Thermomonas haemolytica gen. nov., sp. nov., a γ -proteobacterium from kaolin slurry

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Four aerobic, Gram-negative bacterial strains isolated from kaolin slurry used in the production of paper were subjected to a polyphasic analysis and characterization to determine their taxonomic position. Analysis of the 16S rDNA sequences of the four strains revealed that they represent a new lineage within the γ -Proteobacteria, related to the genera *Xanthomonas*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Luteimonas*, *Xylella* and *Rhodanobacter*. Analysis of the quinone system, the polyamines, the fatty acids and the polar lipids revealed a combination of characteristics that is unique and not described for the phylogenetic relatives. The four strains contain a ubiquinone Q-8, spermidine as the major polyamine, diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as the predominant polar lipids, and a fatty acid profile with predominantly iso-branched fatty acids. The G+C content of the genomic DNA was determined to be within the narrow range 67.1–68.7 mol%. Determination of DNA relatedness, as well as riboprint band patterns and amplified fragment length polymorphism profiles, clearly demonstrated that the four strains are members of a single species. Antibiotic-susceptibility patterns were identical for the four strains. Although showing a high degree of similarities in physiological and biochemical patterns, each of the four strains could be distinguished from the others on the basis of a few biochemical characteristics. On the basis of the estimates of phylogenetic relationships derived from the 16S rDNA sequence analyses, the observed chemotaxonomic characteristics and other phenotypic traits, a new genus, *Thermomonas* gen. nov., and species, *Thermomonas haemolytica* sp. nov., are proposed for the strains A50-7-3^T (= DSM 13605^T = LMG 19653^T), B 50-7-1 (= DSM 13598 = LMG 19655), D50-7-1 (= DSM 13610 = LMG 19656) and B50-8-1 (= DSM 13599 = LMG 19654), with strain A50-7-3^T as the type strain.

Keywords: *Thermomonas haemolytica* gen. nov., sp. nov., γ -proteobacterium, 16S rDNA sequence, chemotaxonomy

INTRODUCTION

Bacteria are ubiquitous in the environment and able to colonize new ecological niches created by man. Recently, a study on the complex diversity in microbial

Abbreviation: AFLP, amplified fragment length polymorphism.

A riboprint and wave diagrams of four strains digested with *EcoRI* and an SDS-PAGE gel are available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>)

The GenBank/EMBL/DBJ accession numbers for the 16S rDNA sequences of *T. haemolytica* strains A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1 are AJ300183/AJ300185, AJ300186, AJ296318 and AJ296319, respectively.

communities colonizing printing paper machines was reported (Väisänen *et al.*, 1998). Several species, including *Acinetobacter radioresistens*, *Methylobacterium mesophilicum*, *Deinococcus geothermalis*, members of the genera *Microbacterium* (formerly *Aureobacterium*; Takeuchi & Hatano, 1998), *Pantoea* and *Ralstonia*, and strains of a taxon tentatively designated as 'Microbulbifer-like', were isolated from commercial brands of kaolin slurry sold as a paper-making chemical. The designation of the latter strains as 'Microbulbifer-like' was based on their fatty acid profiles, which were most similar to those of the recently described species *Microbulbifer hydrolyticus*

(González *et al.*, 1997). These strains were capable of growing at temperatures as high as 50 °C and of decomposing chemicals such as starch, casein and carboxymethyl cellulose (used in the production of paper). Since these strains could not be clearly assigned to any existing genus, we have characterized them by employing a set of different methods to determine their taxonomic position. Here, we report the taxonomic characterization of four strains of the 'Microbulbifer-like' bacteria and present the description of a new genus and new species, namely *Thermomonas haemolytica* gen. nov., sp. nov.

METHODS

Bacterial strains. The strains A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1 were isolated on Tryptic Soy Agar (TSA; Becton Dickinson), at 50 °C, from two different commercial brands of kaolin slurry, sold as a paper-making chemical (Väisänen *et al.*, 1998). The strains were maintained at -70 °C in nutrient broth (Merck) containing 15% glycerol. The following reference strains were included for comparison: *Xanthomonas campestris* DSM 3586^T, *Stenotrophomonas maltophilia* DSM 50170^T (= LMG 958^T), *S. maltophilia* LMG 10872, *S. maltophilia* LMG 10994, *S. maltophilia* LMG 11089 and *Microbulbifer hydrolyticus* DSM 11525^T.

Morphological, physiological and biochemical characterization. Cell morphology was investigated, by phase-contrast microscopy, on cells grown for 24 h at 37 °C or 50 °C in Luria-Bertani broth (LB) on a rotary shaker (300 r.p.m.). The flagellar arrangement was examined by using the staining procedure of Kodaka *et al.* (1982). For electron microscopy, the cells were grown on TSA for 3 d at 38 °C. Ultrathin sections were prepared as described previously (Andersson *et al.*, 1995). Catalase activity and the Gram reaction were analysed by staining and by using the KOH-lysis test, as described previously (Smibert & Krieg, 1994), and by using Bactident-Aminopeptidase Strips (Merck). Oxidase activity was determined using Bactident-Oxidase strips (Merck).

Growth was investigated on TSA (Oxoid), nutrient agar (Merck), LB agar, MacConkey agar (Oxoid), Cetrimide agar (Merck), Czapek-Dox agar (Oxoid) and R2A agar (Oxoid). Haemolytic activity was tested on Columbia Agar Base (Oxoid) supplemented with 10% defibrinated sheep blood, at 37 °C. For determination of the temperature growth range, cells were grown in duplicate on half-strength TSA [full-strength TSA (Difco) diluted 1:1 with water]; agar (YA-KEMIA, Ph.Eur.) was added to 1.5%, w/v. Growth at 14, 18, 28, 37, 45, 50 and 53 °C was assessed, using a precision incubator, as the development of visible colonies. At 14 and 18 °C, strains were grown for 6 d, and at 53 °C, for 3 d. *Microbulbifer hydrolyticus* DSM 11525^T (grown on DSM medium 514) and *S. maltophilia* DSM 50170^T were used as positive controls for growth at low temperatures.

Biochemical tests were performed at 37 and 50 °C for all strains, as described previously (Kämpfer *et al.*, 1997). Gelatin hydrolysis, nitrate reduction, urease and arginine dihydrolase were tested using the API 20NE system. Additional enzyme activities were tested using the API ZYM system. API tests were performed according to the instructions of the manufacturer (API BioMérieux). Supplemen-

tary phenotypic characteristics, including nitrate reduction, indole production, the production of H₂S, citrate utilization, and the methyl red/Voges-Proskauer test, were carried out as described by Smibert & Krieg (1994). The metabolism of glucose was determined by using the oxidative/fermentative test, according to the method of Hugh & Leifson (1953), after incubation for 7 d at 37 and 50 °C. Other physiological tests were performed with all strains, at 37 and 50 °C, as described previously (Kämpfer *et al.*, 1997). Inocula for the supplementary phenotypic tests were performed with cultures grown on TSA for 24 h at 37 and 50 °C, unless indicated otherwise.

Antimicrobial susceptibility. Susceptibility to antimicrobial substances was tested on Iso-Sensitest agar (Oxoid), using antimicrobial-agent-impregnated disks (Oxoid). Any sign of growth inhibition, after 24 h incubation at 37 °C, was scored as sensitivity to the respective antimicrobial. The following antibiotics (Oxoid) were tested: amoxicillin/clavulanic acid (30 µg), ampicillin (10 µg), cephalixin (30 µg), chloramphenicol (30 µg), colistin sulphate (10 µg), erythromycin (30 µg), fusidic acid (10 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (15 µg), neomycin (30 µg), nitrofurantoin (100 µg), penicillin G (10 IU), polymyxin B (300 IU), streptomycin (10 µg), sulphamethoxazole/trimethoprim (25 µg) and tetracycline (30 µg).

Chemotaxonomic analyses. For fatty acid analyses, strains were grown in duplicate on half-strength TSA medium for 96 h at 23 °C and for 48 h at the other temperatures. Fatty acid methyl esters were extracted and prepared by using the standard protocol of the Microbial Identification System (MIDI; Microbial ID). Extracts were analysed using a model HP6890A gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector, automatic sampler, integrator and computer, as described previously (Kämpfer & Kroppenstedt, 1996; Kämpfer *et al.*, 1997). Polyamines were extracted and analysed according to the method of Busse & Auling (1988) and Busse *et al.* (1997). The quinone system and the polar lipids were determined by TLC, as described previously (Tindall, 1990; Ventosa *et al.*, 1993).

Determination of the genomic DNA G + C content. DNAs were extracted according to the method of Auling *et al.* (1986). After enzymic hydrolysis, the G + C content was determined by HPLC analysis of the component nucleosides (Kaneko *et al.*, 1986). The HPLC apparatus was equipped with a Waters pump (model 510), a model U6K injector, a column oven, a UV detector (Waters 484) and a reversed-phase column (250 × 4 mm; hypersil C18; 5 µm particles). The analyses were performed at 30 °C, employing an eluent consisting of 0.2 M (NH₄)H₂PO₄/CH₃CN (20:1, v/v). Nucleosides were measured at 270 nm. The values were calibrated using digested lambda DNA (49.8 mol% G + C) as a standard. DNA-DNA hybridizations were performed at 73 °C (30 degrees below the melting temperature of their genomic DNA) as described previously (Lind & Ursing, 1986; Ziemke *et al.*, 1998).

Riboprint analysis. Bacteria were grown at 37 °C on 0.3% peptone from casein, 0.3% yeast extract and 1.5% agar, pH 7.2 (PYE). Riboprinting was conducted using an automated system (Qualicon), according to the instructions of the manufacturer, and was treated as described elsewhere (Bruce, 1996).

Amplified fragment length polymorphism (AFLP) genomic fingerprinting. Strains were cultured for 48 h on solid

medium containing 3 % trypticase soy broth (BBL) and 2 % technical agar. Isolation and purification of DNA, preparation of template DNA for the PCR, primer labelling, selective PCR amplification and electrophoresis were performed as described by Hauben *et al.* (1999). The AFLP fingerprints were visualized autoradiographically by exposing the dried gels to Hyperfilm-MP (Amersham International) for 5–24 h, depending on the amount of radiation determined with a standard Geiger–Müller counter. Following the exposure, the film was developed with a model RGII X-ray film processor (Fuji). Autoradiograms were scanned using a high-resolution densitoscanner (model RayVen RSU1; X-Ray Scanner Corp.). Transmission image data were stored as TIFF files and processed further using GELCOMP software, version 4.1 (Applied Maths).

16S rDNA sequence analysis. Nucleic acid sequences were determined from PCR-amplified 16S rRNA genes, using the protocols as described previously (Kämpfer *et al.*, 1997) and the sequencing primers described by Lane (1991). Sequences were compared against those in the EMBL/GenBank databases, using FASTA (Pearson & Lipman, 1988). Sequences were aligned, using conserved primary sequence and secondary structure as a reference (Gutell *et al.*, 1985). Evolutionary distances were calculated from pairwise dissimilarities (Jukes & Cantor, 1969), excluding non-homologous positions and ambiguous nucleotide determinations. Estimates of phylogenetic relationships and bootstrap (Felsenstein, 1985) values of branching confidence (derived from 1000 resamplings) were calculated using programs in the Phylogeny Inference Package (PHYLIP; Felsenstein, 1993).

RESULTS

Morphological characteristics and growth requirements

The four strains (A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1) were observed to be Gram-negative, rod-shaped organisms (Fig. 1) which were motile by means of polar, monotrichous flagellation. The type of flagellation could only be visualized by staining, not by electron microscopy (Fig. 1), and motility appeared to be present only when cells were grown at high temperatures (37 °C and higher). Tumbling motility was often observed when cells were studied under phase-contrast microscopy, whereas directed movement could be seen only rarely. A large curved periplasmic layer was observed in many cells. A similar structure was observed recently in cells of the moderately thermophilic α -proteobacterium *Porphyrobacter tepidarius* (Hanada *et al.*, 1997). The cell sizes of the strains were as follows: strain 1.0–12.5 $\mu\text{m} \times 0.5$ –0.8 μm (strain A50-7-3^T), 1.0–10.0 $\mu\text{m} \times 0.5$ –0.8 μm (strain B50-7-1), 1.0–10.0 $\mu\text{m} \times 0.5$ –0.8 μm (strain D50-7-1), and 1.0–3.5 $\mu\text{m} \times 0.5$ –0.8 μm (strain B50-8-1). Filamentous cells (Fig. 1, top) were observed for all strains, although strain B50-8-1 appeared to generate longer filaments. Endospores were not observed. The strains grew well on TSA and other common bacteriological media. Smooth, circular, entire, convex, translucent and non-pigmented colonies as large as 1 mm in diameter grew on TSA and LB agar after 48 h incubation at 37 or 50 °C. After prolonged

Fig. 1. Electron micrographs of ultrathin sections of strain A50-7-3^T grown for 72 h at 38 °C. The filamentous (top) and rod-shaped (bottom) forms are shown. Bars, 200 nm.

incubation (2–3 d) at 50 °C on TSA, the strains produced a diffusible brownish discoloration of the medium and a characteristic ammonium-like odour.

Physiological and biochemical characteristics

Strains A50-7-3^T, B50-8-1, B50-7-1 and D50-7-1 grew in the temperature range between 18 and 50 °C. None of the strains showed growth at 14 or 53 °C. The optimum temperature range for growth was observed between 37 and 50 °C.

No growth was observed on MacConkey agar, Cetrimide agar or Czapek–Dox agar. All strains were able

Table 1. Physiological and biochemical characteristics of strains A50-7-3^T, B50-7-1, D50-7-1 and B50-8-1

Assimilation was determined after 7 d incubation, and hydrolysis of chromogenic substrates was read after 24 h. No differences in the characteristics of the strains were obtained when they were incubated at 37 or 50 °C. All four strains assimilated DL-3-hydroxybutyrate and L-leucine, hydrolysed pNP- α -D-glucopyranoside, pNP-phenyl-phosphonate, 2-deoxythymidine-5'-pNP-phosphate, L-alanine-pNA, L-glutamate- γ -3-carboxy-pNA and L-proline-pNA and showed a positive reaction in the tests for the enzymes catalase, oxidase, alkaline phosphatase, esterase (C₄), esterase (C₈), lipase (C₁₄), leucine arylamidase, valine arylamidase, chymotrypsin, phosphatase acid and naphthol-AS-BI-phosphohydrolase. The four strains were also positive in the test for urease activity and showed a strong β -haemolytic activity. None of the strains assimilated *N*-acetyl-D-glucosamine, L-arabinose, *p*-arbutin, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-mannose, D-maltose, α -D-melibiose, L-rhamnose, D-ribose, sucrose, salicin, D-trehalose, D-xylose, adonitol, i-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, fumarate, glutarate, itaconate, DL-lactate, suberate, β -alanine, L-histidine, L-ornithine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate or phenylacetate. None hydrolysed aesculin, pNP- β -D-galactopyranoside, pNP- β -D-glucuronide, pNP- β -D-glucopyranoside, bis-pNP-phosphate or pNP-phosphoryl-choline, reduced nitrate to nitrite or nitrogen and all were negative in the methyl red/Voges-Proskauer test, and were negative for citrate utilization, indole production and H₂S production.

Characteristic	A50-7-3 ^T	B50-7-1	D50-7-1	B50-8-1
Assimilation of:				
Acetate	+	—	+	—
Propionate	+	+	—	—
<i>cis</i> -Aconitate	+	+	—	—
L-Malate	+	—	—	—
Mesaconate	+	+	—	—
Oxoglutarate	+	—	—	—
Pyruvate	+	+	+	—
L-Alanine	+	+	—	—
L-Aspartate	+	+	—	—
L-Phenylalanine	+	—	—	—
L-Proline	—	+	—	—
L-Serine	+	—	—	—
Hydrolysis of:				
Gelatin	+	+	—	+

to grow on R2A agar. After 24 h incubation at 37 °C on Columbia blood agar, the four strains demonstrated marked β -haemolytic activity. The strains showed an inert reaction in the oxidative/fermentative test after 7 d incubation at 37 and 50 °C. Alkalinization of the medium was observed under aerobic conditions. All strains were negative for the following tests: the methyl red test, the Voges-Proskauer reaction, nitrate reduction, citrate utilization, indole production, and H₂S production. Each of the strains A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1 was positive for the following tests: oxidase; catalase; urease; alkaline monophosphoesterase; esterase (C₄); esterase (C₈); lipase (C₁₄); leucine arylamidase; valine arylamidase; chymotrypsin; acid monophosphoesterase; naphthol-AS-BI-phosphohydrolase; assimilation of DL-3-hydroxybutyrate and L-leucine; and hydrolysis of the chromogenic substrates pNP- α -D-glucopyranoside, pNP-phenyl-phosphonate, 2-deoxythymidine-5'-pNP-phosphate, L-alanine-pNA, L-glutamate- γ -3-carboxy-pNA and L-proline-pNA (Table 1). At least

one out of the four strains was positive for gelatin liquefaction and for the utilization of acetate, propionate, *cis*-aconitate, L-malate, mesaconate, oxoglutarate, pyruvate, L-alanine, L-aspartate, L-phenylalanine, L-proline or L-serine as sole source for carbon and energy (Table 1). Very weak positive reactions were observed with all the assimilation tests.

Susceptibility to antimicrobial compounds

The four isolates were analysed for their susceptibility to 17 antimicrobial compounds. All four strains displayed susceptibilities to the same antimicrobial compounds, varying only in the diameters of the zones of inhibition. They were susceptible to amoxycillin/clavulanic acid, ampicillin, cephalixin, chloramphenicol, colistin sulphate, erythromycin, fusidic acid, gentamicin, kanamycin, neomycin, penicillin G, polymyxin B, streptomycin and tetracycline. No inhibition zones were detected around the disks containing lincomycin, nitrofurantoin or sulphamethoxazole/trimethoprim.

Table 2. Whole-cell fatty acid composition (% of total) of strains A50-7-3^T, B50-8-1, B50-7-1 and D50-7-1 grown at different temperatures

Fatty acid	A50-7-3 ^T			B50-8-1			B50-7-1			D50-7-1		
	23 °C	37 °C	50 °C	23 °C	37 °C	50 °C	23 °C	37 °C	50 °C	23 °C	37 °C	50 °C
Branched-chain saturated												
10:0 iso	0.2	0.3	0.3	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
11:0 iso	6.2	6.7	10.2	6.0	7.6	10.5	5.2	6.7	8.5	6.1	6.2	9.9
12:0 iso	0.2			0.3	0.2					0.1		
13:0 iso	0.5	0.3	0.2	0.4	0.5	0.3	0.3	0.4	0.3	0.2	0.3	0.2
14:0 iso	3.3	3.6	2.9	4.6	3.3	2.7	2.0	3.7	2.7	1.6	3.5	2.5
15:0 iso	46.4	53.7	55.7	41.1	55.3	54.7	45.6	56.3	61.0	42.1	54.1	54.5
15:0 anteiso	0.2	0.3	1.4	0.2	0.3	0.8	0.3	0.3	0.9	0.1	0.4	1.0
16:0 iso	6.4	12.7	7.5	11.4	9.5	8.7	5.1	9.8	8.8	7.7	12.0	9.7
17:0 iso	2.4	2.9	2.3	3.0	2.5	2.9	3.2	2.5	2.9	4.5	2.8	3.0
Branched-chain unsaturated												
15:1 iso F*	4.2	1.4	0.3	2.9	1.4	0.4	3.3	1.6	0.5	2.0	1.4	0.6
16:1 iso H*	0.3	0.2		0.4			0.2			0.3	0.2	
17:1 iso <i>ω</i> 9 <i>cis</i>	18.3	8.0	2.3	17.6	7.5	2.2	23.5	8.9	2.3	22.8	8.4	2.4
Straight-chain												
14:0	0.4	0.2	0.5	0.3	0.2	0.4	0.2	0.2	0.4	0.2	0.3	0.5
15:0			2.0	0.2		1.8			0.7			1.5
15:1 <i>ω</i> 6 <i>cis</i>				0.1	0.5						0.8	
16:0	0.6	0.6	1.7	0.8	0.4	1.2	0.6	0.6	1.1	0.6	0.4	1.9
Hydroxylated												
11:0 iso 3-OH	8.4	7.8	12.6	8.0	9.7	12.6	7.4	8.2	9.9	8.5	7.9	12.0
12:0 iso 3-OH	0.1	0.3		0.4	0.2	0.3				0.2	0.2	
12:0 2-OH		0.2			0.1	0.2					0.2	0.2
15:0 iso 3-OH				0.2			0.5			0.3		
Group A†	0.8	0.5		0.9	0.5		0.8	0.4		1.0	0.5	
Group B†	0.6	0.4		0.7	0.3		0.8	0.4		1.2	0.4	
Other‡	0.4		0.2	0.2			0.8			0.3		
Unsaturated, % of total§	22.8	9.6	2.5	21.0	9.4	2.6	27.0	10.5	2.8	25.0	10.7	3.0

* The position and configuration of the double bond is not known.

† This group consists of fatty acids not resolved by the chromatographic conditions used. Group A includes sum of 15:1 iso H, 15:1 iso I and/or 13:0 3-OH. Group B includes 16:1 *ω*7*cis* and/or 15:0 iso 2-OH.

‡ Includes the sum of the following fatty acids, each of which is present as less than 0.5 % of total fatty acids: 15:1 iso G, 16:1 *ω*7*trans* alcohol, unknown fatty acid or alcohol with equivalent chain-length of 14-971, 16:1 *ω*9*cis*, 16:1 *ω*5*cis*, 15:0 2-OH, 17:1 iso I and 17:1 anteiso B.

§ Sum of fatty acids 17:1 iso *ω*9*cis*, 15:1 iso F, 15:1 *ω*6*cis* and 16:1 iso H.

Chemotaxonomic investigations

Strains A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1 displayed relatively uniform chemotaxonomic characteristics. The quinone system of the four strains was characterized exclusively by the presence of ubiquinones. Ubiquinone Q-8 was observed to be the predominant quinone (96.3–99.2 %), whereas ubiquinone Q-9 was detected only in minor amounts (0.8–3.7 %).

Spermidine was observed to be the major polyamine compound (comprising more than 90 % of the total polyamine content), and spermine was detected in minor concentrations. The diamines 1,3-diamino-

propane, putrescine and cadaverine were found only in trace amounts.

In the polar lipid profiles, diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol were detected in two-dimensional thin-layer chromatograms (results not shown). An additional small spot, which could be stained with molybdenum blue reagent (indicating that it represented an unknown phospholipid; PL1), was detected in extracts from strain B50-8-1. A similar polar lipid profile was detected for *S. maltophilia* DSM 50170^T, whereas *X. campestris* DSM 3566^T was characterized by the additional appearance of phosphatidylmonomethylethanolamine and another unknown phospholipid (PL2). The fatty

Table 3. Sequence similarities between the 16S rRNA genes of strain A50-7-3^T and selected species of the γ -subclass of the *Proteobacteria*

Species*	GenBank/EMBL/DDBJ accession number	16S rDNA sequence similarity (%) with A50-7-3 ^T , AJ300185
<i>Thermomonas haemolytica</i> B50-7-1	AJ300186†	100
<i>Thermomonas haemolytica</i> B50-8-1	AJ300188†	100
<i>Thermomonas haemolytica</i> D50-7-1	AJ300189†	99.9
<i>Xanthomonas campestris</i> LMG 568 ^T	X95917	94.3
<i>Xanthomonas albilineans</i> LMG 494 ^T	X95918	93.4
<i>Pseudoxanthomonas broegbernensis</i> B1616/1 ^T	AJ012231	93.6
<i>Stenotrophomonas maltophilia</i> LMG 958 ^T	X95923	93.4
<i>Stenotrophomonas africana</i> MGB ^T	U62646	91.7
<i>Luteimonas mephitis</i> B1953/27.1 ^T	AJ012228	93.1
<i>Xylella fastidiosa</i> ATCC 35879 ^T	AF192343	93.0
<i>Rhodanobacter lindaniclasticus</i> RP5557 ^T	AF039167	89.3
<i>Pseudomonas aeruginosa</i> LMG 1242 ^T	Z76651	85.2
<i>Microbulbifer hydrolyticus</i> IRE-31-T	U58338	84.3

* Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; NCTC, National Collections of Type Cultures and Pathogenic Fungi, London, UK.

† Partial sequences (see text).

acid profiles (Table 2) of the four strains were characterized by the presence of, mainly, iso-branched acids. The predominant fatty acid was observed to be 15:0 iso, while significant concentrations of 11:0 iso, 11:0 iso 3OH, 14:0 iso, 15:1 iso F, 16:0 iso and 17:1 iso ω 9c fatty acids also were detected. With increasing growth temperature, from 23 to 50 °C, the proportion of unsaturated fatty acids decreased from 24 to 2.7% (mean of the four strains) of total fatty acids. This was due, mainly, to a decrease in the content of 17:1 iso ω 9c. The relative amounts of 17:0 iso remained constant in cells growing over the whole range of growth temperature, which indicates, for all four strains, that adaptation to growth at high temperatures does not occur by increasing the ratio of 17:0 iso to 17:1 iso ω 9cis. Instead, the relative proportion of the major fatty acid, 15:0 iso, increased with increasing growth temperature. Interestingly, the relative amounts of short-chain fatty acids (11:0 iso and 11:0 iso-3-OH) also increased slightly at 50 °C. Comparison of the protein patterns obtained after SDS-PAGE demonstrated a high degree of similarity within the four strains (see supplementary data in IJSEM Online, <http://ijs.sgmjournal.org>).

16S rDNA sequence analysis

The nearly complete (i.e. 1500 nucleotide positions, an estimated 97% of the complete sequence) 16S rDNA sequence for strain A50-7-3^T was determined, including positions 28–1525 (*Escherichia coli* 16S rRNA

gene-sequence numbering), while less complete sequences were determined for strains B50-7-1 (positions 28–932), B50-8-1 (positions 28–1017) and D50-7-1 (positions 28–932). However, the sequences determined for the four strains are so similar (Table 3) that further sequencing of the 16S rDNAs of strains B50-7-1, B50-8-1 and D50-7-1 was considered to be unnecessary.

The 16S rDNA sequence determined for strain A50-7-3^T was compared with those of all validly published species of *Stenotrophomonas*, *Xanthomonas* and *Xylella* (Wells *et al.*, 1987; Drancourt *et al.*, 1997; Hauben *et al.*, 1997; Moore *et al.*, 1997; Hauben *et al.*, 1999; Trébaol *et al.*, 2000), as well as strains comprising distinct genomic species of *Stenotrophomonas* but which are not differentiated taxonomically (Hauben *et al.*, 1999) (Table 3). The 16S rDNA sequences of species of the relatively new genera *Luteimonas* and *Pseudoxanthomonas*, also related to *Stenotrophomonas*, *Xanthomonas* and *Xylella*, described by Finkmann *et al.* (2000), were also observed to be no more similar to the sequence of strain A50-7-3^T than were the sequences of species of *Stenotrophomonas*, *Xanthomonas* and *Xylella* (Table 3).

Comparative evolutionary distance analyses of the 16S rDNA sequence of strain A50-7-3^T demonstrated that it clusters within the γ -*Proteobacteria*, most closely to the species of *Stenotrophomonas*, *Xanthomonas*, *Xylella*, *Pseudoxanthomonas*, *Luteimonas* and *Rhodano-*

Fig. 2. Estimated 16S rDNA phylogenetic relationships among the strains A50-7-3^T, B50-7-1, B50-8-1, D50-7-1, related genera of the *Xanthomonas-Stenotrophomonas* phylogenetic lineage, and species representing distinct lineages within the γ -subclass of the *Proteobacteria*. The dendrogram was generated from evolutionary distances, calculated from pairwise dissimilarities, using the FITCH program contained in the PHYLIP package. Bootstrap estimates of confidence, derived from 1000 resamplings, are shown for branchings with values greater than 80%, within the *Xanthomonas-Stenotrophomonas* phylogenetic lineage. The sequence data for the reference organisms were obtained from the GenBank/EMBL/DDJB databases, as listed in Table 3. The bar represents the scale of estimated evolutionary distance (i.e. a mean of five substitutions at any nucleotide position, per 100 nucleotide positions) which has occurred from the points of sequence divergence.

bacter (Fig. 2). It is clear that strain A50-7-3^T (as well as strains B50-7-1, B50-8-1 and D50-7-1) comprise a distinct phylogenetic branch of the *Stenotrophomonas-Xanthomonas-Xylella* lineage (Fig. 2). The topography of the branching order of the *Stenotrophomonas-Xanthomonas-Xylella* phylogenetic lineage within the dendrogram of Fig. 2 was supported by identical branching orders generated using an al-

ternative treeing algorithm of maximum-likelihood (fastDNAml, version 1.0) (data not shown) and by estimated high bootstrap proportions of confidence for the respective branchings (Fig. 2).

Riboprints and AFLP genomic fingerprinting

The riboprints obtained after digestion of genomic DNA displayed a homogeneous pattern. Relative to the other three strains, in B50-7-1 one band in the area of 5 kbp was missing in the riboprint (available as supplementary data in IJSEM Online; <http://ij.sgmjournals.org>).

Strains A50-7-3^T and B50-8-1 were observed to be almost indistinguishable from each other, on the basis of their AFLP fingerprint patterns, whereas strains B50-7-1 and D50-7-1 displayed some genetic variability, as indicated by the slightly different AFLP band patterns (Fig. 3). The level of reproducibility was determined by electrophoretically separating independently prepared AFLP samples, in duplicate. Intragel-correlation levels between duplicates from the same strains varied from 92% to 97%. Four strains of *S. maltophilia*, from a study presented recently by Hauben *et al.* (1999), were included to demonstrate the heterogeneity of AFLP band patterns in a single species. The four strains, A50-7-3^T, B50-8-1, B50-7-1 and D50-7-1, displayed similar profiles, which were different from the *S. maltophilia* profiles. None of the bands characterizing *S. maltophilia* was found in AFLP profiles of the four strains analysed, indicating that the four strains are quite distinct from any of the known *S. maltophilia* genomic groups.

DNA relatedness and G + C content of genomic DNA

Labelled DNA of strain A50-7-3^T was hybridized with DNAs of strains B50-7-1, B50-8-1 and D50-7-1 as well, and resulted in 100% relatedness in each experiment. The pooled standard deviation of the experiment was 1.2%. This high degree of DNA relatedness unambiguously demonstrates that the four isolates are members of a single species. The G + C contents of the genomic DNAs of the four strains A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1 were

Fig. 3. UPGMA (unweighted pair group arithmetic average-linkage algorithm) clustering of AFLP patterns of the four *Thermomonas* strains and four *Stenotrophomonas maltophilia* strains representing different genomic groups of the species as defined by Hauben *et al.* (1999). Similarity between AFLP densitometric profiles was obtained using the Pearson product-moment correlation coefficient.

determined to be 68.5, 68.7, 67.1 and 68.1 mol%, respectively.

DISCUSSION

The 16S rDNA sequence analyses of the four strains A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1 and their alignment with other sequences demonstrated that they represent a new taxon which is distinct from all other validly described taxa. The closest, albeit relatively distant (i.e. with 16S rDNA sequence similarities of less than 95%; Table 3), phylogenetic relatives are the species of the genera *Xanthomonas*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Luteimonas* and *Xylella*, within a distinct lineage of the γ -subclass of the *Proteobacteria* (Fig. 2). Organisms related to the genera *Stenotrophomonas*, *Xanthomonas* and *Xylella*, possessing a wide range of metabolic activities, are being detected increasingly in a variety of environmental and clinical environments (Drancourt *et al.*, 1997; Finkmann *et al.*, 2000; Hauben *et al.*, 1999; Nalin *et al.*, 1999). It is clear that the diversity of such organisms is much greater than previously recognized and that the limited, traditional taxonomic organization of the '*Stenotrophomonas*–*Xanthomonas*–*Xylella* phylogenetic lineage' will be markedly extended in the near future.

On the basis of the 16S rDNA sequence dissimilarities (Table 3), the calculated phylogenetic distances to the species with the most similar sequences, and the estimated bootstrap levels of confidence for the dendrogram topography (Fig. 2), strains A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1 are recognized to represent a new genus within the *Stenotrophomonas*–*Xanthomonas*–*Xylella* lineage of the γ -*Proteobacteria*. The high sequence similarities of 16S rRNA genes and, in particular, the high degrees of similarities based upon genomic DNA AFLP and riboprint analysis, indicate that the four strains are members of a single species. This assumption is clearly confirmed by the results obtained from DNA–DNA hybridizations and comparison of protein patterns.

The observed homogeneities in the polyamine patterns, polar lipid profiles, fatty acid profiles and the quinone system are in accordance with the observed close genotypic relationships between the four isolates. Differences in chemotaxonomic traits (Table 4) characterize the strains and clearly distinguish them from the nearest phylogenetic relatives.

Although strain B50-8-1 is somewhat distinct from the other three strains, on the basis of certain biochemical differences and the DNA G+C content (mol%), DNA-relatedness and genotypic analyses group this strain together with the others in a single species. However, as demonstrated previously (Janssen *et al.*, 1996), the combination of *ApaI* and *TaqI* as restriction enzymes, and guanine as a single extension base for amplification, reveal complex AFLP patterns which correlate perfectly with species affiliation. The

suitability of this approach has been demonstrated for *S. maltophilia* (Hauben *et al.*, 1999). Furthermore, riboprint analyses also have been shown to reflect recognized species affiliations if a certain degree of similarity is observed in the banding patterns (Grimont & Grimont, 1986; Busse *et al.*, 2000). In our study, both AFLP genomic fingerprints and riboprint patterns, together with the other data presented, appear to be excellent tools for replacing the analysis of DNA relatedness based on the results of DNA–DNA hybridizations.

On the basis of the phylogenetic placement, estimated from 16S rDNA-sequence levels of dissimilarity with related taxa, together with unique chemotaxonomic characteristics, DNA G+C contents (mol%), riboprint patterns, AFLP profiles, DNA relatedness, antibiotic susceptibilities, and physiological and biochemical traits, we propose a new genus for strains A50-7-3^T, B50-7-1, B50-8-1 and D50-7-1, namely *Thermomonas* gen. nov., and the type species *Thermomonas haemolytica* sp. nov.

Description of *Thermomonas* gen. nov.

Thermomonas (Ther.mo.mo'nas. Gr. n. *therme* heat; Gr. n. *monas* a unit, monad; N.L. fem. n. *Thermomonas* a thermophilic monad).

Gram-negative, non-spore-forming rods, usually occurring in filaments. The temperature range for growth is 18–50 °C, optimal growth occurring between 37 and 50 °C. Cells are oxidase- and catalase-positive. The following polar lipids are present: diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The fatty acid profile mainly contains iso-branched fatty acids with the predominant acid 15:0 iso; other characteristic fatty acids are 11:0 iso, 11:0 iso 3-OH, 16:0 iso and 17:1 iso (ω 9c). Spermidine is the predominant polyamine, and the major quinone is ubiquinone Q-8. The type species of the genus is *Thermomonas haemolytica*.

Description of *Thermomonas haemolytica* sp. nov.

Thermomonas haemolytica (hae.mo.ly'ti.ca. Gr. n. *haema* blood; Gr. adj. *lyticus* dissolving, loosening; N.L. fem. adj. *haemolytica* blood-dissolving).

The Gram-negative, rod-shaped cells are 1.0–12.5 μ m long $\times$ 0.5–0.8 μ m wide. The cells are motile by means of polar monotrichous flagellation or are non-motile. Non-spore-forming. Colonies on complex standard media at 50 °C are whitish/translucent (at 37 °C they are transparent), circular, smooth and convex with an entire edge. Growth occurs in the temperature range 18–50 °C, optimum growth occurring between 37 and 50 °C. Cells are oxidase- and catalase-positive. Nitrate is not reduced, and the cells give negative results in the methyl red/Voges–Proskauer test and are negative for citrate utilization, and indole and H₂S production. DL-3-Hydroxybutyrate and L-leucine are assimilated.

Table 4. Differential characteristics of *T. haemolytica* compared to representatives of the genera *Xanthomonas*, *Stenotrophomonas*, *Pseudoxanthomonas*, *Luteimonas*, *Xylella* and *Microbulbifer*

Organisms: 1, *Thermomonas haemolytica*; 2, *Microbulbifer hydrolyticus*; 3, *Luteimonas mephitis*; 4, *Stenotrophomonas maltophilia*; 5, *Xanthomonas campestris*; 6, *Pseudoxanthomonas broegbernensis*; 7, *Xylella fastidiosa*. Data for *Thermomonas haemolytica* and polar lipids generated in this study; all other data taken from previous studies (Oyaizu & Komagata, 1983; Palleroni, 1984; Wells *et al.*, 1987; Busse & Auling, 1988; Auling *et al.*, 1991; Stead, 1992; Palleroni & Bradbury, 1993; Yang *et al.*, 1993a, b; Vauterin *et al.*, 1995, 1996; González *et al.*, 1997; Finkmann *et al.*, 2000). Abbreviations: Amp, ampicillin; Pen G, penicillin G; Ery, erythromycin; Kan, kanamycin; Neo, neomycin; Strep, streptomycin; v+, most of the strains are not susceptible; v–, most of the strains are susceptible; ND, not determined; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unknown phospholipid; PME, phosphatidylethanolamine.

Species	1	2	3	4	5	6	7
Temp. optimum (°C)	37–50	37	28	35	28	28	26–28
Nitrate reduction	–	–	–	+	–	–	–
Aesculin hydrolysis	–	ND	–	+	+	–	–
Susceptibility to:							
Amp	+	+	–	–	–	–	+
Pen G	+	–	–	–	–	–	–
Ery	+	ND	–	–	–	–	ND
Kan	+	+	v+	+	+	–	+
Neo	+	ND	v–	–	–	+	ND
Strep	+	–	–	–	+	–	–
Major fatty acids	15:0 iso, 16:0 iso	15:0 iso, 17:1 iso	15:0 iso, 17:1, 17:0 iso	15:0 iso, 15:0 anteiso	15:0 iso, 16:1, 15:0 anteiso	15:0 iso, 15:0 anteiso, 16:0, 16:1, 17:1 iso	16:0, 16:1, 17:0
Major hydroxy fatty acids	11:0 iso 3-OH	11:0 iso 3-OH, 15:0 iso 2-OH	11:0 iso 3-OH	13:0 iso 3-OH, 12:0 3-OH, 11:0 iso 3-OH, 13:0 2-OH	13:0 iso 3-OH, 12:0 3-OH, 11:0 iso 3-OH	11:0 iso 3-OH, 11:0 iso 2-OH	10:0 2-OH
Major polar lipids§	DPG, PE, PG	ND	ND	DPG, PE, PG	DPG, PE, PG, PME, PL	ND	ND
Quinone system	Q-8	ND	Q-8	Q-8	Q-8	Q-8	ND
Major polyamines	SPD	ND	ND	CAD, SPD	SPD	ND	ND

pNP- α -D-glucopyranoside, pNP-phenyl-phosphonate, 2-deoxythymidine-5'-pNP-phosphate, L-alanine-pNA, L-glutamate- γ -3-carboxy-pNA and L-proline-pNA are hydrolysed. Compounds that are not assimilated or hydrolysed by any of the strains are listed in Table 1. The strains are positive in the tests for the enzymes alkaline phosphatase, esterase (C₄), esterase (C₈), lipase (C₁₄), leucine arylamidase, valine arylamidase, chymotrypsin, phosphatase acid and naphthol-AS-BI-phosphohydrolase and urease activity, and show strong β -haemolytic activity. Some strains assimilate acetate, propionate, *cis*-aconitate, L-malate, mesaconate, oxoglutarate, pyruvate, L-alanine, L-aspartate, L-phenylalanine, L-proline and L-serine, and hydrolyse gelatin. The G+C content of the DNA is 67.1–68.7 mol%. The chemotaxonomic properties and other characteristics are as for the genus. The type strain, *T. haemolytica* A50-7-3^T, has been deposited at the Deutsche Sammlung von Mikroorganismen und

Zellkulturen and the Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent under the accession numbers DSM 13605^T (= LMG 19653^T). Other strains of this species are *T. haemolytica* B50-7-1 (= DSM 13598 = LMG 19655), *T. haemolytica* B50-8-1 (= DSM 13599 = LMG 19654) and *T. haemolytica* D50-7-1 (= DSM 13610 = LMG 19656). The strains were originally isolated from kaolin slurry used in the production of paper.

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