. nov., two mesophilic species isolated from a denitrification reactor with poly(ϵ -caprolactone) plastic granules as fixed bed, and emended description of the genus *Thermomonas*

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Previously, 22 aerobic Gram-negative bacteria were isolated from biofilms growing on granules of the synthetic polyester poly(ϵ -caprolactone); the granules were used as a fixed bed in a denitrification reactor. All the strains showed similar fatty acid profiles. The 16S rRNA gene sequences of five strains were phylogenetically related to *Thermomonas* spp. Repetitive extragenic palindromic DNA-PCR (REP-PCR) fingerprinting revealed four groups, and DNA hybridizations between representative strains showed that the strains belonged to two new species within the genus *Thermomonas*, for which the names *Thermomonas fusca* (type strain LMG 21737^T = DSM 15424^T) and *Thermomonas brevis* (type strain LMG 21746^T = DSM 15422^T) are proposed. Both species are able to grow at low temperatures, but not at 50 °C, and are non-haemolytic. Both species can be differentiated by several other phenotypic features from earlier described species of the genus *Thermomonas*. Cell extracts contain mainly branched fatty acids, with C_{15:0} iso, C_{17:1} iso ω 9c, C_{11:0} iso 3OH and C_{11:0} iso as main constituents. The G + C content of the DNA of the novel species is between 67.6 and 68.7 mol%.

Conventional techniques for heterotrophic denitrification of drinking water consist of using fixed or fluidized bed reactors often with a sand packing as a biofilm carrier and soluble organic carbon sources dosed to the raw water stream. An alternative approach is to use natural solid organic substrates that act as biofilm carriers as well as organic carbon sources. The development of water-insoluble biodegradable polymers led to the idea to use such materials in denitrification reactors. Denitrification processes were developed that used the biodegradable bacterial polyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (Müller *et al.*, 1992) and the synthetic polymer poly(ϵ -caprolactone) (Boley *et al.*, 2000) as sources of

carbon for denitrification and electron donor. Both polymers have been shown to be biodegradable by many bacteria (Mergaert & Swings, 1996; Mergaert *et al.*, 2000).

Previously, we characterized the biofilm on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) granules used in a continuous-upflow fixed-bed reactor, by isolation and identification of the dominant microflora (Mergaert *et al.*, 2001b). In a parallel experiment, a reactor with poly(ϵ -caprolactone) with a similar set-up was investigated and large numbers of bacteria were isolated from the biofilm on the granules and characterized by fatty acid analysis (Boley *et al.*, 2003). While most organisms were identified as *Acidovorax* spp., a group of 22 strains with similar fatty acid profiles remained unidentified; representative strains of this group were found to be phylogenetically related to *Thermomonas haemolytica* (type species) and *Thermomonas hydrothermalis*, two recently described, slightly thermophilic species isolated from kaolin slurry (Busse *et al.*, 2002) and a hot spring (Alves *et al.*, 2003), respectively. The purpose of the present study was to further characterize these 22 isolates by using a polyphasic taxonomic approach. On the basis of the results presented here, two new species of the genus *Thermomonas* are proposed.

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Abbreviation: REP-PCR, repetitive extragenic palindromic DNA-PCR.

The EMBL accession numbers for the 16S rRNA gene sequences of *Thermomonas fusca* LMG 21736, LMG 21737^T, LMG 21738 and LMG 21739 and *Thermomonas brevis* LMG 21746^T are AJ519985, AJ519986, AJ519987, AJ519988 and AJ519989, respectively.

Table 1. REP-PCR fingerprint types of the new *Thermomonas* strains investigated

LMG, BCCM/LMG Culture Collection, Universiteit Gent, Ghent, Belgium; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. Numbers prefixed by 'R-' refer to the strain numbers as preserved in the research collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium.

REP-PCR type	Strains
<i>T. fusca</i>	
A	LMG 21736, LMG 21737 ^T (=DSM 15424 ^T), LMG 21741, R-13284, R-13285, R-13288
B	LMG 21742, LMG 21747, R-13286, R-13289, R-13294, R-13295, R-18581, R-18583
C	LMG 21738, LMG 21739, LMG 21740, R-18582
<i>T. brevis</i>	
D	LMG 21743, LMG 21744, LMG 21745, LMG 21746 ^T (=DSM 15422 ^T)

Strains were isolated from biofilms growing on poly(ϵ -caprolactone) granules (Union Carbide), which were used as a fixed bed in a continuous-upflow denitrification reactor, according to methods described previously (Mergaert *et al.*, 2001b). In brief, the granules were taken aseptically from the reactor, the biofilm was washed off, and serial dilutions were plated onto R2A agar (Difco) and incubated aerobically at 20 °C. The 22 strains investigated in this study were all isolated from granules sampled at the anoxic top of the fixed bed in June 1998 and August 1999. The strain designations are listed in Table 1. As reference strains, *T. haemolytica* LMG 19653^T, LMG 19654 and LMG 19656 were included in some experiments. Strains were pre-cultured on R2A agar for 3 days at 28 °C, unless mentioned otherwise.

To investigate the phylogenetic affiliations of the isolated strains, the almost-complete 16S rRNA gene sequences of five representative strains were determined. DNA preparation and 16S rRNA gene sequence analysis were carried out as described previously (Mergaert *et al.*, 2001a). Phylogenetic analysis was performed using the BIONUMERICS software package (Applied Maths; <http://www.applied-maths.com/>),

taking into account the homologous nucleotide positions after discarding all unknown bases and gaps. Using the same software package, a neighbour-joining dendrogram (Saitou & Nei, 1987) was constructed based on global alignment of the sequence similarities (Fig. 1). Strains LMG 21738 and LMG 21739 showed a pairwise sequence similarity of 99.7 %, and the similarity between strains LMG 21736 and LMG 21737^T was 99.8 %. Sequence similarity between these two couples was 97.7–97.9 %, and with strain LMG 21746^T the sequence similarity was 97.9–98.0 % and 96.2 %, respectively. The latter percentage is a strong indication that the sequenced strains belong to more than one species (Stackebrandt & Goebel, 1994). After a FASTA search, the sequences of the isolates were compared to related sequences available from the EMBL database. In the phylogenetic dendrogram generated, the sequences from the strains grouped with those of *T. haemolytica* LMG 19653^T (95.0–97.2 % pairwise sequence similarity) and *T. hydrothermalis* SGM-6^T (95.0–96.6 %) within the *Xanthomonas* branch of the γ -Proteobacteria. Strains LMG 21736 and LMG 21737^T showed highest sequence similarity (99.6 %) to an unidentified Fe(II)-oxidizing bacterial strain, BRG3, isolated from freshwater sediment after

Fig. 1. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of *Thermomonas* strains and nearest members of the γ -Proteobacteria. *Fulvimonas soli* was included as an outgroup. Bootstrap values of the branches are shown in percentages of 1000 replicates, when less than 100 %. Bar, sequence divergence.

anaerobic chemolithoautotrophic enrichment conditions using Fe(II) as the sole electron donor and nitrate as the electron acceptor (Buchholz-Cleven *et al.*, 1997).

Repetitive extragenic palindromic DNA-PCR (REP-PCR), based on primers targeting the repetitive extragenic palindromic sequence, allows the rapid grouping of strains that are genomically highly related to each other (Versalovic *et al.*, 1991; Rademaker *et al.*, 2000) and to select representative strains for further study by DNA–DNA hybridization analysis. REP-PCR genomic fingerprints were prepared for all strains listed in Table 1, using the primers REP1R-I and REP2-I (Versalovic *et al.*, 1991), as described by Rademaker & de Bruijn (1997) and Rademaker *et al.* (2000). Numerical analysis was carried out using the BIONUMERICS software package, as described by the same authors. On the basis of numerical and visual comparisons, four groups, each consisting of strains with almost identical profiles, could be distinguished (Table 1). Representatives from each REP-PCR cluster were compared by DNA–DNA hybridization analysis and their G+C contents (mol%) were determined (Table 2). DNA was prepared according to the method of Pitcher *et al.* (1989), with the modifications described by Logan *et al.* (2000). Alternatively, the method of Marmur (1961) was applied. DNA base content was determined using a HPLC method. DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The obtained nucleoside mixture was then separated by HPLC using a Waters Symmetry Shield C8 column thermoregulated at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ 1⁻¹ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. DNA from the strains had a G+C content of 67.6–68.7 mol%, which is in the range reported for *T. haemolytica* (67.1–68.7 mol%; Busse *et al.*, 2002) and slightly higher than that reported for *T. hydrothermalis* (64.7 mol%; Alves *et al.*, 2003).

DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Willems *et al.* (2001), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 50 °C. Reciprocal experiments were performed for every pair of strains and the results given are the means. The relative DNA binding between the strains showing REP-PCR fingerprint types A, B and C was 82–102 %. This confirms the findings of Stackebrandt & Goebel (1994) that 16S rRNA gene sequence similarities down to 97 % are found between strains showing DNA reassociation values of more than 70 %, a percentage generally accepted as the minimum for an intraspecies genomic relationship (Wayne *et al.*, 1987). These strains showed 49–57 % relative DNA binding with strain LMG 21746^T, which represented REP-PCR fingerprint group D. Strain LMG 21737^T, representing REP-PCR cluster A, and strain LMG 21746^T, representing REP-PCR cluster D, showed 40 and 41 % DNA binding, respectively, to *T. haemolytica* LMG 19653^T. Thus, the 22 strains isolated from a denitrification reactor belong to two new species (Wayne *et al.*, 1987) within the genus *Thermomonas*. For the 18 strains from REP-PCR clusters A, B and C, the name *Thermomonas fusca* is proposed; for the four strains showing REP-PCR profile type D, the name *Thermomonas brevis* is proposed.

Phenotypic characteristics were determined for all strains of *T. fusca*, *T. brevis* and *T. haemolytica*. Most methods, including API 20NE and API ZYM tests, have been described by Mergaert *et al.* (2002), and the incubation temperature was 28 °C. Growth at 4, 14, 20, 28, 37 and 50 °C was assessed on R2A agar after 5 days incubation. Haemolysis was tested on Columbia agar base (Oxoid) supplemented with 10 % defibrinated horse blood after 2 days incubation at 37 °C. Reduction of nitrate was tested as described by Mergaert *et al.* (2001b). Cell morphology

Table 2. DNA–DNA relatedness and G+C contents of *Thermomonas* strains

Strain: 1, *T. fusca* LMG 21737^T; 2, *T. fusca* LMG 21741; 3, *T. fusca* LMG 21747; 4, *T. fusca* LMG 21738; 5, *T. fusca* LMG 21739; 6, *T. brevis* LMG 21746^T; 7, *T. haemolytica* LMG 19653^T. DNA reassociation values are given as the means of reciprocal values. ND, Not determined.

Strain	REP-PCR type	G+C content (mol%)	DNA reassociation (%)						
			1	2	3	4	5	6	7
1	A	67.6	100						
2	B	68.5	92	100					
3	B	68.7	102	ND	100				
4	C	68.2	ND	86	ND	100			
5	C	68.3	ND	80	82	ND	100		
6	D	68.4	49	52	57	ND	51	100	
7	ND	68.5*	40	ND	ND	ND	ND	41	100

*Value from Busse *et al.* (2002).

was investigated by phase-contrast microscopy and flagella staining was performed using the method of Heimbrook *et al.* (1989).

Cells of *T. fusca* were straight, filament-forming rods, while cells of *T. brevis* were non-filamentous. Motile cells from both species showed single polar flagella. The strains grew well on R2A agar and tryptic soy agar (TSA; BBL), between 10 and 37 °C, and under anoxic conditions in nutrient broth supplemented with nitrate. At 50 °C, no growth was detected, and the inocula were killed off, as evidenced by further incubation at 28 °C. At 14 or 20 °C, the colonies of *T. fusca* were brown in colour with a dark centre, while at 28 °C two strains, LMG 21741 and LMG 21742, failed to produce the brown pigment, but were faint-yellow. At 37 °C, only strains showing REP-PCR profile type C (Table 1) were able to produce brown-pigmented colonies, while the other strains remained non-pigmented. Strains of *T. haemolytica* and *T. brevis* were non-pigmented regardless of the incubation temperature.

Biochemical and physiological characteristics of the strains tested are given below. Different reactions were obtained for gelatin liquefaction with strains of *T. haemolytica*, and for C14-lipase, trypsin and chymotrypsin with strains of *T. fusca*. The species can be differentiated from each other

and from *T. hydrothermalis* (Alves *et al.*, 2003) by several features, which are listed in Table 3.

For fatty acid analysis, strains were grown for 48 h on TSA and their fatty acid methyl esters were extracted and separated by GLC using the MIDI system (MICROBIAL ID), as described previously (Mergaert *et al.*, 1993). For all *Thermomonas* strains tested, cell extracts contained mainly branched fatty acids, with C_{15:0} iso, C_{17:1} iso ω9c, C_{11:0} iso 3OH and C_{11:0} iso as main constituents (Table 4). The fatty acid composition of *T. haemolytica* was similar to those reported by Busse *et al.* (2002), although the latter authors used different temperatures of incubation for cultivation of cells. Within *T. fusca* broad ranges of relative amounts were found for the fatty acids C_{14:0} iso and C_{16:0} iso, but there was no clear-cut correlation with genomic data and phylogenetic similarity. The three species differed from each other in their relative amounts of the main fatty acids C_{15:0} iso and C_{17:1} iso ω9c. The fatty acid compositions were also similar for those reported for *T. hydrothermalis* strains (Alves *et al.*, 2003), except for lower amounts of C_{17:0} iso ω9c detected in the latter species, most probably as a result of the higher growth temperature (50 °C) used by Alves *et al.* (2003), a phenomenon demonstrated for *T. hydrothermalis* and discussed by Busse *et al.* (2002).

Table 3. Biochemical and physiological characteristics of *Thermomonas* strains

Species: 1, *T. fusca* (n=18); 2, *T. brevis* (n=4); 3, *T. haemolytica* (n=3); 4, *T. hydrothermalis* (n=2). n, Number of strains investigated. +, Positive reaction; –, negative reaction; ±, weak positive reaction; ND, not determined. Where two reactions are given, the most frequent reaction is given first; the reaction of the type strain of the species is given in parentheses. Data for *T. fusca*, *T. brevis* and *T. haemolytica* were generally obtained after incubation at 28 °C, those for *T. hydrothermalis* are from Alves *et al.* (2003) and were generally obtained at 50 °C.

Test	1	2	3	4
Nitrate reduction	+	+	–	+
Nitrite reduction	+	+	–	+
Aesculin hydrolysis	–	+	–	+
Growth on glucose	–	+	–	+
Growth on mannose	–	+	–	–
Growth on N-acetylglucosamine	–	+	–	–
Growth on maltose	–	+	–	+
C14 Lipase	– or + (–)	–	–	+
Chymotrypsin	+ or – (–)	–	+	+
β-Glucosidase	–	–	–	+
N-Acetyl-β-D-glucosaminidase	–	+	–	–
Cystine arylamidase	+	+	+	–
Brown pigment on R2A at 14 or 20 °C	+	–	–	ND
Growth at 10 and 14 °C	+	+	–	–
Growth at 50 °C, 6 days	–	–	+	+
Haemolysis 37 °C, 3 days	–	–	+	±
Filamentous cells	+	–	+	–
Motility	+	+	+	–

Table 4. Fatty acid compositions of *Thermomonas* strains

n, Number of strains investigated; TR, trace amount ($\leq 1.0\%$ of total). Also in trace amounts of total in some strains: C_{10:0}, C_{10:0} 3OH, C_{10:0} iso, C_{10:0} anteiso, C_{12:0} iso, C_{12:0} iso 3OH, C_{13:0} anteiso, C_{14:1} ω 5c, C_{15:0}, C_{15:0} iso 3OH, C_{15:1} ω 6c, C_{16:1} iso H, C_{16:1} ω 9c, C_{18:0}, summed feature 1 and summed feature 4. Summed feature 1 comprises any combination of C_{13:0} 3OH, C_{15:1} iso H and C_{15:1} iso I (capital letters refer to unknown positions of the double bonds). Summed feature 3 comprises C_{15:0} iso 2OH or C_{16:1} ω 7c or both. Summed feature 4 comprises C_{17:1} iso I or C_{17:1} anteiso B or both.

Fatty acid	Range as a percentage of total fatty acids		
	<i>T. fusca</i> (<i>n</i> =18)	<i>T. brevis</i> (<i>n</i> =4)	<i>T. haemolytica</i> (<i>n</i> =3)
C _{11:0} iso	5.8–9.1	7.2–10.1	7.9–8.4
C _{11:0} iso 3OH	6.5–10.5	7.8–11.2	9.2–10.0
C _{13:0} iso	0.6–4.6	TR	TR
C _{14:0}	1.4–2.8	1.1–1.3	TR
C _{14:0} iso	0.9–8.1	TR	2.9–5.2
C _{15:0} anteiso	0.3–2.2	TR	TR
C _{15:0} iso	30.9–44.8	45.3–48.7	49.5–55.2
C _{15:1} iso F	2.5–5.9	1.4–1.6	3.0–3.7
C _{16:0}	0.7–2.3	2.6–3.0	TR
C _{16:0} iso	0.6–11.2	TR	4.3–7.6
C _{17:0} iso	0.8–2.3	2.2–2.8	1.5–2.1
C _{17:1} iso ω 9c	14.4–23.3	20.1–22.5	11.3–12.3
Summed feature 3	2.7–7.0	2.8–3.1	TR

Description of *Thermomonas fusca* sp. nov.

Thermomonas fusca (fus'ca. L. fem. adj. *fusca* dark, tawny).

Cells are rods that form filaments, and are 0.7 μ m wide by 3–10 μ m long. Motile by means of a single polar monotrichous flagellum or non-motile. Aerobic, but anoxic growth occurs with nitrate as electron acceptor. Growth occurs on complex medium at 4–37 °C, but not at 50 °C; optimum growth occurs at 28–37 °C. On R2A agar, convex, circular, smooth, translucent colonies of about 2 mm diameter and with entire margins are formed after 5 days incubation at 28 °C. At 20 °C, brown colonies with a dark centre are produced, but at higher temperatures some strains remain non-pigmented or are faint-yellow in colour. Nitrate and nitrite are reduced. Non-haemolytic. Positive for the following enzymes: oxidase, catalase, alkaline phosphatase, C4-esterase, C8-esterase, valine arylamidase, cystine arylamidase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase. Negative for the following enzymes: arginine dihydrolase, urease, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase, *N*-acetyl-glucosaminidase and α -fucosidase. Aesculin is not hydrolysed. Gelatin is liquefied. No growth occurs on glucose, mannose, *N*-acetylglucosamine, maltose, arabinose, mannitol, gluconate, caprate, adipate, malate, citrate or phenylacetate. Indole is not produced. Other characteristics are as for the genus. The G + C content of the DNA is 67.6–68.7 mol%. Isolated from biofilm grown on poly(ϵ -caprolactone) plastic granules that were used as a fixed bed in a denitrification reactor.

The type strain is *Thermomonas fusca* LMG 21737^T (=DSM 15424^T).

Description of *Thermomonas brevis* sp. nov.

Thermomonas brevis (bre'vis. L. fem. adj. *brevis* short).

Cells are non-filamentous rods that are 0.7 μ m wide by 3–10 μ m long. Motile by means of a single polar monotrichous flagellum. Aerobic, but anoxic growth occurs with nitrate as electron acceptor. Growth occurs on complex medium at 4–37 °C, but not at 50 °C; optimum growth occurs at 28–37 °C. On R2A agar, convex, circular, smooth, translucent colonies of about 2 mm diameter and with entire margins are formed after 5 days incubation at 28 °C. Regardless of the incubation temperature, non-pigmented colonies are formed. Nitrate and nitrite are reduced. Non-haemolytic. Positive for the following enzymes: oxidase, catalase, alkaline phosphatase, C4-esterase, C8-esterase, valine arylamidase, cystine arylamidase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase and *N*-acetyl-glucosaminidase. Negative for the following enzymes: arginine dihydrolase, urease, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase. Aesculin is hydrolysed. Gelatin is liquefied. Growth occurs on glucose, mannose, *N*-acetylglucosamine and maltose, but not on arabinose, mannitol, gluconate, caprate, adipate, malate, citrate or phenylacetate. Indole is not produced. Other characteristics are as for the genus. Isolated from biofilm grown on poly(ϵ -caprolactone) plastic granules that were used as a fixed bed in a denitrification reactor.

The type strain is *Thermomonas brevis* LMG 21746^T (=DSM 15422^T). The G+C content of its DNA is 68.4 mol%.

Emended description of the genus *Thermomonas*

The inclusion of two additional species (this report) as well as *T. hydrothermalis* (Alves *et al.*, 2003) in the genus requires an emendation of the description of *Thermomonas*. The genus is as described by Busse *et al.* (2002) with the following modifications and emendations. Phylogenetically, the genus belongs to the *Xanthomonas* branch within the γ -Proteobacteria. Cells are either rods or filaments. Species differ with regard to maximum and minimum growth temperatures and are thus either mesophilic or slightly thermophilic. The G+C content of the DNA is between 64.7 and 68.7 mol%.

The type species of the genus is *Thermomonas haemolytica*.

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